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## ► To cite this version:

Anthony Kwasiborski, Franck Bastide, Bruno Hamon, Pascal Poupard, Philippe Simoneau, et al.. In silico analysis of RNA interference components and miRNAs-like RNAs in the seed-borne necrotrophic fungus *Alternaria brassicicola*. *Fungal Biology*, 2022, 126 (3), pp.224 - 234. 10.1016/j.funbio.2021.12.004 . hal-03595685

**HAL Id: hal-03595685**

**<https://hal.science/hal-03595685>**

Submitted on 11 Mar 2022

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# Fungal Biology

## In silico analysis of RNA interference components and miRNAs-like RNAs in the seed-borne necrotrophic fungus *Alternaria brassicicola*.

--Manuscript Draft--

<b>Manuscript Number:</b>	FUNBIO-D-21-00092R1
<b>Article Type:</b>	Original Research
<b>Section/Category:</b>	Genomics, genetics and molecular genetics
<b>Keywords:</b>	Pathogen fungi <i>Alternaria brassicicola</i> smallRNA sequencing RNAi component miRNA identification
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<b>Abstract:</b>	<p>RNA interference is a mechanism of suppressing gene expression in plants, animals and fungi. This regulation mechanism involves three main enzymes, Dicers (Dcr), Argonautes (Ago) and RNA Dependent RNA Polymerases (Rdrp) allowing to produce smallRNAs. RNA interference and smallRNAs have a role in the plant-microorganisms interaction, either in a pathogenic or in a symbiotic relationships. <i>Alternaria brassicicola</i> is a pathogenic fungus of the Brassicaceae plants. During plant infection, it is able to transmit itself vertically and horizontally, giving advantages for new infection and dissemination. To investigate RNA interference and the presence of smallRNAs in <i>A. brassicicola</i>, an in silico analysis was achieved. Two DCR, 4 AGO and 3 RDRP genes were identified confirming the presence of smallRNAs in <i>A. brassicicola</i>. SmallRNA sequencing from wild-type strain and DCR deleted mutants allowed the identification of 17 miRNAs in <i>A. brassicicola</i>. The synthesis of these miRNAs is only weakly influenced by the inactivation of DCR genes suggesting the possible existence of an alternative Dicer-independent miRNA synthesis pathway. Target's prediction of <i>A. brassicicola</i> miRNAs identified genes in the fungus and in the plant model <i>Arabidopsis thaliana</i>. Some miRNAs were predicted to target <i>A. thaliana</i> genes involved in the methylation of histone and in the disease resistance.</p>

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1 ***In silico* analysis of RNA interference components and miRNAs-like RNAs in the seed-borne**  
2 **necrotrophic fungus *Alternaria brassicicola*.**

3

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22

23

24 **Abstract**

25 RNA interference is a mechanism of suppressing gene expression in plants, animals and fungi. This  
26 regulation mechanism involves three main enzymes, Dicers (Dcr), Argonautes (Ago) and RNA  
27 Dependent RNA Polymerases (Rdrp) allowing to produce smallRNAs. RNA interference and  
28 smallRNAs have a role in the plant-microorganisms interaction, either in a pathogenic or in a  
29 symbiotic relationships. *Alternaria brassicicola* is a pathogenic fungus of the *Brassicaceae* plants.  
30 During plant infection, it is able to transmit itself vertically and horizontally, giving advantages for  
31 new infection and dissemination. To investigate RNA interference and the presence of smallRNAs  
32 in *A. brassicicola*, an *in silico* analysis was achieved. Two *DCR*, 4 *AGO* and 3 *RDRP* genes were  
33 identified comforting the presence of smallRNAs in *A. brassicicola*. SmallRNA sequencing from  
34 wild-type strain and *DCR* deleted mutants allowed the identification of 17 miRNAs in *A.*  
35 *brassicicola*. The synthesis of these miRNAs is only weakly influenced by the inactivation of *DCR*  
36 genes suggesting the possible existence of an alternative Dicer-independent miRNA synthesis  
37 pathway. Target's prediction of *A. brassicicola* miRNAs identified genes in the fungus and in the  
38 plant model *Arabidopsis thaliana*. Some miRNAs were predicted to target *A. thaliana* genes  
39 involved in the methylation of histone and in the disease resistance.

40

41

42

43 **Keywords**

44 Pathogen fungi / *Alternaria brassicicola* / smallRNA sequencing / RNAi component / miRNA  
45 identification

46

47

48

49 **Abbreviations**

50 miRNA: micro RNA

51 *DCR* : *Dicer* gene

52 *AGO*: *Argonaute* gene

53 *RDRP*: RNA dependent RNA polymerase

54 RNAi: RNA interference

55 nt-sRNA: non-transcribed small RNAs

56 *AbmiRNA*: *Alternaria brassicicola* micro RNA

57 *AbmiRNA-C*: *Alternaria brassicicola* micro RNA candidate

58

59

60

## 61 **Introduction**

62 In eukariota, from animals to fungi, gene expression is regulated by a large panel of mechanisms.  
63 Amongst them, RNA interference is a conserved regulatory mechanism (Nakayashiki, 2005) that  
64 induces gene silencing by binding smallRNAs to mRNA with complementary sequences (Zamore *et*  
65 *al.*, 2000). RNA interference mechanism involves three main enzymes: RNase III proteins called  
66 Dicers (Dcr) that synthesize smallRNAs, Argonaute proteins (Ago) that help the recognition of  
67 smallRNAs and the sequence-specific target and RNA-dependent RNA polymerases (Rdrp) that  
68 generate double stranded RNAs from aberrant RNAs (Torres-Martinez *et al.*, 2017). SmallRNAs  
69 are a large group of endogenous non-coding RNAs of about 18 to 30 nt mainly classified as short  
70 interfering RNAs (siRNAs), microRNAs (miRNAs) and piwi-interacting RNAs (piRNAs)  
71 (Carreras-Villaseñor *et al.*, 2013). A lot of studies are focusing on identifying miRNA-like RNAs by  
72 sequencing with bioinformatic approaches in fungi with economical interest, such as the industrial  
73 strain *Trichoderma reesei* (Kang *et al.*, 2013) or in pathogenic fungi *Metarhizium anisopliae* (Zhou  
74 *et al.*, 2012) and *Sclerotinia sclerotiorum* (Zhou *et al.*, 2012). Advances in next-generation  
75 sequencing technologies has greatly accelerated the identification of smallRNAs and allowed to  
76 access less represented smallRNAs. However, these studies used complete genomes and adapted  
77 available bioinformatic tools from plants and animals for which miRNAs are largely studied. In  
78 fungi, RNA interference mechanism and smallRNAs were described to be involved in development  
79 (Carreras-Villaseñor *et al.*, 2013), antiviral defense (Segers *et al.*, 2007) and pathogenicity (Campo  
80 *et al.*, 2016). Interestingly, recent works demonstrated that smallRNAs are also involved in cross-  
81 kingdom communication. Indeed, plants can transfer smallRNAs in pathogen fungi to silence  
82 virulence genes (Cai *et al.*, 2018; Rajwanshi *et al.*, 2019) and inversely some fungi are able to  
83 transfer their own smallRNAs to the host plant to silence host immunity genes (Weiberg *et al.*,  
84 2013). Identifying RNAi components and exploring the involvement of smallRNAs in the life cycle

85 of pathogenic fungi could be of great interest notably for the development of innovative disease  
86 control alternatives.

87 The necrotrophic plant pathogen *Alternaria brassicicola* is the causative agent of the black spot  
88 disease in *Brassicaceae* plants including many economically important crop species as oilseed and  
89 cabbage and the plant model *Arabidopsis thaliana* (Lawrence et al., 2008). This pathogen may be  
90 responsible for high yield losses. During its life cycle, this fungus can transmit itself vertically, from  
91 leaf to seeds, and horizontally, from plant to plant giving significant advantages for progeny  
92 infection and long-distance dissemination (Baker and Smith, 1966; Elmer, 2001). Then the fungus  
93 can resist to very low water availability (Pochon *et al.*, 2013) and to chemical stresses through the  
94 plant production of defense molecules (Pedras *et al.*, 2011). Moreover, as necrotrophic pathogen, *A.*  
95 *brassicicola* secretes an arsenal of host cell wall-degrading enzymes and diverse secondary  
96 metabolites, especially toxins, required for plant penetration and nutrient consumption in order to  
97 colonize and kill host tissue (Cho *et al.*, 2007; Meena *et al.*, 2017). While pathways involved in the  
98 virulence of *A. brassicicola* have been extensively studied, little is known on regulatory  
99 mechanisms. As RNAi and smallRNAs are described as involved in the pathogeny of fungus, to  
100 study these two mechanisms could be new ways to increase our knowledge about *A. brassicicola*  
101 virulence. The Abra43 strain was isolated in 1999 from radish seeds during quality controls of  
102 French commercial lots. Recently, a new genome sequence of *A. brassicicola* Abra43 has been  
103 reported (Belmas *et al.*, 2018). Obtained from a 7-day-old colony growing on potato dextrose agar  
104 at 24°C, the genome was sequenced using a combination of PacBio and HiSeq4000 technologies.  
105 The draft genome consisted of 29 contigs for a total length of 31,036,461bp with a G+C content of  
106 50.85%. *A. brassicicola* genome was estimated to code 12,456 proteins (Belmas et al., 2018).

107 In this study, we first used the new draft genome of *A. brassicicola* in order to characterize the  
108 major components of the RNA interference machinery in the necrotrophic fungus *A. brassicicola*.  
109 Second, we aimed to identify and characterize *A. brassicicola* miRNA-like RNAs population from

110 the vegetative state of the fungus.

111

## 112 **Materials and methods**

### 113 ***Identification of RNAi machinery components***

114 For the identification of RNAi components, *Neurospora crassa* homologs of Dcr, Ago and Rdrp  
115 genes were searched in the published genomes of *A. brassicicola* strain Abra43 (Belmas *et al.*,  
116 2018) using BLASTP. An E-value of 8e-10 was used as a cutoff in BLASTP searches and the hits  
117 were filtered to keep only those with at least 25% identity to the query sequence. Protein domains  
118 were screened using Pfam v.33.1 (<http://pfam.xfam.org/>, El-Gebali *et al.*, 2019) and compared to  
119 those of the DCR, Ago and Rdrp proteins from *N. crassa*. Then, we looked for the Helicase3, Dcr  
120 dimer and Ribonuclease3 domains in Dcr putative proteins, the Argo, PAZ and Piwi domains in Ago  
121 putative sequences and the RDRP domain in the putative Rdrp proteins and only proteins containing  
122 these domains were conserved. To strengthen the identification, Dcr, Ago and Rdrp specific  
123 domains were directly searched in the protein database of *A. brassicicola* using ScanProsite tool  
124 (<https://prosite.expasy.org/scanprosite/>). The amino acid sequences of potential candidates were  
125 then aligned with sequences from Ascomycota fungi summarized in table S1 using Muscle (Edgar  
126 RC., 2004) with default parameters. The alignments were subsequently processed using MEGA  
127 v10.1.8 (Kumar *et al.*, 2018) for phylogenetic analysis. The LG+F model associated with gamma  
128 distributed in 5 categories was used as criteria for the maximum likelihood phylogenetic tree  
129 building. The robustness of each tree was estimated by performing 1000 bootstrap replicates.

130

### 131 ***Strain and mutant generation***

132 The *A. brassicicola* wild-type strain (Abra43) has previously been described (Sellam *et al.*, 2007)  
133 and was used to generate the various deletion mutants (Table S3). Loss-of-function mutants were  
134 generated by homologous integration of replacement constructs generated by double-joint PCR and



135 containing a selectable marker flanked by sequences specific to the targeted genes (Yu *et al.*, 2004).  
136 The selectable marker corresponded to the Hygromycin-resistant gene from pCB1636 plasmid  
137 (Sweigard *et al.*, 1995) and the Nourseothricin-resistant gene from pNR plasmid (Malonek *et al.*,  
138 2004). For each targeted gene, two sets of primers (Table S1) were designed to amplify 908 bp  
139 (*DCR1*) and 668 bp (*DCR2*) from the 5' flanking regions and 870 bp (*DCR1*) and 800 bp (*DCR2*)  
140 from the 3' regions. All amplifications were performed with Phusion™ Hot Start High-Fidelity  
141 DNA Polymerase (Finnzymes, Espoo, Finland). The final double-joint PCR were carried out using  
142 the NF-NR primer pairs to have a fragment including 5' and 3' complementary flanking regions  
143 surrounding the selectable marker. Products were purified and used to transform *A. brassicicola*  
144 protoplasts as described by Cho *et al.* (2006). Hygromycin-resistant transformants were prescreened  
145 by PCR with relevant primer combinations to confirm integration of the replacement construct at  
146 the targeted locus. Deletion mutants were further purified by two rounds of single-spore isolation  
147 and then confirmed by RT-PCR after a retrotranscription using the RevertAid Reverse Transcriptase  
148 (ThermoFisher Scientific, Massachusetts, USA) . The double mutant (*DCR1DCR2ΔΔ*) was  
149 obtained using the same method and primers except the *DCR1* mutant was used as recipient initial  
150 strain. For each genotype, (*DCR1Δ.1*, *DCR1Δ.3*, *DCR2Δ.6*, *DCR2Δ.7*, *DCR1DCR2ΔΔ.2* and  
151 *DCR1DCR2ΔΔ.4*) two mutants were generated during two independent transformation experiments.

152

### 153 ***Growth conditions***

154 A 10<sup>4</sup> conidia/mL culture in PDB (Difco, Faverges, France) liquid medium was prepared from a 7-  
155 days PDA culture of *A. brassicicola* and its mutants. After 18h incubation at 22°C under shaking in  
156 the darkness, suspensions were filtered and mycelium were immediately frozen in TRI Reagent  
157 solution (Ambion, Foster, USA) and stored at -80°C until used.

158

### 159 ***Small RNA extraction, sequencing and quantification***

160 Total small RNAs were extracted from frozen mycelium using NucleoSpin miRNA kit (Macherey-Nagel,  
161 Düren, Germany) according to the manufacturer's instructions followed by Turbo DNA-free Kit  
162 (Ambion) treatment. RNA quantity and quality were controlled using the Qubit Fluorometric  
163 Quantitation (ThermoFisher Scientific) and the Experion Automated Electrophoresis System (Bio-  
164 Rad, California, USA) respectively. RNAs below 45bp size were selected to create sRNA libraries.  
165 Small RNA sequences were generated using Illumina HiSeq 2500 and the Basecalling pipeline  
166 HiSeq Control Software 2.2.58, RTA 1.18.64.0 and CASAVA-1.8.2 at the Fasteris Company  
167 (Geneva, Switzerland). Each sequencing was carried out in duplicates.

168

#### 169 ***miRNA-like RNAs identification***

170 Small RNA sequencing was cleaned to remove adaptors and low-quality tags. Only unique  
171 sequences along with at least 10 associated read counts were conserved in order to avoid  
172 contaminants such as reads derived from degradation products. After removing ribosomal RNA  
173 (rRNA), transfer RNA (tRNA), small nuclear RNA (snRNA) and small nucleolar RNA (snoRNA)  
174 by aligning unique sequences to Rfam v12.0 (Gardner *et al.*, 2009), the prediction of miRNA was  
175 carried out using the miRDeep v2.0.0.8 algorithm with default values (An *et al.*, 2013). The  
176 conservation status of miRNA was analyzed by comparison with mature miRNAs and stem-loop  
177 recorded in miRbase v22.1 using a cut-off of value of 15, a match score of 5 and a mismatch  
178 penalty value of -4.

179

#### 180 ***miRNA-like RNAs target prediction***

181 The prediction of target genes for *A. brassicicola* miRNAs was carried out using the miRanda v3.3  
182 software with score and energy threshold of 155 and -23 kcal.mol<sup>-1</sup> respectively and the  
183 psRNATarget software with default values on *A. brassicicola* and *A. thaliana* genome as references.  
184 Only target genes predicted by the two softwares were conserved.

185

## 186 *Statistical analysis*

187 Statistical analyses were done with the R software package (version 3.2.3). Small RNA abundances  
188 were analysed using EdgeR (McCarthy *et al.*, 2012) and DEseq2 (Love *et al.*, 2014). Only  
189 sequences with significant values in both softwares were used for further analyses.

190

## 191 **Results**

### 192 *Identification of RNAi components*

193 Using the recently published genome of *A. brassicicola* Abra43 (Belmas *et al.*, 2018), the  
194 identification of RNAi machinery components was investigated. Using Dcr, Ago and Rdrp proteins  
195 from *N. crassa* in a blast analysis, 24, 14 and 28 predicted proteins were respectively identified, in  
196 the *A. brassicicola* genome that could be candidates to RNAi machinery components. In order to  
197 decrease the number of candidates, we searched for protein domains using Pfam v33.1 database (El-  
198 Gebali *et al.*, 2019). The presence of two RNaseIII, one helicase C and one Dcr\_dimer domains was  
199 necessary for Dcr proteins selection (MacRae, 2006). The presence of a Piwi domain and a RDRP  
200 domain was required for the Ago and Rdrp protein selection, respectively (Lyer *et al.*, 2003;  
201 Silvestri *et al.*, 2019) (Figure 1). Then, 2 proteins AbraP02176 and AbraP09650 from *A.*  
202 *brassicicola* could be candidates for Dcr proteins, 4 proteins AbraP00219, AbraP00596,  
203 AbraP02566 and AbraP04899 could be candidates for Ago proteins and finally 3 proteins  
204 AbraP01134, AbraP04622 and AbraP08397 could be candidates for Rdrp. Except for Rdrp, these  
205 results were confirmed by the search of specific domains in the protein database of *A. brassicicola*  
206 using Prosite scan. Then, using this Prosite analysis, 2 proteins, AbraP02176 and AbraP09650,  
207 presented the Dcr specific domains and 4 proteins, AbraP00219, AbraP00596, AbraP02566 and  
208 AbraP04899, presented the specific piwi domain of Ago. By contrast, this method did not allow to  
209 identify Rdrp protein candidates.

210 To further characterize the Dcr, Ago and Rdrp proteins in *A. brassicicola*, phylogenetic analyses  
211 were carried out. The analysis of Dcr proteins phylogeny revealed a highly conserved evolution of  
212 these protein in the Ascomycota. Then, two Dcr proteins were identified in *A. brassicicola*:  
213 AbraP02176 and AbraP09650 that were determined as the homologs of Dcr1 and Dcr2, respectively  
214 (Figure 2). The same analysis using Ago proteins from *Ascomycota* and the 4 Ago candidates from  
215 *A. brassicicola* revealed a higher disparity in the phylogeny (complementary figure 1).  
216 AbraP00219, AbraP00596, AbraP02566 and AbraP04899 could not be assigned to a specific Ago  
217 due to low bootstrap values. However, we could confirm that *A. brassicicola* has 4 different Ago. In  
218 the same way, we could confirm that the three Rdrp candidates from *A. brassicicola* were Rdrp  
219 proteins, but it was not possible to identify Rdrp1 and Rdrp3 (complementary figure 2). However,  
220 AbraP04622 could be predicted as Rdrp2.

221 According to the protein domain comparison and the phylogenic analysis, *AbDCR*-deleted mutants  
222 were created. The RT-PCR (Complementary figure 4), carried out in order to control the deletion of  
223 *DCR* genes, showed that *DCR1* was expressed in the wild-type Abra43 strain and in *DCR2* deleted  
224 mutant and conversely *DCR2* was expressed in the wild-type Abra43 strain and in *DCR1* deleted  
225 mutant. As expected, no *DCR* genes were expressed in the double mutant (Complementary figure  
226 4).

227

### 228 ***Overview of the small RNA sequencing results***

229 Two small RNA libraries were constructed from fungal wild-type and Dcr-deleted mutants and  
230 sequenced. After removal of adapters and low-quality sequences, we obtained an average of  
231  $18'012'460 \pm 1'973'950$  reads per condition with an average quality score (Q30) of  $94.4 \pm 0.3$  %  
232 which represented  $14'611'200 \pm 1'484'632$  unique reads. Most of sequenced reads had between 17  
233 to 30 bp size long representing 95.3 % of unique reads. Within the small RNA libraries, coding  
234 RNAs, transfert RNAs and ribosomal RNAs, represented respectively 7 %, 5 % and 38 % of unique

235 reads while non-transcribed small RNAs (nt-sRNAs) represented 50 % of unique reads (Figure 3A).  
236 Small RNAs size being evaluated between 18 and 25 bp, the following analysis was executed on  
237 this size range. DESeq2 statistical analysis on small RNAs between 18 and 25 bp size long and  
238 identified as nt-sRNAs showed that a total of 2566 unique sequences were differentially represented  
239 according to *DCR1*, *DCR2* and double *DCR* deletion mutants (Figure 3B). In details, 36, 381 and  
240 827 nt-sRNA sequences were under-represented in *DCR1*, *DCR2* and double *DCR* deleted mutants,  
241 respectively. On the other hand, 618, 66 and 638 nt-sRNA sequences were over-represented in  
242 *DCR1*, *DCR2* and double *DCR* deleted mutants, respectively.

243 The evaluation of small RNAs length distribution according to the deletion gave an interesting  
244 result. Indeed, 18 and 19 bp size long small RNAs were more abundant in the *DCR2* and double  
245 *DCR* deleted mutants while higher size small RNAs, from 20 to 25 bp size long, were over-  
246 represented in wild-type and in *DCR1* deleted mutant (Figure 4A). The 5'-terminus nucleotide of  
247 small RNA was not influenced by dicer deletion (Figure 4B). Indeed, in the wild-type strain as in  
248 the simple and double mutants, the 5'-terminus nucleotide of small RNAs presented a light  
249 preference for the uridine with a mean proportion of  $31.8 \pm 1.4$  % of small RNAs whereas guanine,  
250 adenine and cytosine at the 5' end represented respectively  $24.8 \pm 0.3$  %,  $23.7 \pm 0.5$  % and  $19.7 \pm$   
251  $0.7$  % of small RNAs (Figure 4B).

252

### 253 ***Identification and characteristics of miRNAs in A. brassicicola***

254 The miRDeep2 algorithm allows the *in silico* identification of known and novel miRNAs by  
255 predicting RNA-hairpin precursors and calculating the energy of the secondary structure. The  
256 miRDeep2 software identified 17 novel putative miRNAs (Table 1) in the small RNAs population  
257 sequenced from *A. brassicicola* with a size between 18 and 21 bp length. Of these 17 miRNAs, 7  
258 highly probable miRNAs (AbmiRNA) presented a high sequencing coverage and 10 miRNA  
259 candidates (AbmiRNA-C) had a low sequencing coverage. Precursors of AbmiRNAs (Figure 5) and

260 AbmiRNA-C can form the characteristic secondary structure in hairpin.

261 Of the 17 putative AbmiRNAs only 6 were influenced by the separate or simultaneous Dcr deletion  
262 (Table 1). Interestingly, miRNAs influenced by *DCR1* deletion were over-represented while those  
263 influenced by *DCR2* were under-represented (Table1). Indeed, AbmiRNA-02 and AbmiRNA-04  
264 were respectively over-represented by a log<sub>2</sub> fold-change of 4.3 and 2.4 in the *DCR1* deleted  
265 mutant and by 3.7 and 1.8 respectively in the double mutant (Table 1). On the contrary, AbmiRNA-  
266 01, AbmiRNA-C5 and AbmiRNA-C8 were under-represented by a log<sub>2</sub> fold-change of -4.9, -3.7  
267 and -6.5 respectively in *DCR2* deleted mutant and by -6.2, -5.7 and -6.6 respectively in the double  
268 mutant (Table 1). AbmiRNA-C7 was an exception because it was only under-represented by a log<sub>2</sub>  
269 fold-change of -4.6 in the double mutant. Finally, AbmiRNA-03, AbmiRNA-05, AbmiRNA-06,  
270 AbmiRNA-07, AbmiRNA-C1, AbmiRNA-C2, AbmiRNA-C3, AbmiRNA-C4, AbmiRNA-C6,  
271 AbmiRNA-C9 and AbmiRNA-C10 seemed to be synthesized in a Dcr-independent pathway.

272 As no algorithm is currently available for the study of fungal miRNAs, target predictions were  
273 carried out using miRanda (written for the prediction of miRNAs binding site in animals) and  
274 psRNATarget (written for prediction in plants). Only the common targets predicted by these two  
275 softwares were retained.

276 For 16 miRNAs, at least one target binding site was identified in the *A. brassicicola* genome (Table  
277 2). Whereas AbmiRNA-C3 had no predicted target, AbmiRNA-05 had the largest number of  
278 putative targets with 8 predicted sites (Table 2). The majority of these miRNA targets included  
279 cytochrome P450, ion or sugar transporters, enzymes of the carbohydrate metabolism, ribosome  
280 constituent proteins and proteins involved in oxidation and desiccation resistance mechanisms  
281 (Table 2). Ten AbmiRNAs had predicted targets that could be involved in responses to either  
282 osmotic, oxidative or desiccation stresses. For example, deletion of *S. cerevisiae* homologs of  
283 AbraP01593 predicted as putative potassium transporter and of AbraP08633 predicted as  
284 carboxypeptidase B precursor induced decrease of desiccation resistance (Borovikova *et al.*, 2014;

285 Ratnakumar *et al.*, 2011).

286 Using the same strategy with *Arabidopsis thaliana* genome, a large number of putative target sites  
287 were predicted. Ten miRNAs had target sites in *A. thaliana*. Interestingly, a same miRNA, as  
288 AbmiRNA-01, AbmiRNA-04, AbmiRNA-06, AbmiRNA-07, AbmiRNA-C1, AbmiRNA-C2,  
289 AbmiRNA-C3, AbmiRNA-C5 and AbmiRNA-C8 presented binding site on several splice variants  
290 of the same gene (Table 2). In particular, 3 miRNAs (AbmiRNA-04, AbmiRNA-06 and AbmiRNA-  
291 C1) had targets of interest. AbmiRNA-04 targeted 5 splice variants of AT1G79580 coding for NAC  
292 domain transcriptional regulator superfamily protein (Table 2). NAC proteins are one of the largest  
293 classes of plant-specific transcriptional regulators and are important in both abiotic and biotic stress  
294 responses as well as in plant development regulation (Christianson *et al.*, 2010; Mondal *et al.*,  
295 2020). AbmiRNA-06 had 5 splice variants of AT1G01920 as targets, identified as a SET domain-  
296 containing protein (Table 2). This protein is involved in histone lysine methylation, an epigenetic  
297 regulatory mechanism (Herz *et al.*, 2013; Ng *et al.*, 2007). AbmiRNA-C1 targeted 3 splice variants  
298 of AT1G58400, identified as disease resistance protein presenting the CC-NBS-LRR pattern (Table  
299 2). This protein family recognized specific pathogen-derived products and initiated a resistance  
300 response (Belkhadir *et al.*, 2004; Moffet *et al.*, 2002).

301

## 302 **Discussion**

303 RNA interference is a cross-kingdom post-transcriptional regulation implicated in numerous  
304 pathways and influencing phenotypes or responses to environmental stimuli (Calkins *et al.*, 2018).  
305 RNAi components comprising Dcr proteins, Argonaute proteins and RNA-dependent RNA  
306 polymerases have been identified in various fungi belonging to Ascomycota, Basidiomycota and  
307 Zygomycota (Nakayashiki, 2005) and presented similarity with plant and animal components. Here,  
308 based on a blast and phylogeny analysis, 2 *DCR* genes (*AbDCR1* and *AbDCR2*), 4 *AGO* genes and 3  
309 *RDRP* genes were identified in the genome of the necrotrophic fungus *Alternaria brassicicola*. This

310 result is in accordance with other data reported in fungi that typically possess 1-3 Dcr, 1-4 Ago and  
311 1-4 Rdrp (Chang *et al.*, 2012), although some fungi, like the arbuscular mycorrhizal fungi  
312 *Rhizophagus irregularis*, possess a single Dcr protein but as much as 40 Ago-like and 21 putative  
313 Rdrp (Silvestri *et al.*, 2019). These putative proteins could be residues of ancestral duplications of  
314 *DCR*, *AGO* and *RDRP* genes. The presence of *A. brassicicola* genes coding for RNAi components  
315 supposed that miRNAs are synthesized in fungal cells (Chen *et al.*, 2014 ; Kang *et al.*, 2013 ; Zhou  
316 *et al.*, 2012). In the present study, we identified 17 miRNAs candidates in *A. brassicicola*  
317 representing 0.15 % of the whole sequencing reads. This result is consistent with the number of  
318 miRNA identified in other fungi. In *Penicillium chrysogenum*, *Trichoderma reesei* and *Sclerotinia*  
319 *sclerotiorum*, 42, 34 and 13 different miRNAs were respectively identified (Dahlmann and Kück,  
320 2015; Kang *et al.*, 2013; Zhou *et al.*, 2012). These numbers of fungal miRNAs represented a  
321 somewhat low number compared to the 893 miRNAs identified in *Brassica napus* (Shen *et al.*,  
322 2014) or the 326 sequences in *A. thaliana* gathered in the miRBase (<http://www.mirbase.org>). We  
323 could then supposed that the accumulation level of miRNAs in fungi is dramatically low and among  
324 these miRNAs only the most abundant were identified in previous study (Kang *et al.*, 2013; Zhou *et*  
325 *al.*, 2012).

326 Here among the 17 identified miRNAs, 2 were over-represented in *DCR1* deleted mutant and  
327 double mutant (AbmiRNA-02 and AbmiRNA-04) and 3 were underrepresented in *DCR2* deleted  
328 mutant and double mutant (AbmiRNA-01, AbmiRNA-C5 and AbmiRNA-C8). These results  
329 suggest that at least two distinct processes could contribute to the AbmiRNA synthesis, a Dcr  
330 dependent and a Dcr independent pathway. Lee *et al.* (2010) revealed the presence of pre-miRNA  
331 and mature miRNA in the *DCR*-deleted mutant of the fungal model *N. crassa* indicating the  
332 existence of Dcr independent miRNA synthesis pathway. Moreover, authors demonstrated also that  
333 the Qde-2 protein, an Ago-like protein, and a Mrp13 homolog of *S. cerevisiae*, a putative RNase III  
334 domain-containing protein, were required to produce both pre-miRNAs and miRNA in the Dcr



335 independent pathway. In *A. brassicicola* genome, AbraP00219 was found to be a *QDE-2* homolog  
336 with 34% of similarity. *QDE-2* is another name of *AGO2* in *N. crassa*. Both *Qde-2* and AbraP00219  
337 presented 5 protein domains: Argo N, Argo L1, PAZ, Argo L2 and piwi (Figure 1). In the same way,  
338 *A. brassicicola* is supposed to possess a *MRPL3* homolog coded by the AbraP11760 gene. The Pfam  
339 domain identification of these 2 proteins gave a putative RNase III domain and a Double stranded  
340 RNA-binding domain (Complementary figure 3). Then, proteins required for the miRNA synthesis  
341 in a Dcr independent pathway are potentially present in *A. brassicicola*. As other fungi presented  
342 evidence of the presence of Dcr independent miRNA synthesis pathway (Dahlmann and Kück,  
343 2015; Trieu *et al.*, 2015), it would be interesting to investigate the function of *Qde2* and *Mrpl3*  
344 homologs in *A. brassicicola*.

345 Using target prediction softwares, it appeared that *A. brassicicola* miRNAs could regulate some  
346 genes involved in several metabolic pathways of fungi and of the host plant (Table 2). Interestingly,  
347 we could hypothetise that *A. brassicicola* could be able to manipulate host metabolism and immune  
348 response either in a direct or indirect manner. Indeed, AbmiRNA-04 and AbmiRNA-C1 targeted  
349 respectively NAC domain transcriptional regulator superfamily protein and Disease resistance  
350 protein (CC-NBS-LRR class) family, two proteins involved in plant biotic stress resistance.  
351 Different studies demonstrated this phenomenon in fungal pathogen. For example, *Botrytis cinerea*,  
352 the causative agent of gray mold disease for more than 200 plant species, can deliver small RNAs in  
353 the host plant to hijack the RNAi machinery inducing the absence of plant response to *B. cinerea*  
354 infection (Baulcombe 2013; Wang *et al.*, 2018; Weiberg *et al.*, 2013). Then by targeting directly the  
355 plant immune system, *A. brassicicola* could have an advantage to easily colonize the host plant. *A.*  
356 *brassicicola* may also hijack the plant metabolism targeting the regulatory mechanisms. AbmiRNA-  
357 06 targeted 5 splice variants of AT1G01920 coding for a SET domain-containing protein often  
358 found in chromatin-associated complexes (Freitag, 2017). These SET proteins are involved in  
359 methylation of different lysines in histones 3 and 4 regulating chromatin conformation and

360 associated gene expression (Herz *et al.*, 2013). Berr *et al.* (2010) demonstrated the implication of  
361 SET domain histone methyltransferase in the induction of the Jasmonate/Ethylene pathway in the  
362 response of necrotrophic fungi infection. Then, by hijacking plant regulatory mechanisms, *A.*  
363 *brassicicola* can suppress or activate different metabolic pathway to favor the infection processes.

364

365 In conclusion, this study allowed to identify the main components of the RNAi mechanism in *A.*  
366 *brassicicola*. This pathogenic fungus possesses the whole machinery for the synthesis of miRNAs,  
367 i.e. 2 Dcr, 4 Ago and 3 Rdrp proteins. In addition, 17 miRNAs putatively involved in the cellular  
368 mechanisms of the fungus were identified in this paper. Some of these miRNAs targeted proteins  
369 involved in oxidation and dessication resistance mechanisms. They could be a regulatory pathway  
370 involved in the *A. brassicicola* virulence. Interestingly, some miRNAs could also target the cellular  
371 mechanisms of the host plants in order to favor the infection.

372

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518



519

520 **Figure captions :**

521

522 **Figure 1: Domains alignment for *Neurospora crassa* and *Alternaria brassicicola* Dcr proteins**

523 **(A), Ago proteins (B) and Rdrp proteins (C).** Alignments were achieved using Pfam 33.1

524 (<https://pfam.xfam.org/>).

525

526 **Figure 2: Phylogenetic tree of putative Dcr proteins from *Alternaria brassicicola* and Dcr**

527 **protein sequences from members of the Ascomycota.** Maximum likelihood tree was generated

528 using MEGA v10.1.8 under the LG +F model, 5 discrete gamma categories and 1000 bootstrap

529 repetitions. The tree was rooted using dicer1 from the basidiomycota *Pleurotus ostreatus*.

530

531 **Figure 3: smallRNAs sequencing analysis in *A. brassicicola* Abra43 isolate.** (A) Repartition of

532 sequenced smallRNAs according to non-transcript RNA database and coding RNAs. rRNA :

533 ribosomal RNA, tRNA : transfert RNA, snoRNA : small nuclear RNA, soRNA : soluble RNA and

534 nt-sRNA : non-transcript small RNA. (B) Number of smallRNAs under-represented and over-

535 represented in Dicer-deletion mutant compared to wild-type Abra43.

536

537 **Figure 4: Small RNA characteristics in the pathogenic fungus *A. brassicicola*.** (A) Repartition

538 of smallRNA size according to strains, Abra43 and the *DCR* deleted mutants. (B) 5'-terminus

539 nucleotide repartition in the smallRNA sequencing from Abra43 and *DCR* deleted mutants.

540

541 **Figure 5: Predicted secondary structures of putative *A. brassicicola* miRNAs.** Red letters

542 represented mature miRNA, yellow letters were terminal loops and blue or purple letters

543 represented consensus stars.

544

545 **Complementary figure 1: Phylogenetic tree of putative Ago proteins from *A. brassicicola* and**  
546 **Ago protein sequences from members of the Ascomycota.** Maximum likelihood tree was  
547 generated using MEGA v10.1.8 under the LG +F model, 5 discrete gamma categories and 1000  
548 bootstrap repetitions. The tree was rooted using Ago protein from the basidiomycota *Dichomitus*  
549 *squalens*.

550

551 **Complementary figure 2: Phylogenetic tree of putative Rdrp proteins from *A. brassicicola* and**  
552 **Rdrp protein sequences from members of the Ascomycota.** Maximum likelihood tree was  
553 generated using MEGA v10.1.8 under the LG +F model, 5 discrete gamma categories and 1000  
554 bootstrap repetitions. The tree was rooted using Rdrp protein from the basidiomycota *Serendipita*  
555 *indica*.

556

557 **Complementary figure 3: Domains alignment for *N. crassa* Mrp13 protein and *A. brassicicola***  
558 **AbraP11760 protein.** Alignments were achieved using Pfam 33.1 (<https://pfam.xfam.org/>).

559

560 **Complementary figure 4: Schematic representation of *DCR1* (A) and *DCR2* (B) loci (gray**  
561 **boxes),** disruption constructs with the HygB resistance gene (HPH) cassette (white boxes) and  
562 flanking sequences (black boxes). RT-PCR arrows represent the position PCR amplification regions  
563 used for PCR screening of deleted mutants. **RT-PCR to confirm the deletion of *DCR1* (A) and**  
564 ***DCR2* (B) genes in *A. brassicicola* Abra43 isolate and mutants.** RNAs were extracted from the  
565 samples used for the small RNA sequencing and retrotranscribed. Specific primers were used to  
566 amplify, respectively, a 347 bp and 293 bp sequences of the gene exons.

567

**Table 1:** Predicted miRNAs, characteristics and expression ratio in mutant compared to Abra43.

Stars represent the statistical significance: \* &lt; 0.05, \*\* &lt; 0.01 and \*\*\* &lt; 0.001.

Name	Sequence	Length	Genomic position	Expression ratio (mutant vs Abra43)								
				-8	-6	-4	-2	0	2	4	6	
AbmiRNA-01	UACAGAAGAAGAAGGAUAA	19	tig00000004_pilon:340077-340096		***	***						
AbmiRNA-02	AGCGGAAGCGCGCCGGGC	18	tig00000034_pilon:473399-473417							***	***	
AbmiRNA-03	GCACCUCCCCUGGAGCACC	19	tig00000029_pilon:1936159-1936178									
AbmiRNA-04	ACUCUUCUGUAGGCAGGCU	19	tig00000029_pilon:2432165-2432184							**	**	
AbmiRNA-05	UGGCGAUGCGGAUGAACU	18	tig00000029_pilon:896291-896309									
AbmiRNA-06	ACCAGGACUUUACUUUGU	19	tig00000006_pilon:92687-92706									
AbmiRNA-07	UUCUAGGCCGUACGCGCGCUA	21	tig00000029_pilon:2438164-2438185									
AbmiRNA-C1	UGCAUGUCUGGAGACGCCAUG	21	tig00000001_pilon:1293393-1293414									
AbmiRNA-C2	CAGGUACUCGUCAAGGAGC	19	tig00000016_pilon:2532350-2532368									
AbmiRNA-C3	UACAUGGUCUUUCUGUAGACG	21	tig00000016_pilon:2978393-2978414									
AbmiRNA-C4	UGUUCGUGUUCUGGAAAGACG	21	tig00000000_pilon:2285665-2285684									
AbmiRNA-C5	UCGGUCGCGAAGAACGCAGAC	21	tig00000016_pilon:2729861-2729882		*	*						
AbmiRNA-C6	UUGGUAUCCAUGGGGCA	18	tig00000025_pilon:135485-135503									
AbmiRNA-C7	UACUACGGCGGAGGAGCUCU	21	tig00000004_pilon:2323807-2323828							*		
AbmiRNA-C8	UUGCGCAUCUGGUCGAAGAUG	21	tig00000004_pilon:28824-28845		***	***						
AbmiRNA-C9	CUGAUGUCUUUGGAAGGA	18	tig00000025_pilon:159717-159735									
AbmiRNA-C10	GCGGAGAGUGUGGACAAA	18	tig00000029_pilon:20406-20424									

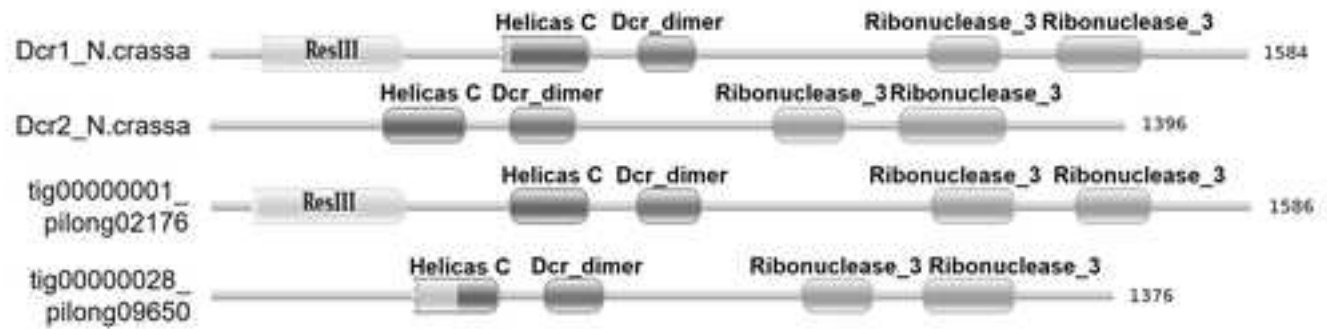
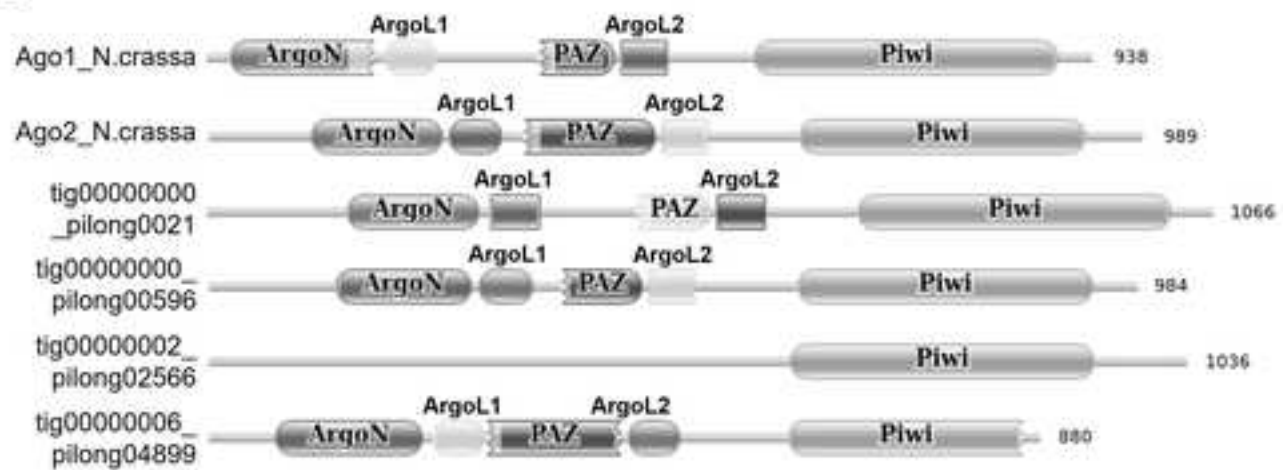
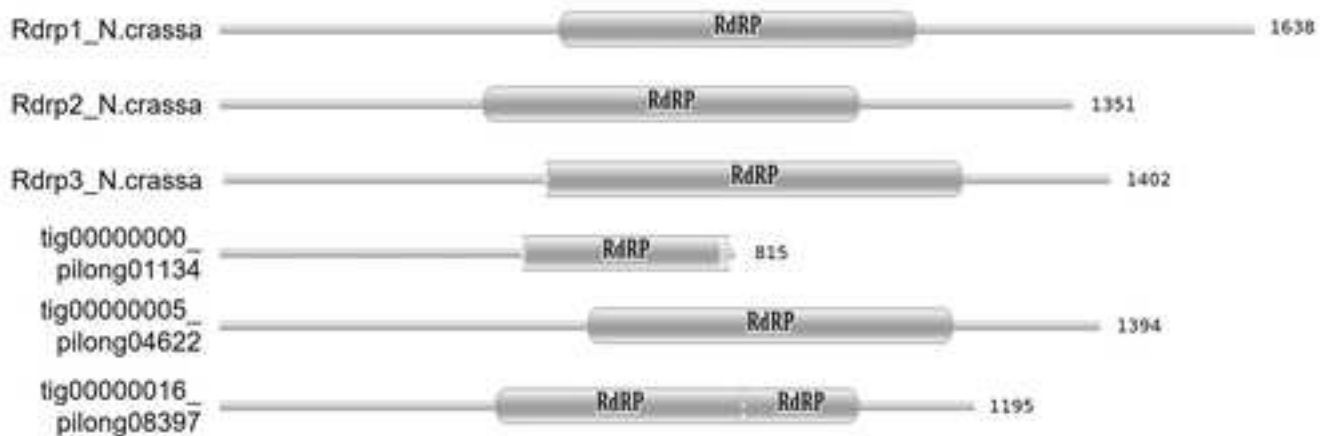
**Table 2:** Predicted miRNAs target genes in *A. brassicicola* and in *A. thaliana*. Prediction were achieved using miRanda v3,3 and psRNATarget software. Only common targets were represented.

miRNA	Targets in <i>Alternaria</i>		Targets in <i>Arabidopsis</i>	
	Genes	Identification	Genes	Identification
AbmiRNA-01	tig00000000_pilong00470	Cytochrome P450	AT1G02020.1 AT1G02020.2 AT1G02020.3 AT1G12640.1 AT1G13950.1 AT1G32361.1	Nitroreductase family protein Nitroreductase family protein Nitroreductase family protein MBOAT (membrane bound O-acyl transferase) family protein Eukaryotic elongation factor 5A-1 RING/U-box superfamily protein
AbmiRNA-02	tig00000001_pilong01593 tig00000001_pilong01882 tig00000006_pilong05145 tig00000007_pilong05448 tig00000008_pilong06792 tig00000025_pilong08633 tig00000028_pilong10008	Similar to potassium ion transporter Ubiquitin system component Cue Hypothetical protein PTT_20330 NADH dehydrogenase Carbohydrate esterase family 4 protein Carboxypeptidase B precursor Cytochrome P450 (Lanosterol 14-alpha-demethylase)		
AbmiRNA-03	tig00000025_pilong09044 tig00000028_pilong10019	Lactam utilization protein LamB Similar to RfeC	AT1G35630.1	Protease-associated (PA) RING/U-box zinc finger family protein
AbmiRNA-04	tig00000001_pilong01280 tig00000001_pilong01553 tig00000004_pilong03235 tig00000004_pilong04006 tig00000016_pilong07313 tig00000026_pilong09563 tig00000036_pilong12022	---NA--- Carboxylesterase type B Similar to thioredoxin Hypothetical protein PTT_13930 Glutamine-dependent NAD(+) synthetase synthase Acyl-CoA N-acyltransferase DNA repair protein Rad26	AT1G10840.1 AT1G10840.2 AT1G47770.1 AT1G68310.1 AT1G68310.2 AT1G79580.1 AT1G79580.2 AT1G79580.3 AT1G79580.4 AT1G79580.5	Translation initiation factor 3 subunit H1 Translation initiation factor 3 subunit H1 Beta-galactosidase related protein MIP18 family protein (DUF59) MIP18 family protein (DUF59) NAC (No Apical Meristem) domain transcriptional regulator superfamily protein NAC (No Apical Meristem) domain transcriptional regulator superfamily protein NAC (No Apical Meristem) domain transcriptional regulator superfamily protein NAC (No Apical Meristem) domain transcriptional regulator superfamily protein NAC (No Apical Meristem) domain transcriptional regulator superfamily protein
AbmiRNA-05	tig00000000_pilong00187 tig00000007_pilong05375 tig00000007_pilong05653 tig00000008_pilong06738 tig00000011_pilong07016 tig00000012_pilong07152 tig00000025_pilong08957 tig00000034_pilong11772	rhamnogalacturonan acetylerase Succinyl-CoA:3-ketoacid-coenzyme A transferase subunit A ---NA--- Hypothetical protein PTT_11535 Similar to catalase Similar to MFS sugar transporter Similar to actin-binding protein Similar to thiazole biosynthetic enzyme		



**Table 2: Continued**

AbmiRNA-C4	tig00000005_pilong04732 tig00000025_pilong08552 tig00000025_pilong08553 tig00000032_pilong11475	Conserved hypothetical protein Dolichyl-phosphate mannosyltransferase Pre-mRNA-splicing factor cwc22 Bicyclomycin resistance	
AbmiRNA-C5	tig00000001_pilong02093 tig00000002_pilong02565	Zinc finger RING-type protein SNARE domain-containing protein	AT3G19870.1 AP-5 complex subunit beta-like protein AT3G19870.1 AP-5 complex subunit beta-like protein AT3G19870.2 AP-5 complex subunit beta-like protein AT3G19870.2 AP-5 complex subunit beta-like protein AT3G57250.1 Emsy N Terminus (ENT) domain-containing protein
AbmiRNA-C6	tig00000004_pilong03919 tig00000008_pilong06264 tig00000023_pilong08508	Oxidoreductase Putative mitochondrial carrier protein pet8 protein Conserved hypothetical protein	
AbmiRNA-C7	tig00000004_pilong03160 tig00000028_pilong09753 tig00000029_pilong10266	---NA--- LSM domain-containing protein (mRNA regulation) ---NA---	
AbmiRNA-C8	tig00000007_pilong05831 tig00000008_pilong06756 tig00000016_pilong08209 tig00000025_pilong08658 tig00000029_pilong11167	Hypothetical protein SETTUDRAFT_112808 Ribosome control protein 1 Hypothetical protein PTT_13169 Ribosome biogenesis protein Pescadillo Predicted protein	AT3G05820.1 Invertase H AT3G05820.2 Invertase H AT5G08470.1 Peroxisome 1 AT5G08470.2 Peroxisome 1 AT5G08470.3 Peroxisome 1 AT5G44440.1 FAD-binding Berberine family protein AT5G44440.2 FAD-binding Berberine family protein
AbmiRNA-C9	tig00000028_pilong10086 tig00000032_pilong11324 tig00000036_pilong12169	Similar to 50S ribosomal protein L24 hypothetical protein SETTUDRAFT_151548 Similar to short chain dehydrogenase/reductase family protein	
AbmiRNA-C10	tig00000005_pilong04516 tig00000005_pilong04773 tig00000016_pilong07313 tig00000029_pilong11182	Conserved hypothetical protein Similar to 37S ribosomal protein Rsm22 Glutamine-dependent NAD(+) synthetase synthase Putative chromo domain-containing protein	

**A****B****C****Figure 1**

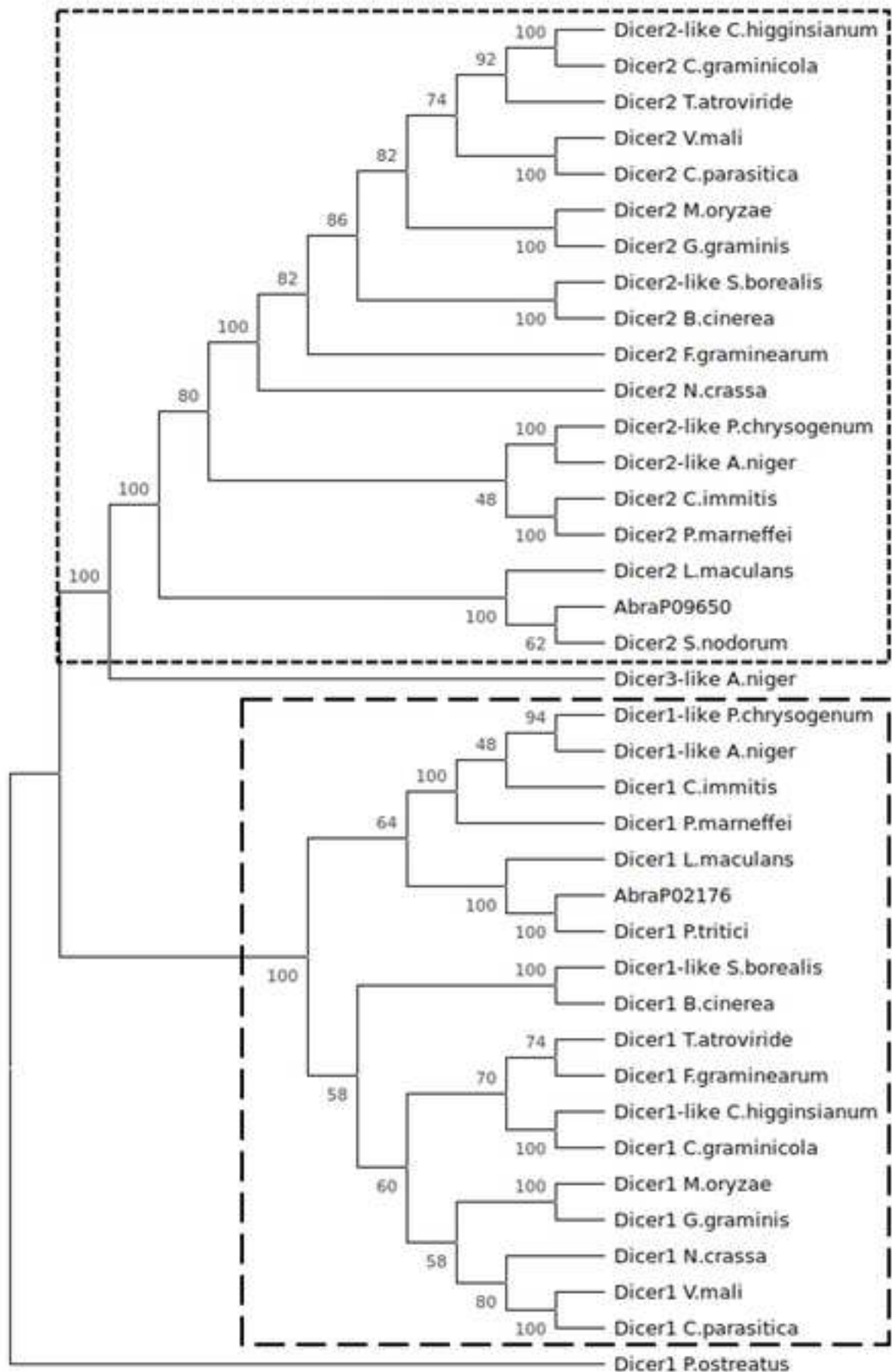


Figure 2



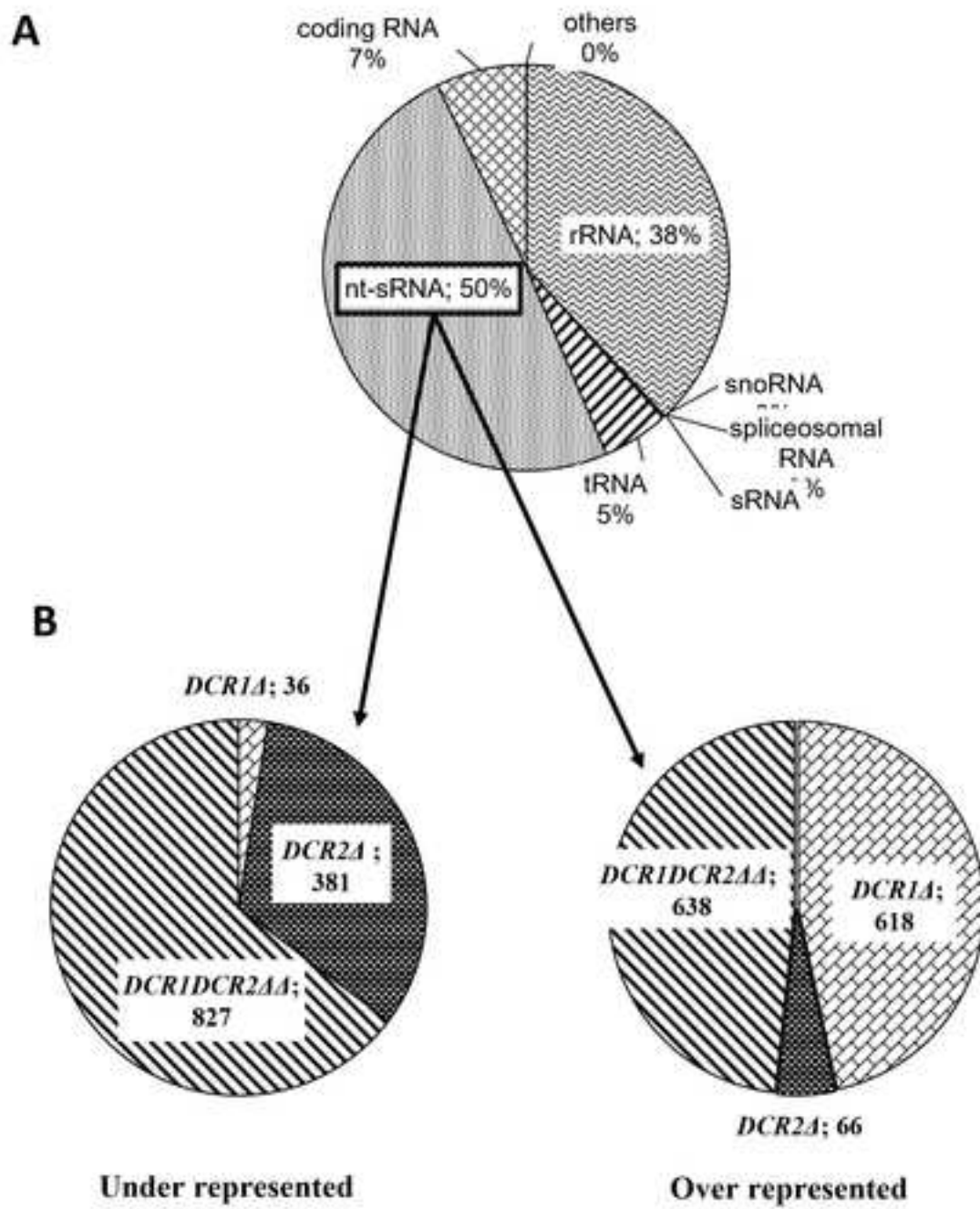


Figure 3

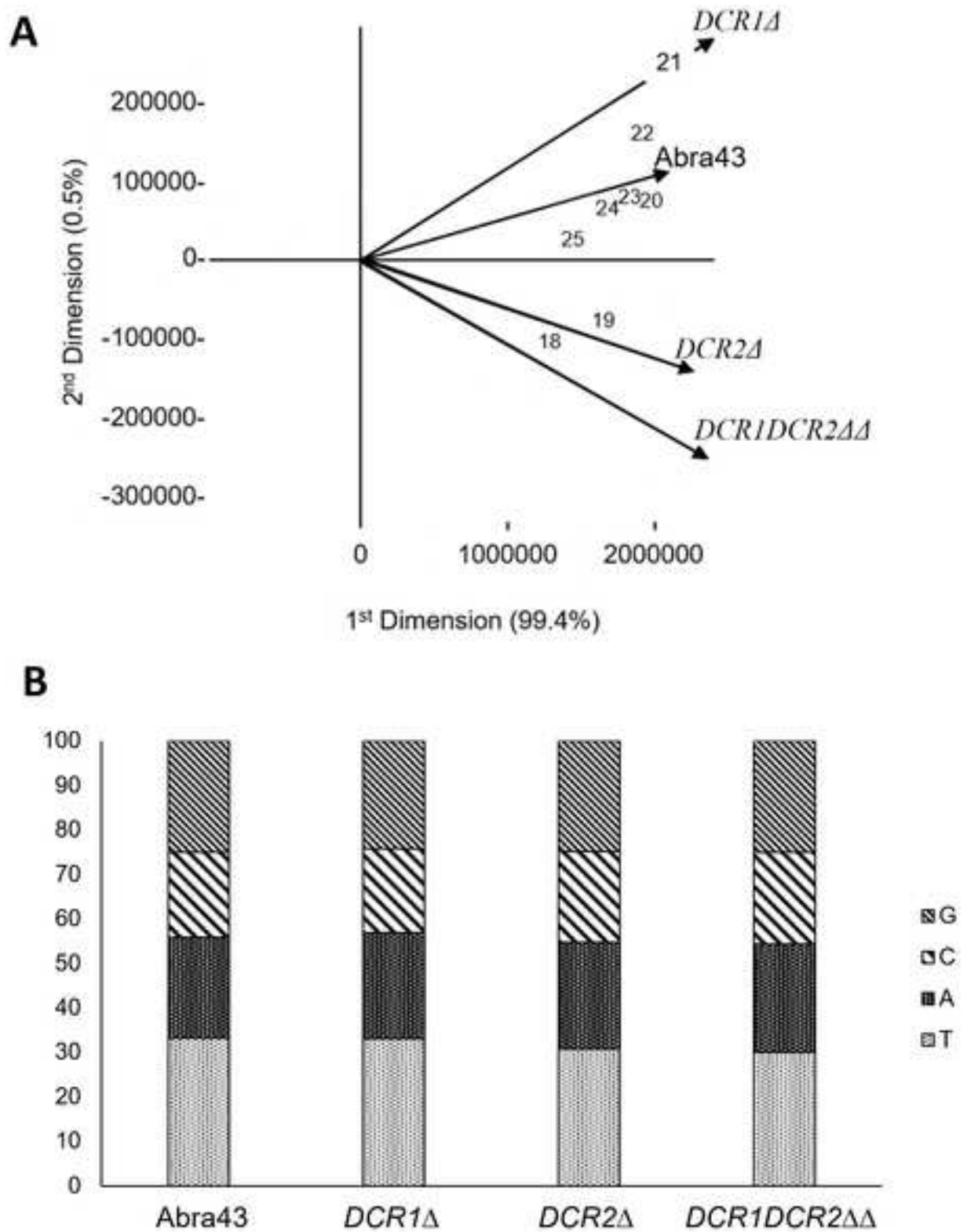
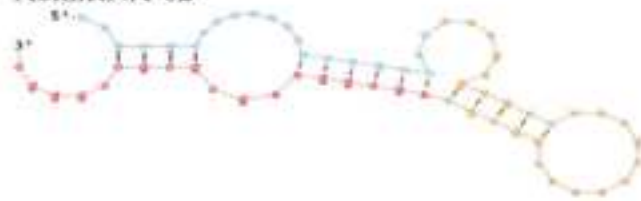


Figure 4

AbmiRNA-01



AbmiRNA-02



AbmiRNA-03



AbmiRNA-04



AbmiRNA-05



AbmiRNA-06



AbmiRNA-07

**Figure 5**

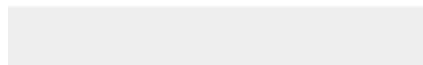




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**Supplementary File**

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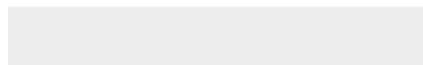




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