Inadvertent gene silencing of argininosuccinate synthase (*bcass1*) in *Botrytis cinerea* by the pLOB1 vector system

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SUMMARY

For several years, researchers working on the plant pathogen *Botrytis cinerea* and a number of other related fungi have routinely used the pLOB1 vector system, based on hygromycin resistance, under the control of the *Aspergillus nidulans* *oliC* promoter and what was reported to be the β -tubulin (*tubA*) terminator. Recently, it has been demonstrated that this vector contains a 446-bp portion of the *B. cinerea* argininosuccinate synthase gene (*bcass1*) rather than the *tubA* terminator. As argininosuccinate synthase is essential for the production of L-arginine, inadvertent gene silencing of *bcass1* may result in partial L-arginine auxotrophy and, indeed, may lead to altered phenotypes *in planta*. In this article, we report our findings relating to possible problems arising from this incorrect plasmid construction. As an absolute baseline, gene disruption of *bcass1* was carried out and generated a strict auxotroph, unable to grow without exogenous arginine supplementation. The knock-out displayed an alteration in host range *in planta*, showing a reduction in pathogenicity on strawberries, French bean leaves and tomatoes, but maintained wild-type growth on grape, which is in accordance with the reported arginine availability in such tissues. Deliberate gene silencing of *bcass1* mirrored these effects, with strongly silenced lines showing reduced virulence. The degree of silencing as seen by partial auxotrophy was correlated with an observed reduction in virulence. We also showed that inadvertent silencing of *bcass1* is possible when using the pLOB1 vector or derivatives thereof. Partial arginine auxotrophy and concomitant reductions in virulence were triggered in approximately 6% of transformants obtained when expressing enhanced green fluorescent protein, luciferase, monomeric red fluorescent protein or β -glucuronidase using the pLOB1-based expression system, which inadvertently contains 446 bp of the *bcass1* coding sequence. We recommend the testing of transformants obtained using this vector system for arginine auxotrophy in order to provide assurance that any observed effects on the

development or virulence are a result of the desired genetic alteration rather than accidental *bcass1* silencing.

INTRODUCTION

Botrytis cinerea is an opportunistic necrotrophic fungus with a broad host range, capable of infecting over 200 plant species worldwide, including ornamentals, vegetables and fruits (Williamson *et al.*, 2007). Various biological and chemical control measures have been developed to limit the damage caused by *B. cinerea*. Because fungicide effectiveness declines soon after field introduction, new targets for control are being sought (Leroux *et al.*, 2002; Smith, 1998). Once potential targets are identified through gene knockout or silencing methodologies, they must be validated by *in planta* growth assays. Molecular genetic approaches have been deployed in the last decade to identify important virulence determinants of *B. cinerea* that may serve as targets for novel control measures (van Kan, 2006; Tudzynski and Siewers, 2004). Gene function can be studied by targeted or random insertional mutagenesis and the evaluation of whether the mutation affects virulence.

pLOB1 (GENBANK Accession No. AJ439603) (Fig. 1A, B) is a hygromycin resistance vector that is frequently utilized for *B. cinerea* molecular manipulations. Since its first publication (van Kan *et al.*, 1997), this hygromycin cassette has been used to generate random insertion mutants and over 30 targeted gene-specific mutants (for example: Brito *et al.*, 2006; Doehlemann *et al.*, 2005, 2006a, b; Espino *et al.*, 2005; ten Have *et al.*, 2010; Hayashi *et al.*, 2002a, b; van Kan *et al.*, 1997; Kars *et al.*, 2005a, b; Reis *et al.*, 2005; Rui and Hahn, 2007a, b; Schoonbeek *et al.*, 2002; Schouten *et al.*, 2002a, b). When the sequence of pLOB1 was compared with the genome sequence of *B. cinerea* strain B05.10 (<http://www.broadinstitute.org/>), it became apparent that the terminator sequence downstream of the hygromycin phosphotransferase (*hph*) gene in pLOB1 was identical to a partial *B. cinerea* endogene (argininosuccinate synthase, AS)

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Fig. 1 Diagrams of the pLOB1 and pLOB1-MCS vectors. pLOB1 (A) and pLOB1-MCS (B) contain the *Aspergillus nidulans* *oliC* promoter and *bcass1* partial gene fragment. pLOB1 contains the hygromycin resistance gene (*hph*), whereas pLOB-MCS contains a 120-bp multiple cloning site (MCS). Transcript and regulatory sequence sizes are shown above each segment, and construct sizes and names are denoted at the end of each plasmid.

instead of the β -tubulin terminator. AS is the enzyme that catalyses the rate-limiting step in the conversion of L-citrulline to L-arginine and has been recognized as one of the key factors regulating L-arginine metabolism. Because the AS sequence present in pLOB1 and its derivatives is identical to that of the endogenous *B. cinerea* gene, transcriptional readthrough into this region could inadvertently induce gene silencing mechanisms. This could result in partial auxotrophy and, potentially, a reduction in virulence *in vivo*. In this article, we investigate the possibility of inadvertent *bcass1* silencing in pLOB1-based transformants, and identify phenotypes associated with partial AS down-regulation *in vitro* and *in vivo*.

RESULTS

Sequence analysis pLOB1-based vectors (Fig. 1A, B) are controlled by the *Aspergillus nidulans* *oliC* promoter and what was previously reported to be the *B. cinerea* *tubA* terminator (van Kan *et al.*, 1997). Sequence analysis indicates that bases 25–471 of the terminator region in pLOB1 (GENBANK Accession No. AJ439603) are identical to bases 771–1218 of the *B. cinerea* AS gene (*bcass1*) coding region (locus BC1G_00121.1, <http://www.broad.mit.edu>). Transcriptional readthrough into the pLOB1 'terminator' region may result in the generation of hybrid mRNAs, including a substantial part of *bcass1*, potentially inducing the gene silencing pathway. The principal aim of this study was to investigate the possibility of inadvertent *bcass1* silencing in pLOB1-based transformants and to identify phenotypes associated with (partial) *bcass1* down-regulation *in vitro* and *in planta*.

bcass1 gene replacement

In order to assess the maximum phenotypic effect of the down-regulation of AS expression *in vitro* and *in vivo*, *bcass1* was

disrupted with the p Δ *bcass1* knockout vector (Fig. 2A). Two mutants, designated Δ *bcass1*-1 and Δ *bcass1*-2, were found to be incapable of growing on plates without L-arginine, but demonstrated wild-type growth rates with L-arginine supplementation (Fig. 3A). The wild-type displayed a small (3.2%) but significant (*t*-test, *F*-value significant at $P = 0.006$) decrease in radial growth in the presence of L-arginine (Table 1). Southern analysis confirmed the knockout of the *bcass1* gene through homologous recombination of knockout vector p Δ *bcass1* (Fig. 2B). Mutant Δ *bcass1*-2 contained a second ectopic integration of the p Δ *bcass1* plasmid. Integration of the complete p Δ *bcass1* knockout vector was confirmed by polymerase chain reaction (PCR) (Fig. 2C).

Wild-type and L-arginine auxotrophic mutants, Δ *bcass1*-1 and Δ *bcass1*-2, were inoculated onto French bean leaves, strawberries, tomatoes and grapes (20 replicates) to determine whether AS gene knockout resulted in a decrease in the observed virulence (Fig. 3B). Lesion sizes on bean leaves were compared at 3 days post-inoculation. On average, lesions from knockout mutants Δ *bcass1*-1 and Δ *bcass1*-2 were 88.4% and 70.2% smaller, respectively, than those observed for the wild-type (data not shown). Mutants also exhibited a reduction in growth on strawberries and tomatoes, but appeared to be unaltered in virulence on grapes.

bcass1 complementation

As knockout mutants Δ *bcass1*-1 and Δ *bcass1*-2 demonstrated a significant reduction in growth during plate-based analysis and virulence on French bean leaves, strawberries and tomatoes, gene complementation was performed to validate the observed phenotypes. The complete *bcass1* region was amplified and used to complement Δ *bcass1*-1 and Δ *bcass1*-2 mutants. As knockout strains Δ *bcass1*-1 and Δ *bcass1*-2 are hygromycin B resistant, a

Fig. 2 (A) *bcass1* loci before and after disruption by homologous recombination of a hygromycin resistance knockout cassette. Start of translation (black arrows), stop codons (asterisk), introns (grey boxes) and restriction sites are also indicated. The hygromycin resistance (*hph*), *oliC* promoter and *trpC* terminator are also labelled on the hygromycin resistance knockout cassette. Southern (B) and polymerase chain reaction (PCR) (C) analyses confirm the integration of the knockout cassette. Wild-type (WT) and two knockout mutants ($\Delta bcass1$ -1 and $\Delta bcass1$ -2) were digested with restriction enzymes *SacI* and *Apal* and probed with partial *bcass1* (B, I) and hygromycin (B, II) gene fragments, respectively. The arrows indicate the size of the ~5.2-kb *SacI* wild-type fragment and the ~9.3-kb *Apal* gene disruption fragment. (C) The amplification of genomic DNA with primers spanning the entire gene disruption vector resulted in a gene fragment of ~6.0 kb for both knockout mutants ($\Delta bcass1$ -1 and $\Delta bcass1$ -2).

second selection marker (pNR2) conferring nourseothricin resistance was used during co-transformation (Hayashi *et al.*, 2002a; Malonek *et al.*, 2004). Regeneration agar and subsequent sub-culturing events were not supplemented with L-arginine to ensure that pBCASS1 had integrated into the fungal genome. The recovered transformants were then plated in triplicate on minimal medium with and without L-arginine. Four recovered complemented transformants (three generated from $\Delta bcass1$ -1 and one generated from $\Delta bcass1$ -2) displayed wild-type growth rates with and without L-arginine supplementation (results not shown). Wild-type and complemented transformants were also grown in triplicate on strawberries and French bean leaves, confirming wild-type growth (results not shown).

Induction of *bcass1* silencing

The reduction in virulence by *bcass1* gene knockout mutants further corroborated our concerns about the inadvertent genera-

tion of partial L-arginine auxotrophs through gene silencing of *bcass1* when using pLOB1-based vectors. As a result, it became necessary to test whether pLOB1-based vectors were able to induce *bcass1* silencing by co-transformation of the unmodified pLOB1-MCS expression vector and pOT-HYG hygromycin resistance vector into strain B05.10. As pLOB1-MCS contains a multiple cloning site (120 bp) flanked by the *Aspergillus nidulans* *oliC* promoter and *bcass1* partial gene fragment, it serves as a sense silencing construct (Fig. 1B). Transformants (49 in total) were tested *in vitro* in triplicate to screen for the occurrence of silencing-induced auxotrophy. Transformants showing a reduction in radial growth of at least 10% on minimal medium lacking L-arginine (in comparison with minimal medium supplemented with L-arginine) were considered to be partially auxotrophic and potentially demonstrating a level of *bcass1* silencing (Table 1; Fig. 4A). Overall, 20 transformants were found to exhibit *bcass1* silencing, averaging a decrease in radial growth by 26.5% in the absence of L-arginine. Integration of the pLOB1-MCS silencing

Fig. 3 (A) Examples of the L-arginine plate-based assay. Mutants and wild-type were plated on minimal medium supplemented with (+Arg) and without (–Arg) L-arginine. The wild-type (WT) maintains its growth under both treatment schemes. The *Δbcass1-1* disruption mutant is incapable of growing without L-arginine, but recovers with L-arginine supplementation. (B) *In planta* testing showing reduced lesion size of L-arginine auxotrophic mutants, *Δbcass1-1* and *Δbcass1-2*. (a) The left side of French bean leaves is infected with the wild-type, whereas the right side is the knockout mutant. Strawberries (b), tomatoes (c) and grapes (d) were sliced in half before being inoculated. The left halves of the strawberry and grape are infected with the wild-type, and the right sides are the knockout mutants. Infections were photographed 3 days post-inoculation and repeated at least 20 times.

cassette was confirmed in transformants exhibiting *bcass1* silencing by PCR analysis with primers OL12 and TUB2 (Table S1, see Supporting Information).

Transformants exhibiting a range of *bcass1* silencing-induced auxotrophy were selected (three high, three intermediate and six low, as judged from plate-based analysis). Transformants exhibiting a 10%–29% decrease in radial growth without L-arginine were considered to demonstrate low levels of silencing. Intermediate levels of silencing were defined as transformants exhibiting a 30%–49% increase in radial growth with nutrient supplementation, whereas highly silenced lines demonstrated a 50%–69% increase in radial growth. Subsequent Northern analysis (probe amplified by primer pair BCASS 1 F and BCASS 1R; Table S1, see Supporting Information) revealed that 11 of the 12 samples exhibited reduced *bcass1* transcript levels in comparison with the wild-type (Fig. 5). Unexpectedly, transformant 54 produced *bcass1* transcript levels higher than that of the wild-type. Northern blots were probed with an actin probe as an indicator of equal loading. Films were overexposed to highlight the degree of silencing displayed by each transformant.

Six transformants (61, 62, 63, 68, 70 and 80) with varying degrees of silencing were subjected to *in planta* analysis to determine whether *bcass1* silencing had an impact on virulence (Fig. 4B). The tested transformants exhibited a reduction in lesion area on French bean leaves in comparison with the wild-type (area determined using ImageJ analysis). The reduction in virulence correlated significantly (*F*-value significant at $R^2 = 0.9338$) with the level of silencing-induced auxotrophy observed during plate-based analysis. Correlation was calculated by comparing the lesion areas *in planta* with the area of growth *in vitro*. The reduction in virulence associated with *bcass1* mRNA silencing calls into question the utility/reliability of the pLOB1-based transformation system.

A clear correlation between the levels of silencing and auxotrophy was not observed in all transformants tested, in contrast with the strict correlation between auxotrophy and reduced virulence.

Table 1 Degree of arginine auxotrophy of pLOB1-MCS(*t-tubA*)-based transformants. Transformants were grown on minimal medium without or with L-arginine supplementation, in all cases in three replicates. Colony diameters were recorded after 3 days. The difference in colony diameter was used to determine the level of auxotrophy, where high auxotrophy was defined as >50% reduction (in growth on medium without relative to with arginine), intermediate as 30%–49% reduction and low as 10%–29% reduction.

Transformant	Average radial growth without L-arginine (mm)	Average radial growth with L-arginine (mm)	Relative growth in the absence or presence of arginine	Level of auxotrophy
WT	34 ± 2	33 ± 2	103	N/A
51	29 ± 3	35 ± 2	83	Low
54	25 ± 5	28 ± 1	89	Low
56	31 ± 1	34 ± 0	90	Low
59	25 ± 4	32 ± 0	78	Low
60	24 ± 4	30 ± 2	80	Low
61	22 ± 2	33 ± 0	67	Intermediate
62	9 ± 1	27 ± 2	33	High
63	12 ± 2	35 ± 3	31	High
64	29 ± 1	32 ± 2	90	Low
65	27 ± 1	30 ± 1	90	Low
66	18 ± 3	32 ± 3	56	Intermediate
67	30 ± 6	33 ± 1	90	Low
68	18 ± 3	33 ± 1	55	Intermediate
70	27 ± 2	34 ± 0	79	Low
75	16 ± 4	32 ± 1	50	High
77	11 ± 1	29 ± 1	38	High
79	21 ± 6	31 ± 2	68	Intermediate
80	18 ± 2	32 ± 2	56	Intermediate
400	27 ± 2	29 ± 2	90	Low
408	26 ± 1	29 ± 4	90	Low

Fig. 4 *In vitro* (A) and *in planta* (B) analysis of pLOB1-MCS-based transformants. (A) Plate-based assays show regeneration of growth for select transformants with L-arginine supplementation (bottom row). Plates without supplementation display a gradient of silencing efficiency/partial auxotrophy (top row). The same transformants were also subjected to *in planta* virulence assays (B). Plate and leaf photographs were taken at 3 days post-inoculation.

Fig. 5 Northern analysis confirming reduced *bcass1* transcript levels in pLOB1-MCS-based transformants. Transformants were probed with a partial *bcass1* gene fragment (A), whereas *bcactin* (probe amplified with primers ACTIN F and ACTIN R) was used to confirm equal loading of mRNA (B). (C) Levels of auxotrophy as determined by plate-based analysis [low ('L'), intermediate ('I') and high ('H')] are also indicated.

Inadvertent *bcass1* silencing

The lower *bcass1* transcript levels observed in the pLOB1-MCS-based transformants can be attributed to gene silencing triggered by the partial AS sequence located downstream of the multiple cloning site. Following these observations, concerns arose regarding previous experiments involving pLOB1, as many of these studies have generated mutants displaying an alteration in phenotype *in vitro* and/or a reduction in virulence. Therefore, the phenotypic screening of previously generated pLOB1-based transformants may determine the potential effects caused by the *bcass1* region in pLOB1-based vectors. Such investigations may prove to be valuable for future molecular manipulations, as well as aid in the re-examination of the results presented previously. To determine the potential for inadvertent *bcass1* silencing, previously developed pLOB1-based transformants (Patel *et al.* 2008a, b) were re-assessed (Table 2). The reporter gene transformants enhanced green fluorescent protein (eGFP), luciferase (LUC), β -glucuronidase (GUS) and monomeric red fluorescent protein (mRFP) (Patel *et al.*, 2008a) and superoxide dismutase (SOD) silencing transformants (Patel *et al.*, 2008b) were obtained by co-transformation with a modified pLOB1-MCS expression vector and pLOB1 hygromycin resistance cassette. All transformants were plated on minimal medium supplemented with and without L-arginine. In total, 7% of reporter gene (three eGFP, two LUC, two GUS and one mRFP) and 6% of SOD-silenced (three sense and three antisense) transformants demonstrated *bcass1* silencing-induced auxotrophy (Fig. 6), representing 6.6% of all tested pLOB1-based transformants.

Transformants that exhibited *bcass1* silencing were also tested *in planta*. Primary leaves of dwarf French bean plants were inoculated with the wild-type, two *bcass1* knockout mutants ($\Delta bcass1-1$ and $\Delta bcass1-2$), eight reporter gene transformants and six SOD-silenced transformants exhibiting *bcass1* silencing (Fig. 7A and data not shown). Leaves were also inoculated with $\Delta bcass1-1$ and each auxotrophant to compare directly the consequences of partial auxotrophy (Fig. 7B). A decrease in virulence was observed in both *bcass1* knockout mutants, as well as all SOD and reporter gene transformants, in comparison with the wild-type. As expected, pLOB1-based transformants were

Table 2 Summary of transformants used during analysis. Samples include SSOD (sense superoxide dismutase), ASOD (antisense superoxide dismutase), eGFP (enhanced green fluorescent protein), GUS (β -glucuronidase), LUC (luciferase) and mRFP (monomeric red fluorescent protein). Vectors used in transformation, total number of transformants made (*n*) and references are also given.

Samples	Vectors transformed	Total (<i>n</i>)	Reference
SSOD	pLOB1 + pLOB1-SSOD	52	Patel <i>et al.</i> , (2008b)
ASOD	pLOB1 + pLOB1-ASOD	48	Patel <i>et al.</i> , (2008b)
eGFP	pLOB1 + pLOB1-eGFP	24	Patel <i>et al.</i> , (2008a)
GUS	pLOB1 + pLOB1-GUS	28	Patel <i>et al.</i> , (2008a)
LUC	pLOB1 + pLOB1-LUC	28	Patel <i>et al.</i> , (2008a)
mRFP	pLOB1 + pLOB1-mRFP	32	Patel <i>et al.</i> , (2008a)

still more virulent than the knockout $\Delta bcass1-1$. The analysis of lesion area revealed that the wild-type lesion was at least 42% larger than those of all tested transformants (Table 3). A significant correlation was observed between *in planta* and plate-based analysis (*F*-value significant at $R^2 = 0.8967$). Analysis was conducted by comparing the area of the lesion *in planta* (determined using ImageJ analysis) with the area of growth observed during plate-based analysis. These results indicate that *bcass1* down-regulation may be induced by any pLOB1-based vectors.

Northern analysis was performed to determine *bcass1* transcript levels (Fig. 8). All reporter gene transformants showed a degree of *bcass1* silencing in comparison with the wild-type, averaging a c. 50% decrease in *bcass1* transcript. These results suggest that the decrease in virulence is caused by the inadvertent silencing of *bcass1*. The majority of SOD-silenced transformants (Patel *et al.*, 2008b) displayed a lesser degree of *bcass1* silencing, as indicated by the levels of *bcass1* transcript compared with the wild-type.

DISCUSSION

Recent sequence annotation of the *B. cinerea* genome (B05.10, Broad Institute) has increased the need for highly efficient expression and resistance vectors for rapid fungal manipulations and gene screening. pLOB1 is one of the most utilized vector systems for *B. cinerea* (Espino *et al.*, 2005; ten Have *et al.*, 2010;

Fig. 6 Examples of the L-arginine plate-based assay. Transformants were plated on minimal medium supplemented with (+Arg) or without (–Arg) L-arginine. The wild-type (WT) maintains its growth under both treatment schemes. The $\Delta bcass1$ -1 disruption mutant is incapable of growing without L-arginine, but recovers with L-arginine supplementation. Samples GFP1 and GUS163 exhibit stunted growth in comparison with the wild-type without L-arginine, but regain colonizing capabilities on plates with L-arginine.

Fig. 7 (A) Examples of *in planta* testing showing reduced lesion size in partial L-arginine auxotrophic transformants (GUS163, GFP155, GFP1 and LUC 84) in comparison with the wild-type. The left side of the leaf is the wild-type (WT) sample, and the right side is the transformant. (B) Transformants demonstrated growth equal to or greater than that of the knockout mutant ($\Delta bcass1$ -2). The left side of the leaf is the knockout mutant (KO), and the right side is the transformant. The transformants shown contain the β -glucuronidase (GUS) (GUS163), green fluorescent protein (GFP) (GFP155, 1) or luciferase (LUC) (LUC84) exogenous gene.

Table 3 Effect of arginine auxotrophy on virulence and radial growth *in vitro*. Lesion areas of transformants and the wild-type strain B05.10 on bean leaves were quantified using ImageJ, and are presented in arbitrary units ($\pm$ standard error of the mean). The relative virulence of the transformants is shown as the proportion of the average lesion area of a transformant relative to the wild-type (100%). *N* indicates the number of lesions measured. The relative colony diameter of the transformants *in vitro* is also indicated. *n* indicates the number of colonies measured.

Strain	Lesion area of mutant	Lesion area of wild-type	Relative virulence of mutant	Relative colony diameter <i>in vitro</i>
$\Delta bcass1$	4300 $\pm$ 1100 (<i>N</i> = 17)	56700 $\pm$ 7800 (<i>N</i> = 17)	7.5%	0 (<i>n</i> = 3)
GFP1	15300 $\pm$ 900 (<i>N</i> = 5)	81200 $\pm$ 6300 (<i>N</i> = 5)	19%	77 $\pm$ 4% (<i>n</i> = 3)
GFP155	7500 $\pm$ 1000 (<i>N</i> = 7)	37700 $\pm$ 4300 (<i>N</i> = 7)	20%	83 $\pm$ 2% (<i>n</i> = 3)
GUS163	6700 $\pm$ 1500 (<i>N</i> = 5)	48000 $\pm$ 18000 (<i>N</i> = 5)	14%	85 $\pm$ 5% (<i>n</i> = 3)
LUC84	33500 $\pm$ 3900 (<i>N</i> = 5)	58700 $\pm$ 1600 (<i>N</i> = 5)	57%	87 $\pm$ 2% (<i>n</i> = 3)

van Kan *et al.*, 1997; Reis *et al.*, 2005; Rui and Hahn, 2007a; Schouten *et al.*, 2002b). Until recently, the terminator region downstream of the *hph* gene was thought to be the *B. cinerea tubA* terminator. However, analysis has revealed that the termi-

nator region is a 446-bp region of the *B. cinerea AS* gene, which is located adjacent to the *tubA* gene. In retrospect, it appears that an error was made in the restriction mapping of the lambda phage on which the *tubA* gene and the *bcass1* gene are located.

Fig. 8 Northern analysis of *bcass1* mRNA in reporter gene (A) and superoxide dismutase (SOD) (B) transformants exhibiting *bcass1* silencing. All reporter gene transformants showed a degree of *bcass1* silencing in comparison with the wild-type (WT). Green fluorescent protein (GFP) samples (G1 and G152) showed the least amount of silencing, whereas GFP (G155), luciferase (LUC) (L80 and L84), β -glucuronidase (GUS) (B23 and B163) and monomeric red fluorescent protein (mRFP) (M33) transformants exhibited high levels of silencing. SOD transformants displayed a lesser degree of *bcass1* silencing. S44, S160, S165, sense SOD transformants; A76, A100, A101, antisense SOD transformants (Patel *et al.*, 2008b). (A1, B1) Transformants were probed with a partial *bcass1* gene fragment (probe amplified by primer pair BCASS 1 F and BCASS 1 R; Table S1); (A2, B2) *bcactin* (probe amplified with primers ACTIN F and ACTIN R) was used to confirm total mRNA levels.

A 2-kbp *EcoRV* fragment that was judged to be a single fragment in reality appeared to be a doublet, and the fragment containing the true *tubA* terminator region was missing from the phage sequence assembly (J. A. L. van Kan, personal observations). As the fragment that serves as terminator in pLOB1 is a substantial portion of the endogenous *bcass1* gene, any transcriptional readthrough may result in unintentional gene silencing of *bcass1*. The generation of a *bcass1* gene replacement mutant helped to characterize the magnitude of the complete auxotrophic effects *in vitro* and *in planta*. This 'maximum phenotype' could then be compared with *bcass1*-silenced lines, where a range of levels of auxotrophy were likely to occur.

The results demonstrated a reduction in growth on French bean leaves, strawberries and tomatoes. Previous studies in other fungi have demonstrated the importance of amino acid biosynthesis to fungal virulence (Divon and Fluhr, 2007). Reduction in virulence has been reported in *Fusarium oxysporum* f. sp. *melonis* mutants defective in AS (Namiki *et al.*, 2001), *Cladosporium fulvum* and *Fusarium graminearum* defective in methionine biosynthesis and *Magnaporthe grisea* defective in histidine biosynthesis (Seong *et al.*, 2005; Solomon *et al.*, 2001; Sweigard *et al.*, 1998). The host-related variation in virulence may be caused by differences in L-arginine concentrations present in each host tissue. In particular, strawberries and tomatoes contain

substantially less L-arginine (0.2617 and 0.2195 mg/g of fruit, respectively) than grapes (0.4875 mg/g of fruit) (Agricultural Handbook, 1–23, US Department of Agriculture). It may be hypothesized that the concentrations of free L-arginine present in strawberries, tomatoes and French bean leaves are not adequate for complete knockout rehabilitation. On the other hand, grapes contain a higher concentration of free L-arginine, which may allow infection by the mutant. Thus, the virulence of $\Delta bcass1-1$ and $\Delta bcass1-2$ may be heavily reliant on host free L-arginine concentrations and may be exploited to discover nutrient uptake complexities during ensuing fungal infections. The addition of arginine to the conidial suspension used for plant inoculation temporarily relieved the phenotype of *bcass1* auxotrophic transformants in a concentration-dependent manner and allowed the formation and outgrowth of lesions into plant tissue where the level of free arginine was insufficient to sustain growth of the auxotrophic transformant. The higher the arginine concentrations provided in the inoculum, the longer the transformant developed lesions as normal, but lesion development eventually always slowed down or stopped entirely (J. A. L. van Kan, unpublished data).

Although it is difficult to link L-arginine auxotrophy to fungal virulence, it has been hypothesized that a high concentration of L-arginine in *Gibberella zeae* is necessary to shift vegetative

growth to sexual reproduction (Kim *et al.*, 2007). Linkages between fungal sexual and asexual development, as well as virulence, have also been observed in *Schizosaccharomyces pombe*, *Saccharomyces cerevisiae*, *Candida albicans*, *Neurospora crassa*, *Aspergillus nidulans*, *Ustilago maydis*, *Cryphonectria parasitica*, *Magnaporthe grisea* and *Cryptococcus neoformans* (Bolker, 1998; Kim *et al.*, 2007). As $\Delta bcass1$ -1 and $\Delta bcass1$ -2 displayed a severe decrease in pathogenicity on French bean leaves, the induction of *bcass1* silencing may also result in transformants exhibiting reduced levels of virulence. The pLOB1-MCS silencing vector was utilized as a sense silencing construct for the induction of AS down-regulation, with the frequency of sense transformants exhibiting *bcass1* silencing-induced auxotrophy at 41%. *In planta* infection assays were assessed and found to produce a range of reductions in virulence, correlating with those observed during plate-based (*in vitro*) and Northern analysis. Thus, evidence shows that *bcass1* is essential for fungal growth when external L-arginine concentrations are limiting, and, when silenced, results in a reduction in hyphal radial growth *in vitro* and host colonization *in planta*.

As the pLOB1-MCS silencing cassette was able to induce *bcass1* silencing, the employment of any pLOB1-based expression or hygromycin resistance vector during *B. cinerea* molecular manipulations may inadvertently induce *bcass1* silencing, and our results on plate-based assays suggest that inadvertent silencing occurs at c. 7% frequency using a wide range of vectors expressing marker genes (eGFP, GUS, LUC and mRFP) or targeting genes for silencing, such as SOD.

The transformants exhibiting *bcass1* silencing were tested *in planta*. All reporter and SOD transformants exhibiting *bcass1* silencing showed reduced lesion development in *in planta* assays. As reporter genes are generally nontoxic exogenous genes, the virulence of reporter strains should be unaffected after transformation. Northern analysis of these transformants demonstrating *bcass1* silencing also exhibited reduced levels of *bcass1* transcript. Hence, *in planta* observations are a result of partial L-arginine auxotrophy. These results also correlate with those observed during plate-based analysis. As *bcsod1* has been found to be a pathogenicity factor (Rolke *et al.*, 2004), the observed reduction in virulence in SOD-silenced lines (Patel *et al.*, 2008b) may be caused by a combination of *bcsod1* and *bcass1* silencing. However, our investigations have shown that SOD knockout mutants exhibit wild-type growth capabilities on French bean leaves (Patel *et al.*, 2008b). As a result, the reduction in virulence by SOD-silenced transformants may be attributed to inadvertent *bcass1* down-regulation.

Our investigations therefore confirm that pLOB1-based vectors have the potential to trigger the silencing of *bcass1*. Some of these silenced lines appear to have significantly reduced levels of virulence, raising the possibility that knockout phenotypes showing reduced levels of virulence could be a result of

inadvertent silencing of *bcass1*, not target disruption. REMI or T-DNA mutants, obtained using pLOB1-based vectors, which display interesting phenotypes, could also be caused by inadvertent *bcass1* silencing rather than insertional mutagenesis. We recommend that previously established transformants based on pLOB1 should be re-evaluated for L-arginine-dependent growth, and that a different vector series should be developed for all subsequent manipulations in *B. cinerea*, such as the pOT vector system (Patel *et al.*, 2008a, b).

EXPERIMENTAL PROCEDURES

Fungal strains and growth conditions

Experimental analyses and transformation were performed on *B. cinerea* strain B05.10. *Botrytis cinerea* conidia were stored as glycerol stocks at -80°C . Two *bcass1* ($\Delta bcass1$ -1 and $\Delta bcass1$ -2) and three *bcsod1* ($\Delta bcsod1$ -1, $\Delta bcsod1$ -2 and $\Delta bcsod1$ -3) knockout mutants served as controls during *in vitro* and *in planta* experimentation. The fungus was grown on malt agar containing 5% (w/v) malt extract and 1.8% (w/v) agar and incubated at 20°C under near-UV light to induce sporulation. Conidia were harvested 7–14 days post-inoculation in 10 mL of water containing 0.05% (v/v) Tween-80, and collected by centrifugation at 3000 g for 5 min. For DNA extraction, 5% (w/v) malt extract broth was inoculated with 5×10^8 spores/L and incubated at 20°C and 180 r.p.m. for 24 h. Mycelia were harvested by centrifugation and lyophilized.

Transformation of *B. cinerea*

Transformants were produced using the methodology described by Hamada *et al.* (1994) with modifications described by van Kan *et al.* (1997). Auxotrophic mutants and silenced transformants were recovered by supplementation of regeneration agar with 1 mM L-arginine. Emerging fungal colonies were subjected to several subculturing events (with L-arginine supplementation) to select against heterokaryons containing mixtures of transformed and untransformed nuclei.

bcass1 gene replacement

The left arm (representing 1591 bases of the 5' untranslated region and 360 bases of the *bcass1* open reading frame) of the knockout vector was amplified with the primer pair LA SACI F and LA SACI R containing *SacI* restriction sites. The right arm (representing 801–2352 bases past the translational stop codon) containing *HindIII* and *XhoI* restriction sites was amplified with the primer pair RA HINDIII F and RA XHOI R (Table S1, see Supporting Information). Fragments were independently ligated into the hygromycin resistance vector pOT-HYG (controlled by

the *Aspergillus nidulans* *oliC* promoter and *Aspergillus nidulans* *trpC* terminator) and digested with the corresponding restriction enzymes (Fig. 2A). The complete knockout vector, p Δ bcass1, was used to transform strain B05.10. The recovered transformants were plated in triplicate on minimal medium with and without L-arginine supplementation.

For the complementation of the knockout mutants, the complete *bcass1* region was amplified from the wild-type strain B05.10 by PCR with primers COMP BCASS1 F and COMP BCASS1 R (Table S1, see Supporting Information) and cloned into the TOPO TA Cloning pCR $\text{\textcircled{R}}$ 2.1-TOPO $\text{\textcircled{R}}$ vector. The subsequent expression cassette, pBCASS1, was used for complementing Δ bcass1-1 and Δ bcass1-2 mutants.

Standard nucleic acid techniques

Fungal genomic DNA was isolated as described by Schouten *et al.* (2002b). PCR analysis was performed as described by Saiki *et al.* (1988) using Reddy Mix (Bioline), and amplification products were separated by gel electrophoresis. The primers used during PCR analysis are listed in Table 1. Purified products (Wizard Gel Extraction Kit, Promega) were cloned using the pCR 2.1 TOPO Cloning Kit (Invitrogen). Ligation strategies were designed using Clone Manager Suite (Sci Ed Central). Sequence analysis and alignment were aided by BLAST analysis at the National Center for Biotechnology Information (NCBI) (Altschul *et al.*, 1990).

Northern and Southern analysis of total RNA and genomic DNA was completed according to Sambrook *et al.* (1989) with slight amendments (Heneghan *et al.*, 2007; Kilaru *et al.*, 2009). Fungal genomic DNA (5 μ g) was digested to completion with *SacI* or *Apal* (50 units). Digested fragments were separated on a 1.0% (w/v) agarose gel. The gel was depurinated with 0.25 M HCl for 15 min and denatured with 0.5 M NaOH for 30 min before being blotted onto Hybond-N membrane (GE Healthcare). Membranes were rinsed with 2 $\times$ standard saline citrate (SSC; 0.3 M NaCl and 0.03 M sodium citrate, pH 7) after RNA or DNA transfer. The RNA or DNA was immobilized by UV cross-linker.

The membrane containing the *SacI*-digested DNA was hybridized with a partial *bcass1* gene fragment (amplified with primers BCASS1 F and BCASS1 R; Table S1, see Supporting Information), and the filter containing *Apal*-digested DNA was hybridized with a partial hygromycin resistance gene fragment (amplified with primers HYG F and HYG R; Table S1, see Supporting Information). Integration of the complete p Δ bcass1 knockout vector was confirmed by PCR with primers LA SACI F and RA XHOI R (Table S1, see Supporting Information).

L-Arginine auxotrophy measurements

L-Arginine auxotrophy was assayed on Gamborg's minimal agar [GM, 3.16 g/L Gamborg's B5 (DUCHEFA Biochemie B.V.,

Haarlem, the Netherlands), 100 mM sucrose, 100 mM sodium phosphate, 18 g/L agar] supplemented with or without filter-sterilized 200 μ M L-arginine. Plates were incubated at 20 °C for 3 days before radial measurements were taken. Silencing frequencies were calculated by comparing the colony diameters in the presence and absence of L-arginine. Transformants exhibiting a $\geq 10\%$ decrease in radial growth without L-arginine (in comparison with minimal medium with L-arginine) were considered to be demonstrating a level of *bcass1* silencing.

Infection assay on French bean, strawberry, tomato and grape

Conidia of sporulating *B. cinerea* wild-type and transformants were harvested and resuspended in H₂O at a working concentration of 1×10^6 conidia/mL. Infection studies were conducted on 14-day-old dwarf French bean plants (Tendergreen) in triplicate. Punctured plant leaves were then inoculated with a single droplet containing 5×10^3 conidia from the wild-type and a transformant. Leaves were incubated over a period of 5 days with diurnal cycles of 16 h light and 8 h darkness and 100% humidity, before being photographed. Areas of infection were then determined with ImageJ software (Abramoff *et al.*, 2004).

Commercially sourced strawberries and grapes were sliced in half and surface sterilized for 5 min in a 5% (v/v) bleach solution and sterilized distilled water. Fruit halves were then arranged in Petri dishes with 10 mL of sterilized distilled water before being punctured with a sterile plastic pipette tip and inoculated with 5 μ L of a spore suspension of the B05.10 wild-type strain (left half of fruit) and appropriate transformant or knockout mutant (right half of fruit). The Petri dishes were incubated at room temperature for 3 days.

ACKNOWLEDGEMENTS

We would like to thank Professor Paul Tudzynski (University of Munster, Germany) for providing the SOD cDNA and knockout mutants. We would also like to acknowledge Dr Michael Pearson (University of Auckland, New Zealand) for his ongoing collaborations and discussions. This research was made possible by the generosity of the LESARS (University of Bristol, UK) funding body. This work was carried out under DEFRA Licence PHL 247A/6129.

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SUPPORTING INFORMATION

Additional Supporting Information may be found in the online version of this article:

Table S1 Primer sequences utilized during experimentation.

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