ISSN 0026-2617, Microbiology, 2014, Vol. 83, No. 1–2, pp. 140–148. © Pleiades Publishing, Ltd., 2014.

EXPERIMENTAL ARTICLES

Investigating the Role of Dicer 2 (*dcr2*) in Gene Silencing and the Regulation of Mycoviruses in *Botrytis cinerea*[1]

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Received May 16, 2013

Abstract—*Botrytis cinerea*, the fungus causing gray mould disease, is usually controlled by cultural and chemical methods. It would be interesting to see if mycoviruses were a feasible method for reducing fungal virulence thus controlling the disease, but first more has to be understood of the RNA silencing mechanism and whether mycoviruses can combat such defences. Analysis of the *B. cinerea* genome data identified two Dicer genes: *dcr1* and *dcr2*. In other fungi, mutation or deletion of *dcr2* usually leads to impaired gene silencing. Targeted gene disruption created two independent *B. cinerea* Δ*dcr2* mutants in a ku70 background. When the Δ*dcr2* mutants were transformed with an argininosuccinate synthetase (*bcass1*) silencing cassette, many of these transformants displayed arginine auxotrophy, suggesting that silencing was still functional in a Δ*dcr2* mutant. Transfection of the wild-type and *dcr2*-disrupted *B. cinerea* lines with *Botrytis* virus F (BVF) gave no readily detectable alteration in fungal growth rate or virulence. Expression of *dcr2*, but not *dcr1*, was suppressed in the wild-type at 7 days post infection with BVF, whereas in a Δ*dcr2* mutant, *dcr1* expression was suppressed. By 28 days post BVF-infection, *dcr1* and *dcr2* were expressed to the elevated levels typically observed when gene silencing is induced. This shows that whilst *dcr2* is not essential for gene silencing or for controlling mycovirus such as BVF, it would appear that the mycovirus BVF is able to suppress the normal expression of genes involved in the silencing pathway, at least during early stages of infection of *B. cinerea*.

Keywords: gene-silencing, mycovirus, *Botrytis cinerea*, dicer

DOI: 10.1134/S0026261714020180

The haploid fungus *Botrytis cinerea*, teleomorph: *Botryotinia fuckeliana* (de Bary Whetzel) is the causal organism of gray mould disease. It is an airborne plant pathogen with a necrotrophic lifestyle attacking over two hundred crop hosts worldwide including a large number of economically important vegetable, flower and fruit crops [1]. Much of the economic loss due to the pathogen is in the reduction in the quality of the crop rather than in the overall yield, however these are usually high-value crops where visual appearance is very important to the consumer, which means that even low levels of infection may not be tolerated. The use of chemical control is the primary way to protect against *B. cinerea* but this has some economical and environmental drawbacks. Crop management practices to reduce the incidence *B. cinerea* usually aim to eliminate conditions that promote fungal growth, such as poor ventilation or high humidity. The use of biological control agents (BCA) to further control *B. cinerea* is an appealing alternative to chemicals, since BCA are in most cases, safer for the environment and have less likelihood for the development of resistance [2].

One of the most successful demonstrations of biological control of a fungal plant pathogen has been against the chestnut blight fungus, *Cryphonectria parasitica*, where use of the CHV1 mycovirus conferred hypovirulence to the fungus, weakening the fungus and allowing the chestnut tree to successfully combat infection [3]. The success of the hypovirulence mycovirus in regulating the effect of *C. parasitica* has created interest in using mycoviruses in an attempt to control the virulence of *B. cinerea*. If mycoviruses are ever to be used as part of control for *B. cinerea*, more understanding is needed about the RNA silencing mechanism in *B. cinerea* [4].

The RNA silencing mechanism acts as a cellular defence system to protect against RNA elements, both of natural origin such as viruses and transposable elements, or exotic structures such as artificial hairpin structure [5]. The mechanism is initiated by Dicer, a protein which recognises dsRNA molecules and cleaves them into short interfering RNA (siRNA) and microRNA (miRNA) of 21–25 bp in size [6]. If the RNA entities that enter the host cytoplasm are single

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stranded (e.g. viruses), then endogenous or virus-encoded RNA dependent RNA polymerase (RdRp) creates dsRNA molecules. When activated, this silencing system has the potential to reduce the titre or even eliminate viral RNA elements from the cytoplasm. This means it could impact on the ability of a mycovirus to replicate and hence its ability to cause hypovirulence in a plant pathogenic fungus [7].

In plants, some viruses have developed ways to suppress the RNA silencing mechanism. For example, *Tobacco etch virus* encodes a viral suppressor of RNA silencing, P1/HC-pro, which suppresses the host silencing mechanism by interfering with activity of RISC, the RNA induced silencing complex [8]. Another RNA silencing suppressor is the 2b protein encoded by *Cucumber mosaic* virus [9]. The 2b protein suppresses the RNA silencing mechanism by binding to siRNAs, interfering with RISC activity [10]. It is possible that similar suppression systems are used by mycoviruses to evade or impair host defences [11]. For this work, the mycovirus, *Botrytisvirus F* (BVF) [12, 13], was investigated to determine whether it could evade or even suppress the RNA silencing mechanism. BVF is a single-stranded RNA mycovirus of the *Gammaflexiviridae* and was selected because it is well characterised and has full genome sequence available [13, 14].

Whilst there are usually two dicer proteins in fungi, in almost all cases it is only Dicer 2 that is needed for effective gene silencing to occur. For example in *C. parasitica, Aspergillus nidulans* and *Magnaporthe oryzae*, two Dicer genes are present but only the Dicer2; *MDL*-2, *dcl-B* and *dcl*-2 respectively, were found to be involved in RNA silencing [7, 11, 15]. In *Neurospora crassa* a different situation was observed where both dicers could facilitate efficient gene silencing and both had to be mutated before silencing was impaired [16]. RNA silencing has been reported in *B. cinerea* [17, 18] however the mechanism has not been investigated. Given the importance of the Dicer proteins in RNA silencing, and its regulation of viral titres in other fungi, we decided to investigate the process in *B. cinerea* to understand the role of the Dicer proteins in silencing and whether this could impact on the interaction between *B. cinerea* and mycoviruses such as BVF.

MATERIALS AND METHODS

Fungal strains and growth conditions. Fungal strains used were *B. cinerea* B05.10 [19], *B. cinerea* Δ*ku70* [20], *B. cinerea* RH106-10 [12] and *S. cerevisiae* BY4742 (*MAT*α, *his3*Δ*1*, *leu2*Δ*0*, *lys2*Δ*0*, *ura3*Δ*0*) [21].

B. cinerea was routinely grown on malt extract agar or broth [22]. SH agar [23] was used for protoplast regeneration. Gamborg media was used for arginine auxotrophy assessment as previously described [17].

Yeast was grown in YPD liquid media (yeast extract 10 g L^{-1}, peptone 20 g L^{-1}, D-glucose 20 g L^{-1}) and YPD agar (YPD with agar 20 g L^{-1}). Yeast synthetic dropout media [23], was used as selection media.

Plasmid construction. The *B. cinerea* Δ*dcr2* disruption cassette pNDT-2 was made by in-yeast recombination as outlined in [25]. Fragments of approximately 2 kb were amplified from upstream and downstream of the *dcr2* gene using primers for left arm (fw-taatgcgatagttttttagccttatttcctctgacatgaaatttaggaactccccatg and rv-acttcggaacctgacactgacccggataca), and right arm (fw-catgcatggttgcctaactcggcgcgccgttagaatcttgctgtcttgctcgccctcgt and rv-atagggacctagacttcaggttgtctaactaaattatgaacggcagcaagagtga), designed to incorporate extensions necessary for recombination. The nourseothricin resistance marker (*nat1*) was PCR amplified from pNR2 [26] using specific primers (fw-cctctagagccgcattcccgattcgggccgg and rv-cggcgcgccgagttaggcaaccatgcatgg) with Phusion High-Fidelity polymerase. The three fragments were co-transformed into yeastalong with *Sal*I, *Sac*I double-digested pYES2-19 and uracil prototrophic colonies selected. Plasmid DNA was isolated from the transformant yeast colonies, rescued into *E. coli* and the correct plasmid assembly confirmed by restriction digests and PCR.

The *bcass1*-silencing plasmid pLOB-bar was made by excision of the *bcass1* silencing cassette from pLOB1-MCS [22] using restriction enzymes *Hin*dIII and *Sac*I and ligation into similarly digested pCB1530 [27].

Fungal transformation. *Botrytis* transformations were performed according to Hamada et al. [23], as modified by others [20, 22, 28]. Transformants were selected on SH agar containing 50 μg mL^{-1} nourseothricin or hygromycin B as appropriateand subcultured at least three times to reduce the likelihood of heterokaryon survival.

Plasmid pNDT-2 was transformed into *B. cinerea* using resistance to 50 μg mL^{-1} nourseothricin for selection. Genomic DNA was extracted from each of 40 transformants and PCR analysis was performed using *dcr2* primers (fw-tagagatgtggtaaagcactgtcaaggtct, rv-cggcgcgccgagttaggcaaccatgcatgg) and nourseothricin primers (fw-cctctagagccgcattcccgattcgggccgg, rv-cggcgcgccgagttaggcaaccatgcatgg) to detect the wild-type or disrupted allele of *dcr2*.

Mycovirus transfection. Protoplast fusion between viral-donor and recipients was selected as the transfection method since it obviated the need for vegetative compatibility between strains.The mycovirus recipient host was the *B. cinerea* Δ*ku70* mutant derived from BO5-10 [20], This was chosen so that growth on media containing hygromycin would allow this genotype to proliferate while selecting against any nuclei remaining from the mycoviral donor. *B. cinerea* RH106-10 [12] was used as the mycoviral donor since it contains BVF and is hygromycin sensitive. Protoplasts of both strains were prepared as for transformation.

RH106-10 protoplasts (6×10^6) were fused with protoplasts (6×10^3) of Δ*ku70* by the addition of 100 μL 25% PEG3350, 50 mM $CaCl_2$, 10 mM Tris-HCl pH 7, followed after 30 min by 500 μL of the same solution. After an additional 30 min, the protoplast mixture was collected by centrifugation and plated as per transformation. An overlay of SH containing 50 μg mL^{-1} of hygromycin was applied after 24 h. 50 emerging colonies were subcultured three times on MEA containing 100 μg mL^{-1} hygromycin.

Southern blot analysis. Genomic DNA of the parent and disrupted strains was prepared by the method of [29] and digested using *Sph*I. Southern blot analysis was carried out according to [30]. Two α^{32}P dCTP probes were used; probe A (generated using primers fw-tcttggttcctaggcgaattcttgggttct and rv-ccggtctgtacacaacacaaccatatagta) was situated to the upstream of the integrated cassette, while probe B (generated using primers fw-acgaagagaacgtgaatgatgaaatgtctc and rv-ttcattgttgaagattcaccagctgccgag) was situated downstream.

Virulence assays. Plants were raised in Levingtons F1 compost and used 7–14 days after germination depending on the species. Fresh fruit and vegetables purchased from a local supermarket were surface sterilised with 1% sodium hypochlorite for 5 min and rinsed in deionised H_2O before being used in bioassays. Detached leaves (*Nicotiana tabacum* "White Burley", Tomato "Moneymaker"), or fruits (tomato, grape and strawberry) were placed in an open petri dish and maintained within a humid chamber at room temperature. Whole plants were maintained in a greenhouse with controlled conditions of 20°C and >70% humidity. The appropriate tissues were punctured using a blunt needle and 10 μL of *B. cinerea* conidial suspension (500 conidia μL^{-1} in water) placed onto the punctured area. Lesion diameters were measured 5–7 days after inoculation.

Real time PCR. For real time PCR amplifications, a Maxima® SYBR green qPCR master mix (Fermentas, UK) was used. A typical qPCR reaction mix included 0.25 mM forward and 0.25 mM reverse primers (0.5 μL each), cDNA template (1 μL), Maxima® SYBR green qPCR master mix (20 μL) and H_2O (18 μL). Reactions were performed on a Stratagene MxPro 3005 with cycle conditions havingan initial denaturing temperature of 95°C for 5 min, followed by 40 cycles of 95°C for 30 s, 55°C for 1 min and 72°C for 1 min. Data were analysed by relative quantitation using β-tubulin for comparison. The cDNA from mycelia of the 5 day old *B. cinerea* cultures was prepared and used as templates for qPCR analysis using primers sets for the *dcr1* (fw-tgcctactatgatcccttgg, rv-ccgtccttgcttgtagtttc), *dcr2* (fw-tagtagagggcggcgttcacat, rv-caccttgccaggcttcatcc), *argo1* (fw-gctacatctgctgctattgg, rv-gcggatctccctttgtaaactt) and β-tubulin (fw-tgggctaagggtcattacac, rv-tatggctcgacaacggtatc), with analysis performed in triplicate. Note that primers were not optimised against each other, so only relative quantification was performed.

For mycovirus transmission, two sets of experiments were carried out, the first set used transfected *B. cinerea* lines harvested after 7 days growth on MEA media and the other set, which was subcultured every 7 days, was harvested at 28 days post-transfection. cDNA was prepared for each BVF-infected line (V-BH-1, V-Δ*ku70*, V-Δ*dcr2*-1, V-Δ*dcr2*-2) and uninfected lines (BO5-10, Δ*ku70*, Δ*dcr2*-1, Δ*dcr2*-2) with three technical replicates.

Phylogenetic analysis. Protein sequences were aligned using ClustalW2 [31]. The alignments were then used to generate boot-strapped trees using the Neighbour-joining method within MEGA.

RESULTS AND DISCUSSION

Identification and Disruption of dcr2

The two publically available *B. cinerea* genome sequences (http://www.broadinstitute.org/annotation/genome/botrytis_cinerea.2/Info.html, http://www.cns.fr/spip/-Botrytis-cinerea-.html) were searched to identify Dicer-like genes, using Dicer genes from selected *Ascomycete* as probes (*Sclerotinia clerotiorum, N. crassa, M. oryzae*, and *A. nidulans*). This identified two possible Dicer genes in *B. cinerea* (*dicr1*; BC1G_10104, *dcr2*; BC1G_10438). After resolving discrepancies with mis-called introns, the predicted proteins for each gene were aligned with those from other fully-sequenced *Ascomycetes* along with other non-fungal model organisms (*Drosophila melanogaster, Caenorhabditis elegans* and *Arabidopsis thaliana*) using Clustal W2 and a phylogenetic tree was generated based on the neighbour joining method [33]. This showed that the fungal Dicer 1 and 2 proteins form distinct clades (Fig. 1). The non-fungal Dicer proteins did not integrate into the fungal Dicer clades. Therefore *B. cinerea* contains two dicer genes, separating into the two distinct dicer clades on phylogenetic analysis, which is typical of the majority of ascomycete fungi sequenced to date.

The *B. cinerea dcr1* gene was targeted for gene disruption since it has been reported that disruption of the Dicer 2 gene inhibited RNA silencing in several fungi including *M. oryzae, C. parasitica* and *A. nidulans* [7, 11, 15]. Transformation of the Δ*dcr2* disruption cassette pNDT-2 into the wild type B05-10 failed to generate any Δ*dcr2* disrupted mutants out of 300 transformants (data not shown). Targeted insertion is reported to be more efficient in Δ*ku70* mutants of *B. cinerea* [20] therefore pNDT-2 was transformed into these mutants. PCR analysis of 40 transformants showed that 38 of the transformants produced PCR products corresponding to the native Δ*dcr2* locus, however two transformants were identified as possible Δ*dcr2* disruption mutants.

Southern blot analysis was used to confirm whether the possible Δ*dcr2* mutants had the desired recombi-

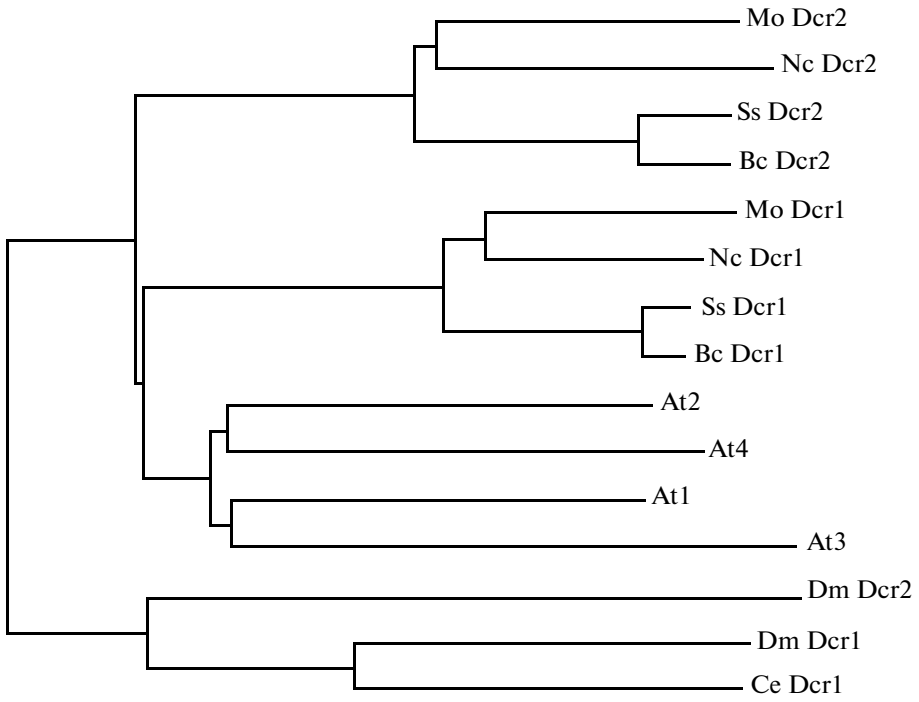

Fig. 1. Neighbour-Joining tree of the Dicer 1 and 2 protein sequences. Dicers of selected Ascomycete fungi (*B. cinerea* (Bc), *S. sclerotiorum* (Ss), *N. crassa* (Nc) and *M. oryzae* (Mo)) form separate clades for Dicer-1 and Dicer-2, while animal and plant Dicers *A. thaliana* (At), *C. elegans* (Ce) and *D. melanogaster* (Dm) are separate.

nation event. Results showed that probe A hybridised to a 4.9 kb fragment for both the putative disrupted mutants compared toa 15.9 kb fragment for the parent, while probe B hybridised to a 10.9 kb fragment for both the putative mutants and 15.9 kb fragment for the parent (Fig. 2). This indicated successful disruption of *dcr2* in both Δ*dcr2*-1 and Δ*dcr2*-2. The use of the Δ*ku70* strain therefore increased the targeting efficiency as has previously been reported in *B. cinerea* [20] and several other fungi including *N. crassa* [34], *M. grisea* [35] and *Aspergillus* sp. [36, 37].

Phenotypic Analysis of dcr2 Mutants

Growth rates of Δ*dcr2*-1 and Δ*dcr2*-2 along with B05-10 and Δ*ku70* were determined on MEA plates after 5 days to assess whether disruption of *dcr2* had any effect on growth, however there was no significant difference between the *B. cinerea* Δ*dcr2* mutants and controls (Student *t*-test, $p < 0.05$). The visual appearance of the disrupted strains was also normal as were conidial size and numbers (data not shown). This is in contrast to *M. oryzae*, where disruption of MDL-2 resulted in reduction in mycelial growth [15], but is similar to the situation in *C. parasitica* [7] and *N. crassa* [16] where there were no observable morphological changes in growth rate with loss of Dicer 2.

Silencing of argininosuccinate synthase (*bcass1*) has previously been shown to result in partial arginine auxotrophy as indicated by reduced growth rates of strains on minimal media in the absence of arginine supplementation [18]. To functionally assess Δ*dcr2*-1 and Δ*dcr2*-2 for silencing ability, the argininosuccinate synthase-silencing plasmid, pLOB-bar, was transformed into Δ*dcr2*-1, Δ*dcr2*-2 and the original Δ*ku70* parent, giving 96, 64 and 75 surviving transformants for Δ*dcr2*-1, Δ*dcr2*-2 and Δ*ku70* respectively. The transformants and Δ*ku70* parent were point inoculated onto Gamborg media with and without arginine supplementation and the radial growth rates were measured after 5 days, with three biological replicates for each (Fig. 3). Transformants that showed a growth reduction of >10% on media without arginine supplementation were considered to indicate silencing of *bcass1*. From the population of pLOB-bar transformants of Δ*dcr2*-1, Δ*dcr2*-2 and Δ*ku70*, 99, 94 and 91% of transformants respectively showed a growth reduction of >10% without arginine supplementation and so were considered to show RNA silencing of *bcass1*. All three populations showed an average of 60% growth in comparison to arginine supplementation. Whilst this is a higher frequency of silencing than that reported by Patel et al. [18], it should be noted that their study involved co-transformation so a proportion of their

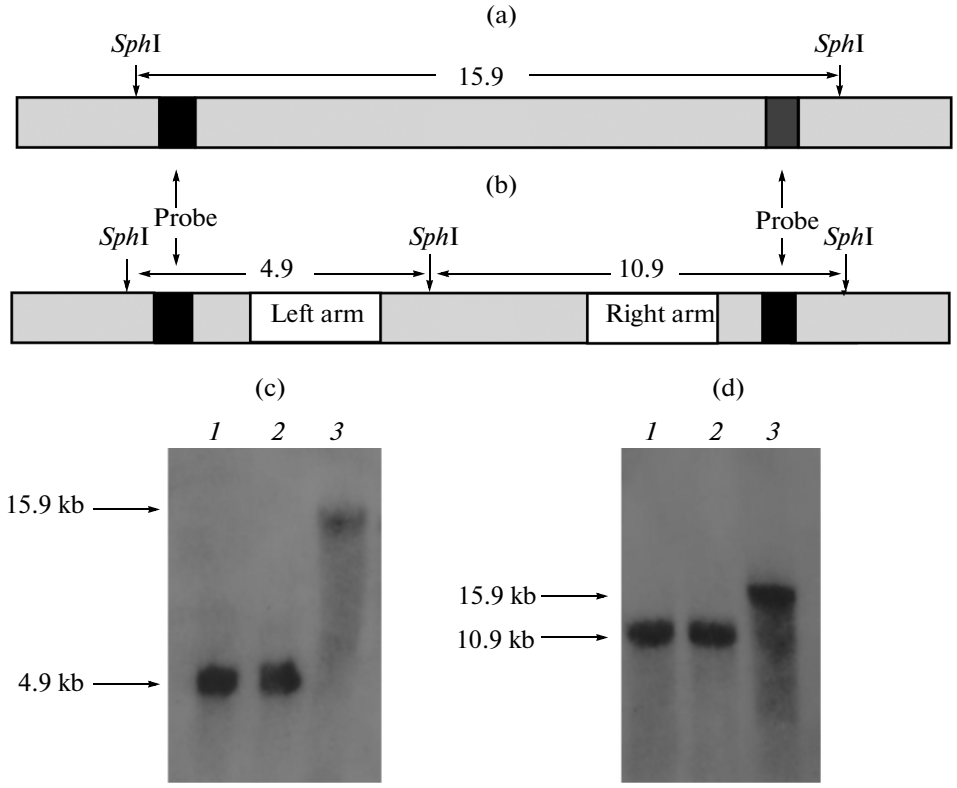

Fig. 2. Southern analysis for *B. cinerea* Δ*dcr2* mutants and wild type. Panel (a), schematic of the wild-type dcr locus. Panel (b), schematic of the dcr2 locus upon replacement with *nat1*. Panel (c), southern analysis using probe 1. Panel (d), southern using probe 2. Lanes (*1*–*3*) are *Sph*I digested DNA of Δ*dcr2*-1 (*1*), Δ*dcr2*-2 (*2*) and Δ*ku70* (*3*) showing that in both cases the disrupted mutants have a single hybridising band, and as predicted these are of a different size to the wild-type signal.

transformants may not have contained the silencing cassette.

This observation of a silencing-phenotype in a *dcr2* mutant is in marked contrast to the majority of ascomycete fungi studied to date, where disruption of *dcr2* leads to complete abolition of post-transcriptional gene silencing. The possibility remains however that the phenotype of reduced growth in absence of arginine supplementation observed in these experiments might be due to altered rates of *bcass1* transcription rather than a post-transcriptional mechanism, however in other fungi it is post-transcriptional mechanisms which are the norm when such a construct has been introduced, so there is no reason to suppose that *B. cinerea* would exceptionally perform silencing by transcriptional means.

The relative expression of *dcr1*, *dcr2* and *argo1* (Argonaut 1 : NCBI accession number: BC1G_00797) were investigated in Δ*ku70*, Δ*dcr2*-1 and Δ*dcr2*-2 and also the *bcass1*-silenced transformants S-Δ*dcr2*-43, S-Δ*dcr2*-21 and silenced S-Δ*ku70*–*60*. These three silenced lines showed 38, 60 and 35% residual growth respectively in comparison to arginine supplementation, so had good, but differing levels of silencing as judged by phenotype. Each line was inoculated onto Gamborg liquid media with and without arginine supplementation. Δ*dcr2* mutants had the same pattern of *dcr1* and *argo1* expression as the wild type irrespective of silencing state, showing no compensatory increase in *dcr1* expression despite the loss of *dcr2*.

When silencing of *bcass1* was induced respective of the *dcr2* background, all lines showed a small but significant (~5%, $p < 0.05$) increase in Δ*dcr1* expression, with a further significant small (~5%, $p < 0.05$) increase when the silenced lines were grown on media lacking arginine supplementation (Fig. 4). *dcr2* expression showed a similar pattern to *dcr1*, but with a 30% increase in expression during active *bcass1*-silencing and a further 70% increase when grown on media without arginine supplementation. In all the lines analysed, relative expression levels for *argo1* increased by ~20% when *bcass1*-silencing was induced, with a further increase ~10% when the lines were grown on media without arginine supplementation. Under such conditions, the fungus needs argininosuccinate synthase for arginine production, so it would be expected to increase transcription of this gene. It is possible that the elevated transcription rate for *bcass1* might result in elevated expression of the

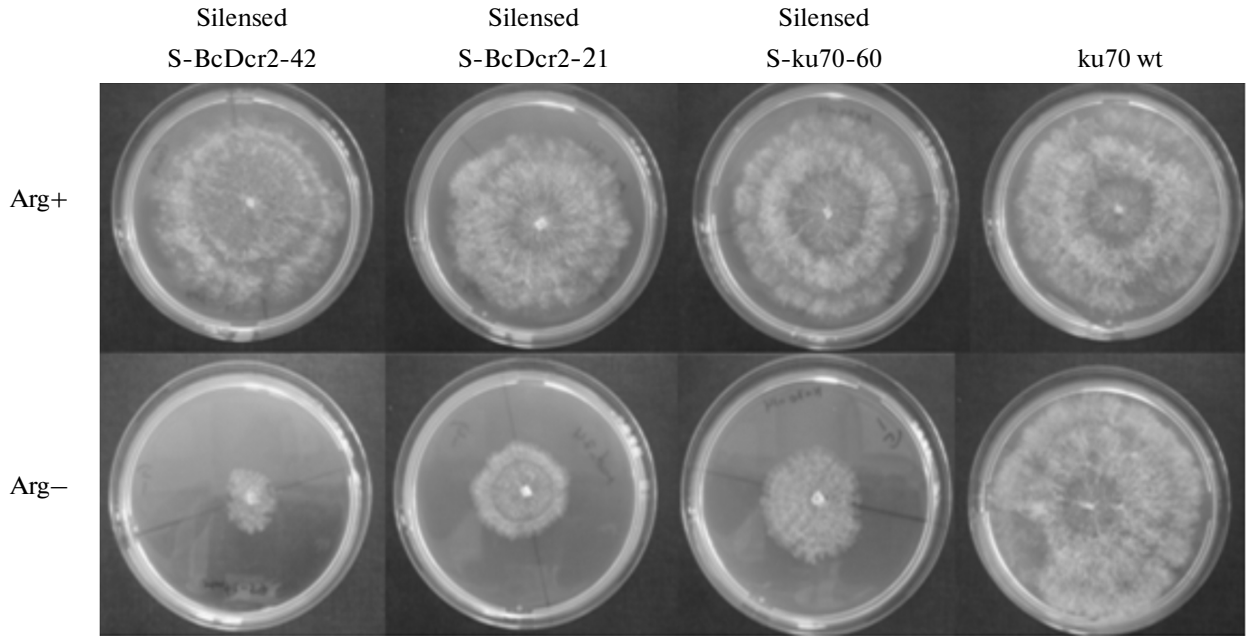

Fig. 3. Examples of the *bcass1*-silenced phenotype indicated by reduced growth in the absence of arginine supplementation. Gamborg minimal media assays with and without arginine for the arginine-silenced *B. cinerea dcr2* mutants (S-Δ*dcr2*-43 and S-Δ*dcr2*-21) and Δ*ku70* (S-Δ*ku70-60*), and non-silenced Δ*ku70* wild type (control).

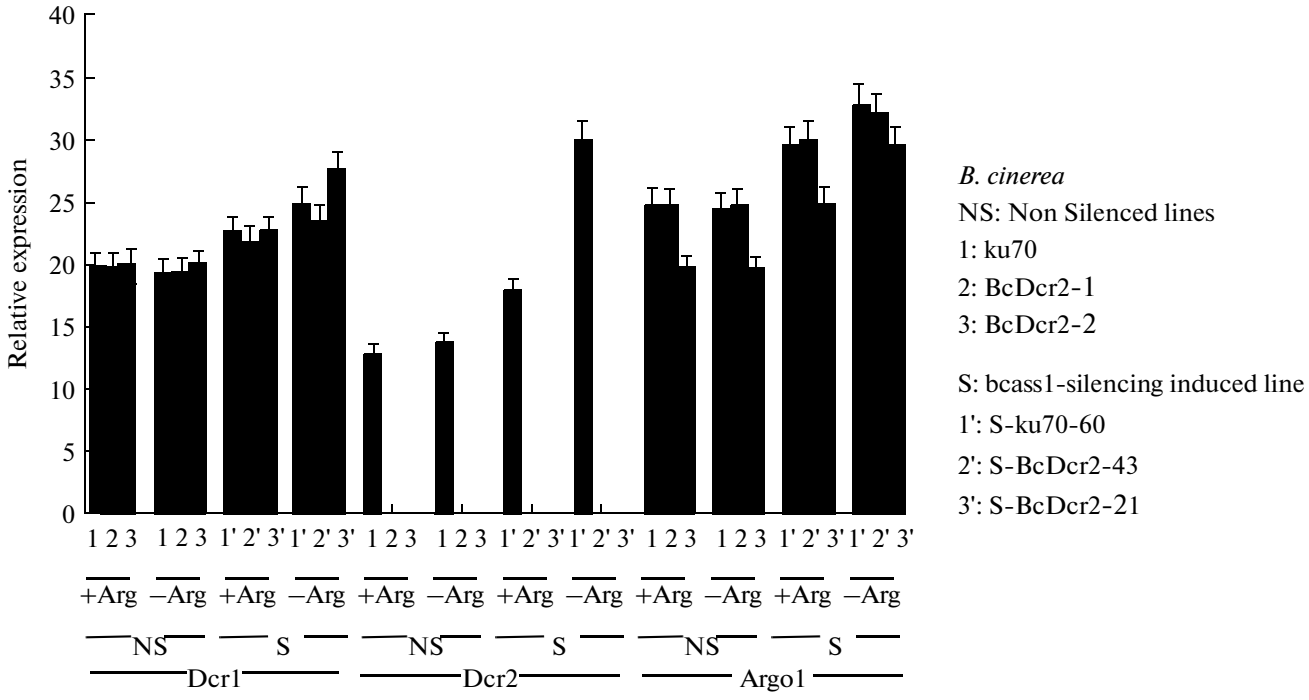

Fig. 4. The relative expression levels for the genes *dcr1*, *dcr2* and *argo1* for *bcass1*-silenced lines, showing an increase in expression when *bcass1*-silencing was induced, and a further increase in expression when lines were grown on media lacking arginine supplementation (–Arg., Non-silenced lines: Δ*ku70*, Δ*dcr2*-1, Δ*dcr2*-2. *bcass1*-silencing induced lines: S-Δ*ku70-60*, S-Δ*dcr2*-43, S-Δ*dcr2*-1.

dicer and argonaut silencing machinery to counter this.

There was no significant difference in relative expression levels of *dcr1*, *dcr2* and *argo1* for the unsilenced lines grown on media with or without arginine supplementation, but the difference for the *bcass1*-silenced lines was significant in all cases.

Of the ascomycete fungi investigated to date, *N. crassa* is the only other which is competent in silencing despite loss of *dcr2* [16], however disruption of both dicer 1 and dicer 2 was sufficient to prevent silencing, both genes being functionally redundant. If like in *N. crassa*, the Dicer genes are redundant in RNA silencing in *B. cinerea*, this may explain why the RNA silencing mechanism would still be functional even with loss of the *dcr2* gene.

The Impact of Mycovirus Infection

Dicer 2 (*dcl*-2) mutants of *C. parasitica* are reported to show a noticeable reduction in mycelial growth when infected with the CHV1-EP713 mycovirus, compared to the wild-type strains [7]. To establish whether BVF could alter the fungal phenotype, Δ*dcr2*-1, Δ*dcr2*-2 and the Δ*ku70* parent were transfected with Botrytis Virus F (BVF), a single-strand positive sense mycovirus, by protoplast fusion with the mycovirus donor RH106-10. Six transfected lines were selected for each BVF recipient and RT-PCR confirmed the successful transmission of BVF. One virus-positive line was identified for each (V-Δ*dcr2*-1, V-Δ*dcr2*-2, V-Δ*ku70*) and used for subsequent analysis. Comparative growth rates and *in planta* virulence assays were carried out to determine the impact of viral transmission into each line. There was no significant difference in growth rate between non-infected and BVF-infected lines irrespective of the *dcr2* genetic background. Lesion size was recorded on French dwarf bean leaves after 5 days but there was no significant difference in virulence between the non-infected and the BVF infected lines irrespective of *dcr2* background (data not shown).

It is possible that BVF cannot confer any hypovirulent traits; that hypovirulence can happen, but infrequently, and so was not observed in the relatively few transmission events analysed here; or alternatively the mycovirus was still effectively controlled by the fungus because RNA silencing was fully functional in *B. cinerea*, even in the Δ*dcr2* mutants. To evaluate these possibilities, the relative expression levels for *dcr1*, *dcr2* and *argo1* in BVF-infected and uninfected lines were quantified to determine whether mycovirus BVF had any impact on the expression of the RNA silencing genes. Two sets of qPCR experiments were carried out, the first set used BVF-infected lines (V-Δ*ku70*, V-Δ*dcr2*-1, N-Δ*dcr2*-2) and uninfected lines (Δ*ku70*, Δ*dcr2*-1, Δ*dcr2*-2) harvested after 7 days growth on MEA media and the second set, which was subcultured every 7 days, was harvested at 28 days post-transfection to compare consistency of results over time andthe presence of BVF was confirmed at each point of the time-course.

At seven days post-infection, the Δ*ku70* strain (effectively wild-type for dicer) showed a small increase in *dcr1* expression, but a 6.5 fold decrease in *dcr2* (Fig. 5). By 28 days, levels of both *dcr1* and *dcr2* were increased compared to the uninfected strain. In contrast the infected *dcr2* mutants consistently showed a 2 fold decrease in *dcr1* expression at seven days, but these were restored to the same elevated levels as infected Δ*ku70* by 28 days. Therefore at the molecular level, the presence of BVF affected the relative expression levels of the RNA silencing genes (*dcr1*, *dcr2*, *argo1*). At 7 days post-infection, *dcr2* levels showed a marked decreased for the infected parental Δ*ku70* line, suggesting thatthere was mycovirus-mediated suppression of *dcr2*, but *dcr1* mRNA levels remained normal. In contrast, the *dcr2* mutants showed a reduction in *dcr1* transcript levels during viral infection, showing that this transcript could also be impacted by viral infection in the absence of *dcr2*. *Argo1* expression was elevated during viral infection at both time points, irrespective of *dcr2* background, showing that whatever the mechanism of suppression, it is specific to particular genes, not the silencing pathway in general.

By 28 days post-infection for the BVF-infected lines, the *dcr1* and *dcr2* expression levels showed complete recovery, to the elevated levels typically observed when silencing is induced. The *argo1* expression levels for the BVF-infected lines likewise remained higher than the uninfected lines. For the BVF-infected *B. cinerea*, the recovery of the suppressed *dcr* transcripts showed that the fungus was seemingly able to counter the initial gene suppression by 28 days, although BVF was not eliminated since the mycovirus remained detectable by RT-PCR. This shows that the interaction between *B. cinerea* and BVF is very complex, with BVF initially seemingly able to suppress silencing, whilst the fungus would appear to then recover from such effects to give a normal phenotype in terms of growth and virulence, and can do this even when *dcr2* has been disrupted.

There remains a possibility that deletion of *B. cinerea dcr1* would eliminate the ability to silence RNA, and as a possible consequence, RNA mycoviruses might be able to replicate to elevated levels and cause effective hypovirulence. The uncertainty with the role of *B. cinerea dcr1* can only be resolved when *B. cinerea dcr1* mutants and *dcr1*, *dcr2* double mutants are created. This work has shown that *dcr2* is not essential for RNA silencing to function in *B. cinerea* and that viral titres are not regulated by *dcr2*.

ACKNOWLEDGMENTS

We thank Dr Geneviéve Billon-Grand for help with the *B. cinerea* transformations. This work was

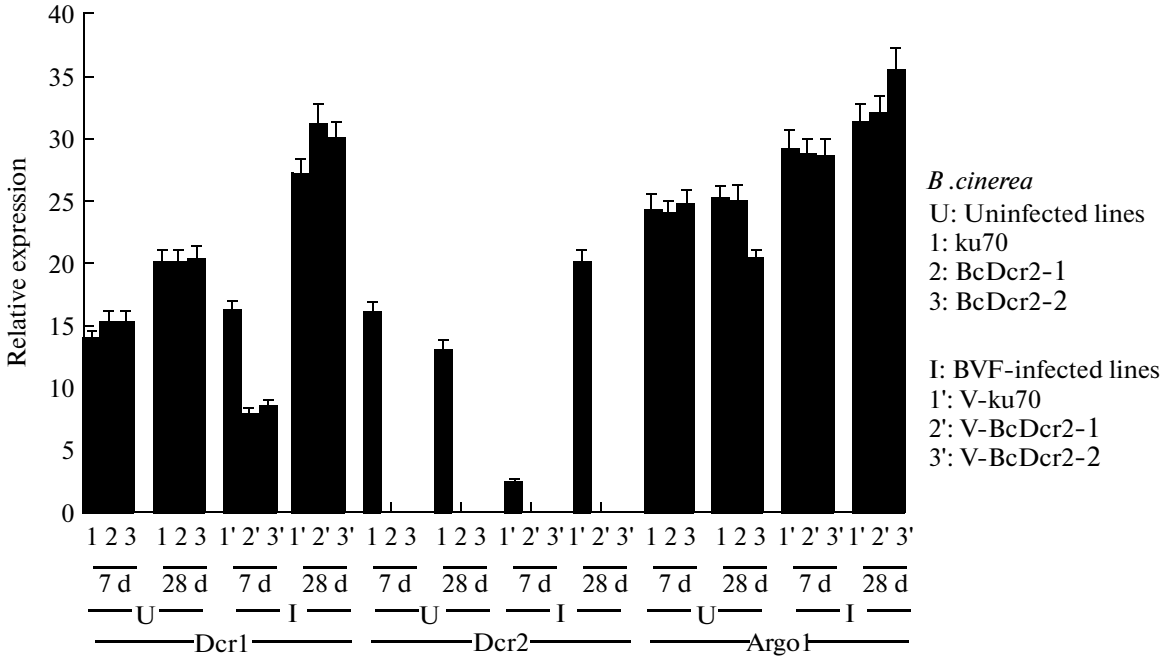

Fig. 5. Relative expression levels for *dcr1*, *dcr2* and *argo1* at 7 and 28 days for BVF-infected (V-Δ*ku70*, V-Δ*dcr2*-1, V-Δ*dcr2*-2) and uninfected (Δ*ku70*, Δ*dcr2*-1, Δ*dcr2*-2) *B. cinerea* lines.

performed under DEFRA plant health licence PHL 247A/6355.

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