



HAL
open science

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

Anthony Kwasiborski, Franck Bastide, Bruno Hamon, Pascal Poupard,
Philippe Simoneau, Thomas Guillemette

► 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

HAL is a multi-disciplinary open access archive for the deposit and dissemination of scientific research documents, whether they are published or not. The documents may come from teaching and research institutions in France or abroad, or from public or private research centers.

L'archive ouverte pluridisciplinaire **HAL**, est destinée au dépôt et à la diffusion de documents scientifiques de niveau recherche, publiés ou non, émanant des établissements d'enseignement et de recherche français ou étrangers, des laboratoires publics ou privés.

Fungal Biology

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

--Manuscript Draft--

Manuscript Number:	FUNBIO-D-21-00092R1
Article Type:	Original Research
Section/Category:	Genomics, genetics and molecular genetics
Keywords:	Pathogen fungi <i>Alternaria brassicicola</i> smallRNA sequencing RNAi component miRNA identification
Corresponding Author:	Anthony Kwasiborski, Ph.D. Université Angers: Université d'Angers Beaucouzé, FRANCE
First Author:	Anthony Kwasiborski, Ph.D.
Order of Authors:	Anthony Kwasiborski, Ph.D. Franck Bastide Bruno Hamon Pascal Poupard, PhD Philippe Simoneau, PhD Thomas Guillemette, PhD
Abstract:	<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>

[Click here to view linked References](#)

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

3

4 Kwasiborski Anthony¹, Bastide Franck¹, Hamon Bruno¹, Poupard Pascal¹, Simoneau Philippe¹,
5 Guillemette Thomas^{1*}

6

7 ¹Univ Angers, Institut Agro, INRAE, *IRHS, SFR QUASAV*, F-49000 Angers, France

8

9 E-mail addresses:

10 Anthony.kwasiborski@gmail.com

11 Franck.bastide@univ-angers.fr

12 Bruno.hamon@univ-angers.fr

13 Pascal.poupard@univ-angers.fr

14 Philippe.simoneau@univ-angers.fr

15 Thomas.guillemette@univ-angers.fr

16

17 Corresponding author:

18 Dr Guillemette Thomas

19 UMR 1345 IRHS, équipe FungiSem

20 42 rue Georges Morel 49 071 Beaucouzé, France

21 Thomas.guillemenette@univ-angers.fr

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 10⁴ 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⁻¹ 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 ± 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 ± 1.4 % of small RNAs whereas guanine,
250 adenine and cytosine at the 5' end represented respectively 24.8 ± 0.3 %, 23.7 ± 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 log₂ 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 log₂ 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 log₂
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

373 **Bibliography**

374

375 An J, Lai J, Lehman ML, Nelson CC, 2013. miRDeep*: an integrated application tool for miRNA
376 identification from RNA sequencing data. *Nucleic Acids Research* **41**(2): 727–737.

377 Baker KF, Smith SH, 1966. Dynamics of seed transmission of plant pathogens. *Annual Review of*
378 *Phytopathology* **14**:311–332.

379 Baulcombe D, 2013. Small RNA-the secret of noble rot. *Science* **342**(6154): 45-46.

380 Belkhadir Y, Subramaniam R and Dangl JL, 2004. Plant disease resistance protein signaling: NBS-
381 LRR proteins and their partners. *Current Opinion in Plant Biology* **7**: 391-399.

382 Belmas E, Briand M, Kwasiborski A, Colou J, N’Guyen G, Iacomi B, Grappin P, Campion C,
383 Simoneau P, Barret M and Guillemette T, 2018. Genome sequence of the necrotrophic plant
384 pathogen *Alternaria brassicicola* Abra43. *Genome Announcement* **6**: e01559-17.

385 Berr A, McCallum EJ, Alioua A, Heintz D, Heitz T and Shen W-H, 2010. Arabidopsis Histone
386 Methyltransferase SET DOMAIN GROUP8 Mediates Induction of the Jasmonate/Ethylene
387 Pathway Genes in Plant Defense Response to Necrotrophic Fungi. *Plant Physiology* **154**: 1403-
388 1414.

389 Borovikova D, Herynkova P, Rapoport A and Sychrova H, 2014. Potassium uptake system Trk2 is
390 crucial for yeast cell viability during anhydrobiosis. *FEMS Microbiology Letters* **350**(1):28-33.

391 Cai Q, Qiao L, Wang M, He B, Lin FM, Palmquist J, 2018. Plants send small RNAs in
392 extracellular vesicles to fungal pathogen to silence virulence genes. *Science* **360**(6393):1126–9.

393 Calkins SS, Elledge NC, Mueller KE, Marek SM, Couger MB, Elshahed MS and Youssef NH,
394 2018. Development of an RNA interference (RNAi) gene knockdown protocol in the anaerobic
395 gut fungus *Pecoramyces ruminantium* strain C1A. *PeerJ* **6**:e4276; DOI 10.7717/peerj.4276.

396 Campo S, Gilbert KB, Carrington JC, 2016. Small RNA-Based Antiviral Defense in the
397 Phytopathogenic Fungus *Colletotrichum higginsianum*. *Plos Pathogens* **12**(6):

398 e1005640.doi:10.1371/journal.ppat.1005640.

399 Carreras-Villaseñor N, Esquivel-Naranjo EU, Villalobos-Escobedo JM, Abreu-Goodger C,
400 Herrera-Estrella A, 2013. The RNAi machinery regulates growth and development in the
401 filamentous fungus *Trichoderma atroviride*. *Molecular Microbiology* **89**: 96–112. doi:
402 10.1111/mmi.12261 PMID: 23672609.

403 Chang SS, Zhang Z, Liu Y, 2012. RNA Interference Pathways in Fungi: Mechanisms and
404 Functions *Annual Review of Microbiology* **66**:305-323.

405 Chen R, Jiang N, Jiang Q, Sun X, Wang Y, Zhang H and Hu Z, 2014. Exploring microRNA-like
406 small RNAs in the filamentous fungus *Fusarium oxysporum*. *PLoS ONE* **9**(8): e104956.
407 doi:10.1371/journal.pone.0104956.

408 Cho Y, Davis JW, Kim KH, Wang J, Sun QH, Cramer RA and Lawrence CB, 2006. A high
409 throughput targeted gene disruption method for *Alternaria brassicicola* functional genomics
410 using linear minimal element (LME) constructs. *Molecular Plant-Microbe Interactions Journal*
411 **19**(1): 7-15.

412 Cho Y, Cramer RA, Kim KH, Davis J, Mitchell TK, Figuli P, Pryor BM, Lemasters E, Lawrence
413 CB, 2007. The Fus3/Kss1 MAP kinase homolog Amk1 regulates the expression of genes
414 encoding hydrolytic enzymes in *Alternaria brassicicola*. *Fungal Genetic and Biology* **44**:543–
415 553.

416 Christianson JA, Dennis ES, Llewellyn DJ and Wilson IW, 2010. ATAF NAC transcription factors:
417 Regulators of plant stress signaling. *Plant Signaling & Behavior* **5**:4, 428-432.

418 Dahlmann TA and Kück U, 2015. Dicer-dependent biogenesis of small RNAs and evidence for
419 microRNA-like RNAs in the penicillin producing fungus *Penicillium chrysogenum*. *PLoS ONE*
420 **10**(5): e0125989. doi:10.1371/journal.pone.0125989.

421 Edgar RC, 2004. MUSCLE: multiple sequence alignment with high accuracy and high throughput.
422 *Nucleic Acids Research* **32**: 1792–1797.

423 El-Gebali S, Mistry J, Bateman A, Eddy SR, Luciani A, Potter SC, Qureshi M, Richardson LJ,
424 Salazar GA, Smart A, Sonnhammer ELL, Hirsh L, Paladin L, Piovesan D, Tosatto SCE RD, The
425 Pfam protein families database in 2019. *Nucleic Acids Research* 2019 doi:
426 10.1093/nar/gky995.

427 Elmer WH, 2001. Seeds as vehicles for pathogen importation. *Biological Invasions* **3**:263–271.

428 Freitag M, 2017. Histone methylation by SET domain proteins in fungi. *Annual Review of*
429 *Microbiology* **71**:413-439.

430 Gardner PP, Daub J, Tate JG, Nawrocki EP, Kolbe DL, Lindgreen S, Wilkinson AC, Finn RD,
431 Griffiths-Jones S, Eddy SR and Bateman A, 2009. Rfam : updates to the RNA families database.
432 *Nucleic Acids Research* **37**: 136-140.

433 Herz HM, Garuss A and Shilatifard A, 2013. SET for life: biochemical activities and biological
434 functions of SET domain-containing proteins. *Cell* **38**(12) ; 621-639.

435 Kang K, Zhong J, Jiang L, Liu G, Gou CY, *et al.*, 2013. Identification of microRNA-Like RNAs in
436 the Filamentous Fungus *Trichoderma reesei* by Solexa Sequencing. *PLoS ONE* **8**(10): e76288.
437 doi:10.1371/journal.pone.0076288.

438 Kumar S, Stecher G, Li M, Knyaz C, and Tamura K, 2018. MEGA X: Molecular Evolutionary
439 Genetics Analysis across computing platforms. *Molecular Biology and Evolution* **35**:1547-1549.

440 Lawrence CB, Mitchell TK, Craven KD, Cho Y-R, Cramer RA, Kim K-H, 2008. At death's door:
441 *Alternaria* pathogenicity mechanisms. *Plant Pathology Journal*. 24:101–111.

442 Lee HC, Li L, Gu W, Xue Z, Crosthwaite SK, Pertsemlidis A, Lewis ZA, Freitag M, Selker EU,
443 Mello CC and Liu Y, 2010. Diverse pathways generate microRNA-like RNAs and Dicer-
444 independent small interfering RNAs in Fungi. *Molecular Cell* **38**:803-814.

445 Love MI, Huber W, Anders S, 2014. Moderated estimation of fold change and dispersion for
446 RNA-seq data with DESeq2. *Genome Biology* **15**: 550.

447 Lyer LM, Koonin EV, Aravind L, 2003. Evolutionary connection between the catalytic subunits of

448 DNA-dependent RNA polymerases and eukaryotic RNA-dependent RNA polymerases and the
449 origin of RNA polymerases. *BMC Structural Biology* **3**:1-23.

450 McCarthy DJ, Chen Y, Smyth GK, 2012. Differential expression analysis of multifactor RNA-Seq
451 experiments with respect to biological variation. *Nucleic Acids Research*,**40**(10): 4288-4297.

452 MacRae IJ, 2006. Structural basis for double-stranded RNA processing by dicer. *Science* **311**:195-
453 198.

454 Malonek S, Rojas MC, Hedden P, Gaskin P, Hopkins P, Tudzynski B, 2004. The NADPH-
455 cytochrome P450 Reductase gene from *Gibberella fujikuroi* is essential for gibberellin
456 biosynthesis. *Journal of Biological Chemistry* **279**:25075–84.

457 Meena MM, Gupta SK, Swapnil P, Zehra A, Dubey MK, Upadhyay RS, 2017. *Alternaria* toxins:
458 potential virulence factors and genes related to pathogenesis. *Front Microbiol* **8**:1451.

459 Moffet P, Farnham G, Peart J and Baulcombe DC, 2002. Interaction between domains of a plant
460 NBS-LRR protein in disease resistance-related cell death. *The EMBO Journal* **21**(17): 4511-
461 4519.

462 Mondal B, Mazumder M, Mukherjee A, Ghosh S, De A, Bose R and Basu D, 2020. Association of
463 *Alternaria barssicicola* induced NAC transcription factors with desiccation and wound responses
464 in Indian mustard. *Physiological and Molecular Plant Pathology* **112**: 101540.

465 Nakayashiki H, 2005. RNA silencing in fungi: Mechanisms and applications. *FEBS Letters* **579**:
466 5950–5957.

467 Ng D WK, Wang T, Chandrasekharan MB, Aramayo R, Kertbundit S and Hall TC, 2007. Plant
468 SET domain-containing proteins: Structure, function and regulation. *Biochimica et Biophysica*
469 *Acta* **1769**: 316-329.

470 Pedras MS, Yaya EE and Glawischnig E, 2011. The phytoalexins from cultivated and wild
471 crucifers: chemistry and biology. *Natural Product Reports* **28**: 1381-1405.

472 Pochon S, Simoneau P, Pigné S, Balidas S, Bataillé-Simoneau N, Champion C, et al., 2013.

473 Dehydrin-like proteins in the necrotrophic fungus *Alternaria brassicicola* have a role in plant
474 pathogenesis and stress response. *PLoS One* **8**:e75143. doi: 10.1371/journal.pone.0075143.

475 Rajwanshi R, Devi KJ, Sharma GR and Lal B, 2019. Role of miRNAs in plant-microbe
476 interaction. In: in vitro plant breeding towards novel agronomic traits, pp. 167-195.

477 Ratnakumar S, Hesketh A, Gkargkas K, Wilson M, Rash BM, Hayes A, Tunnacliffe A and Oliver
478 SG, 2011. Phenomic and transcriptomic analyses reveal that autophagy plays a major role in
479 desiccation tolerance in *Saccharomyces cerevisiae*. *Molecular Biosystems* **7**(1):139-49.

480 Segers GC, Zhang X, Deng F, Sun Q, Nuss DL, 2007. Evidence that RNA silencing functions as
481 an antiviral defense mechanism in fungi. *Proceedings of the National Academy of Sciences USA*
482 **104**: 12902–12906. PMID: 7646660.

483 Sellam A, Iacomi-Vasilescu B, Hudhomme P, and Simoneau P, 2007. In vitro antifungal activity of
484 brassinin, camalexin and two isothiocyanates against the crucifer pathogens *Alternaria*
485 *brassicicola* and *Alternaria brassicae*. *Plant Pathology* **56**: 296-301.

486 Shen D, Suhrkamp I, Wang Y, Liu S, Menkhaus J, Verreet JA, Fan L, Cai D, 2014. Identification
487 and characterization of microRNAs in oilseed rape (*Brassica napus*) responsive to infection with
488 the pathogenic fungus *Verticillium longisporum* using Brassica AA (*Brassica rapa*) and CC
489 (*Brassica oleracea*) as reference genomes. *New Phytologist* **204**: 577–594.

490 Silvestri A, Fiorilli V, Miozzi L, Accotto GP, Turina M, Lanfranco L, 2019. *In silico* analysis of
491 fungal small RNA accumulation reveals putative plant mRNA targets in the symbiosis between
492 an arbuscularmycorrhizal fungus and its host plant. *BMC Genomics* **20**:169-187.

493 Sweigard JA, Carroll AM, Kang S, Farrall L, Chumley FG and Valent B, 1995. Identification
494 cloning and characterization of PWL2, a gene for host species specificity in the rice blast fungus.
495 *Plant Cell* **7**: 1221–1233.

496 Torres-Martinez S, Ruiz-Vasquez RM, 2017. The RNAi universe in fungi : a varied landscape of
497 small RNAs and biological functions. *Annual Review of Microbiology* **1**:371-391.

498 Trieu TA, Calo S, Nicolás FE, Vila A, Moxon S, Dalmay T, Torres-Martinez S, Garre V and Ruiz-
499 Vasquez RM, 2015. A non-canonical RNA silencing pathway promotes mRNA degradation in
500 basal fungi. *PLoS Genetics* **11**(4): e1005168.doi:10.1371/journal.pgen.1005168.

501 Wang Q, An B, Hou X, Guo Y, Luo H and He C, 2018. Dicer-like Proteins Regulate the Growth,
502 Conidiation, and Pathogenicity of *Colletotrichum gloeosporioides* from *Hevea brasiliensis*.
503 *Frontiers in Microbiology* **8**:2621.doi: 10.3389/fmicb.2017.02621.

504 Weiberg A, Wang M, Lin F-M, Zhao H, Zhang Z, Kaloshian I, Huang HD and Jin H, 2013. Fungal
505 Small RNAs Suppress Plant Immunity by Hijacking Host RNA Interference Pathways. *Science*
506 **342**: 118–123. doi: 10.1126/science.1239705 PMID: 24092744.

507 Yu JH, Hamari Z, Han KH, Seo JA, Reyes-Dominguez Y and Scazzocchio C, 2004. Double-Joint
508 PCR: a PCR-based molecular tool for gene manipulations in filamentous fungi. *Fungal Genetics*
509 *and Biology* **41**: 973–981.

510 Zamore PD, Tuschl T, Sharp PA and Bartel DP, 2000. RNAi: Double-Stranded RNA Directs the
511 ATP-Dependent Cleavage of mRNA at 21 to 23 Nucleotide Intervals. *Cell* **101**:25-33.

512 Zhou J, Fu Y, Xie J, Li B, Jiang D, Li G and Cheng J, 2012. Identification of microRNA-like
513 RNAs in a plant pathogenic fungus *Sclerotinia sclerotiorum* by high-throughput sequencing.
514 *Molecular Genetics and Genomics* **287**:275-282.

515 Zhou Q, Wang Z, Zhang J, Meng H and Huang B, 2012. Genome-wide identification and profiling
516 of microRNA-like RNAs from *Metarhizium anisopliae* during development. *Fungal Biology*
517 **116**:1156-1162

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* Mrpl3 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: * < 0.05, ** < 0.01 and *** < 0.001.

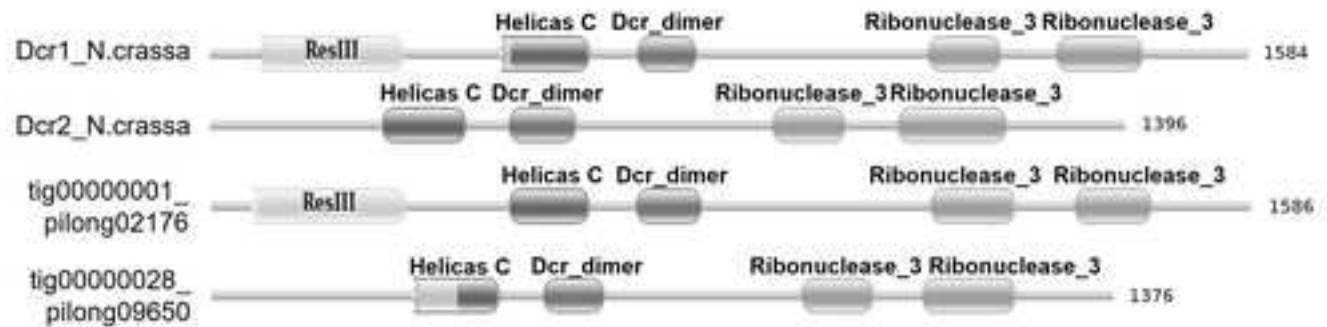
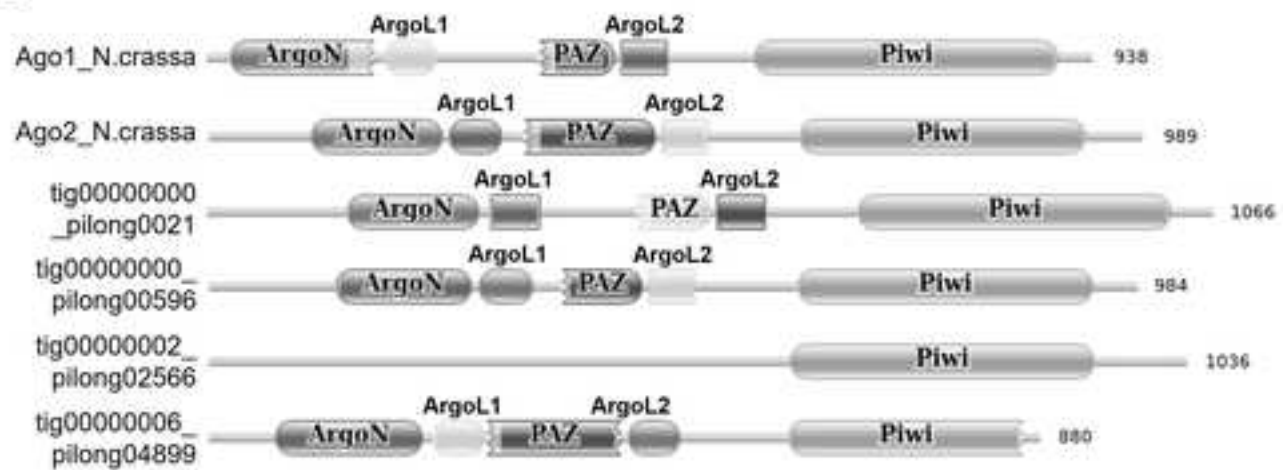
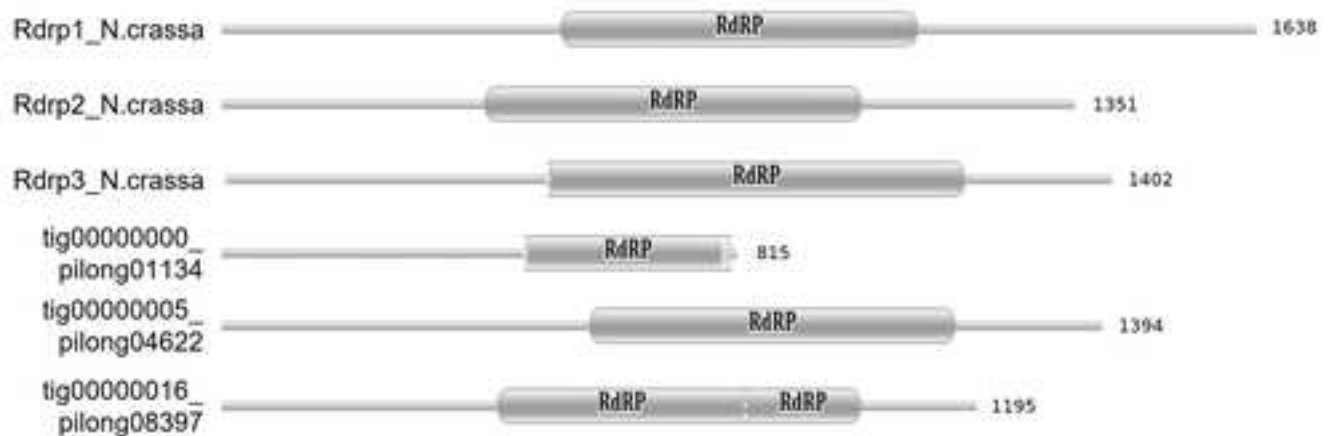
Name	Sequence	Length	Genomic position	Expression ratio (mutant vs Abra43)								
				-8	-6	-4	-2	0	2	4	6	
AbmiRNA-01	UACAGAAGAAGAAGGAUAA	19	tig00000004_pilon:340077-340096		***	***						
AbmiRNA-02	AGCGGAAGCGCGCCGGGC	18	tig00000034_pilon:473399-473417							***	***	
AbmiRNA-03	GCACCUCCCCUGGAGCACC	19	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**

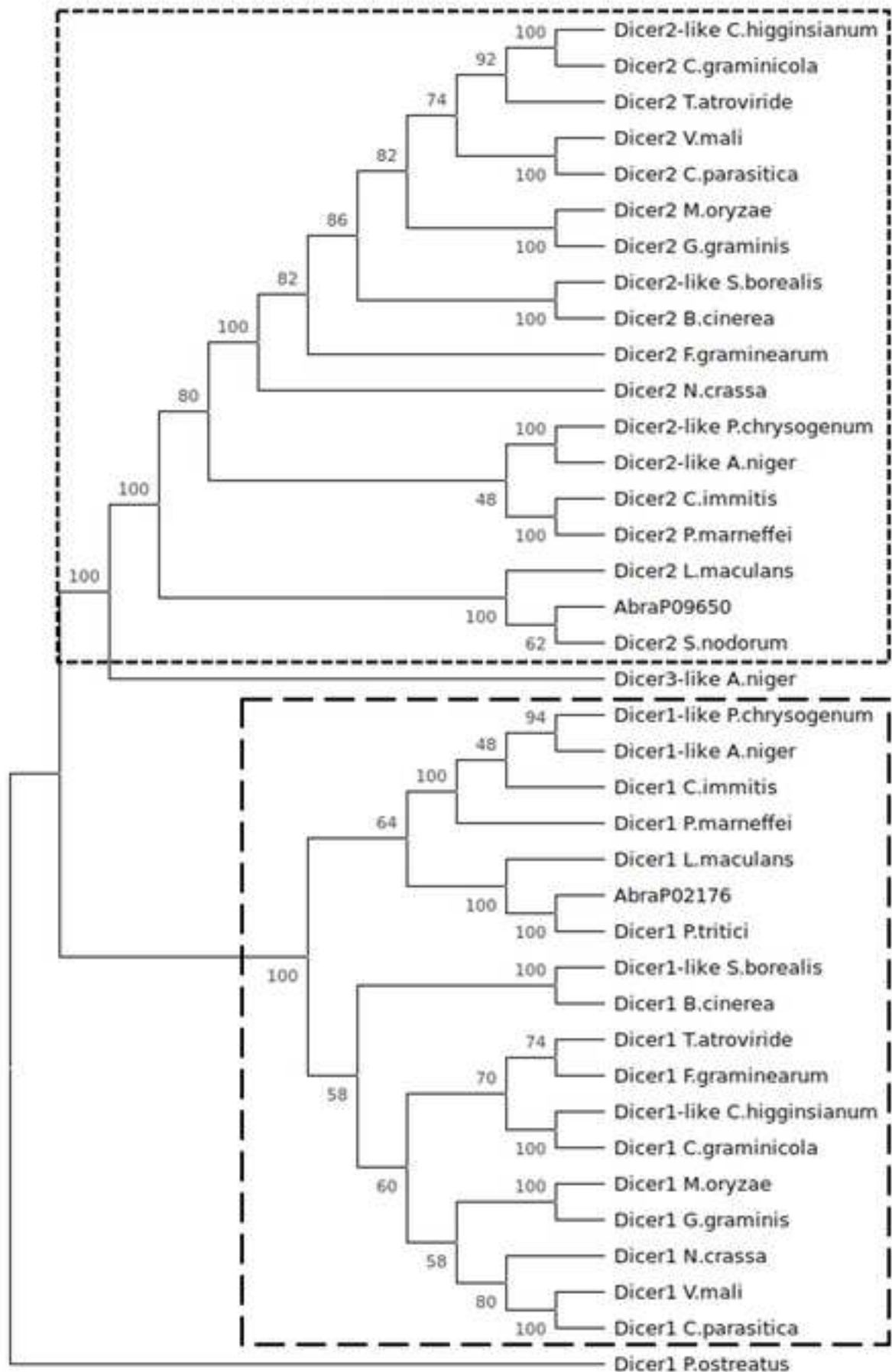


Figure 2

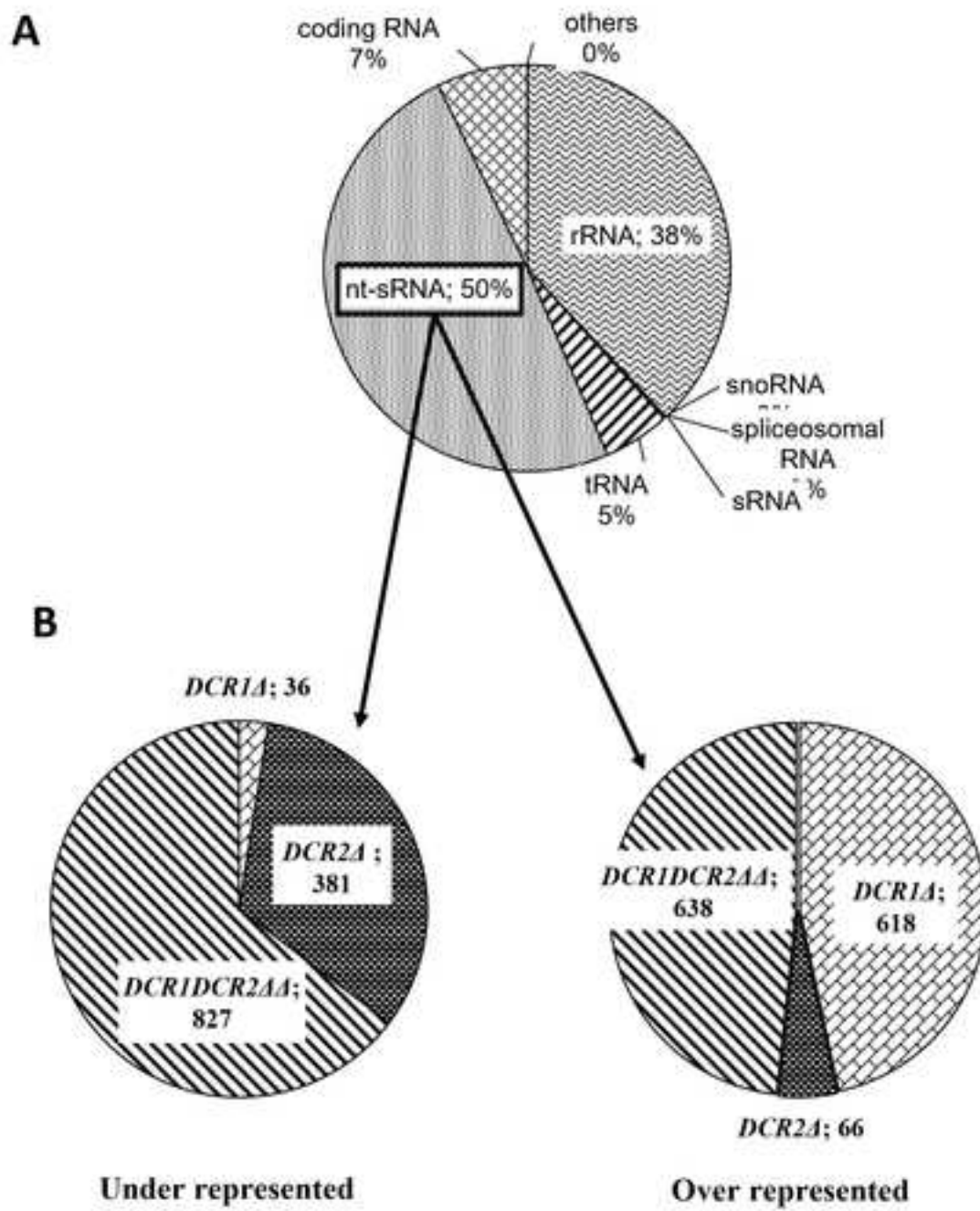


Figure 3

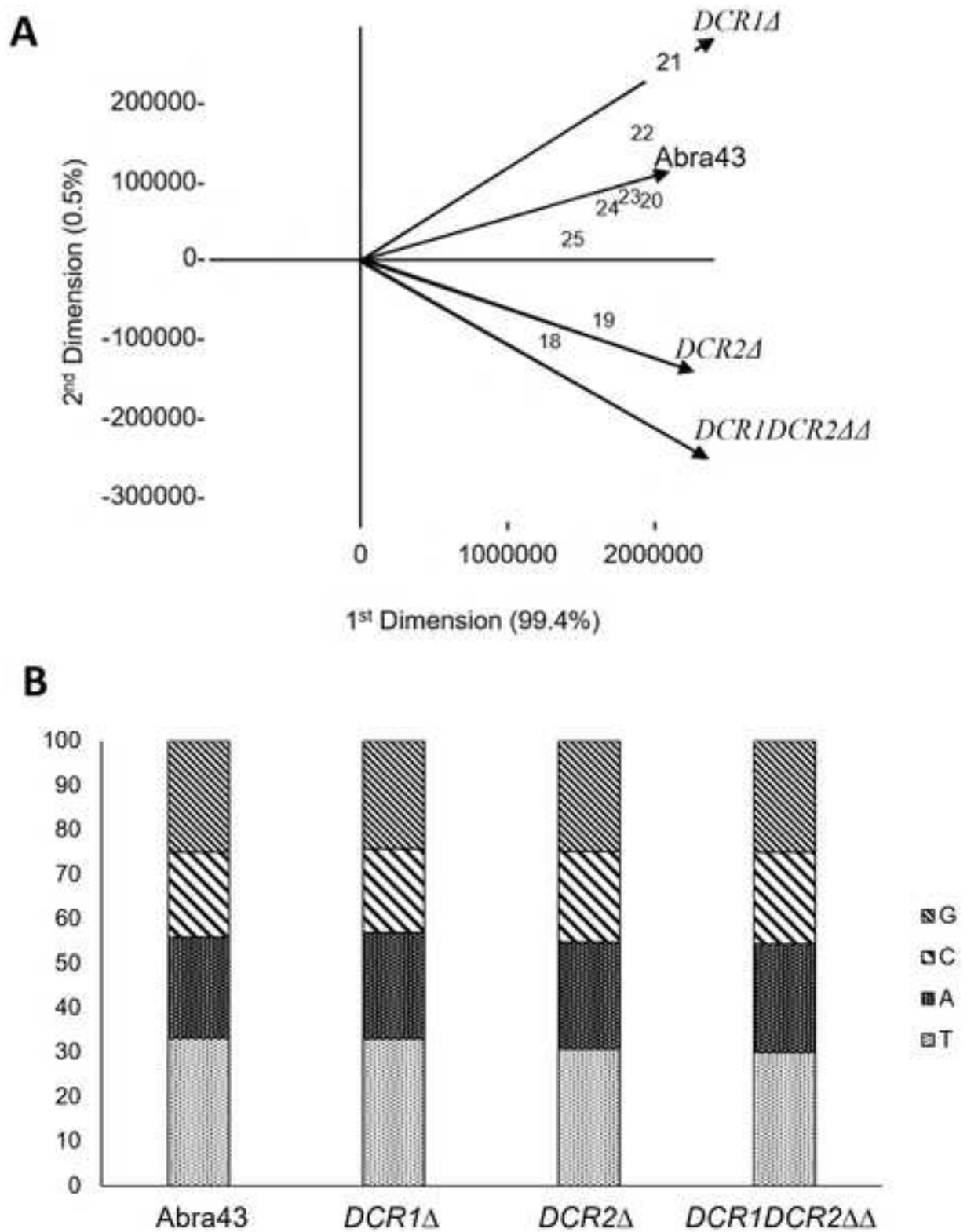


Figure 4

AbmiRNA-01



AbmiRNA-02



AbmiRNA-03



AbmiRNA-04



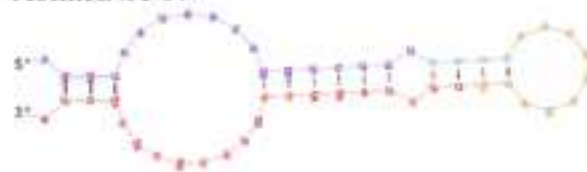
AbmiRNA-05



AbmiRNA-06



AbmiRNA-07

**Figure 5**

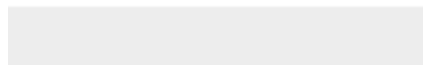




Click here to access/download

Supplementary File

Corr_Complementary_Table-1.pdf

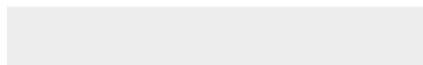




Click here to access/download

Supplementary File

Corr_Complementary_Table-2.pdf

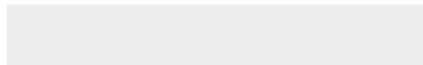




Click here to access/download

Supplementary File

Corr_Complementary_Table-3.pdf

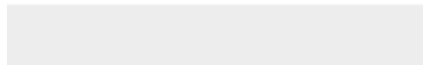




Click here to access/download

Supplementary File

Corr_Complementary_Table-4.pdf







Click here to access/download
Supplementary File
Complementary_Fig2.jpg



