Review of: "Multiple knockout mutants reveal a high redundancy of phytotoxic compounds that determine necrotrophic pathogenesis of Botrytis cinerea"

Nélida Brito[1]

1 University of La Laguna

Potential competing interests: The author(s) declared that no potential competing interests exist.

The manuscript submitted by Leisen et al. describes the generation of multiple knockout mutants of the phytopathogenic fungus Botrytis cinerea using the CRISPR/Cas system. The authors used an efficient and selectable-marker free method to mutate up to 12 genes coding for proteins able to induce plant cell death or involved in the phytotoxins botrydial and botcinin synthesis. This strategy was used to address the functional redundancy of virulence factors of this fungus and confirm the existence of yet to be discovered proteins involved in the infection process. Moreover, the authors propose a mutated version of the Bcpos5 gene conferring resistance to the fungicide cyprodinil as a new selection marker in Botrytis.
All experimental steps were performed logically, and the manuscript is well-written and illustrated. However, there are some considerations that I would like the authors to take into consideration and discuss.

- The new resistance cassette generated in this work contains a mutated version of the B. cinerea Bcpos5 gene coding for a mitochondrial NADPH kinase flanked by the trpC promoter of A. nidulans and the niaD terminator of B. cinerea. This cassette confers resistance to cyprodinil, a fungicide frequently used in agriculture to fight against Botrytis. However, the appearance of cyprodinil resistant strains is well-documented, even associated with multi-resistance events. The authors should discuss the following issues related to this new selective marker:
  - The low and highly variable transformation efficiency depending on the targeted gene (e.g., 24.4 to 0.6 transformants per microgram of DNA for the spl1 and glx1 genes, respectively).
  - The high frequency of spontaneous cyprodinil resistant strains: more than 20% of the cypR transformants generated using the knockout constructs for deletion of the xyn11A, ieb1, and spl1 genes were spontaneous resistant strains. It seems a very high frequency for a useful selective marker.
  - The ectopic integration of the knockout constructs for the nep2 and spl1 genes occurred in more than 50% of the transformants. The use of the Botrytis Bcpos5 gene fused with a terminator fragment of another Botrytis gene (the niaD gene) increases the frequency of the ectopic integration of the knockout constructs.
  - Taking into account these results, the authors should discuss or reconsider the claim that the cypR

cassette is as efficient as others already tested and commonly used.
    - Please, discuss whether this new resistance marker could result in a higher rate of spontaneous fungicide resistance in field strains.
    - Clarify if homokaryons of the single mutants were isolated for further phenotypic analysis.
    - Clarify the statistical analysis performed.

- The Crispr/Cas system used to generate the marker-free multiple knockout mutants includes an unstable telomere vector which confers transient resistance to the fungicide fenhexamid (the pTEL-Fen vector). Therefore, the initial selection of transformants should be in a medium supplemented with fenhexamid. Please correct/precise in the material and methods section.
- The new Crispr/Cas system allows targeting simultaneously two genes. The transformation of the 3x strain with specific sgRNAs targeting the xyn11A and ieb1 genes did not generate any double mutant strain. However, the 6x strain was successfully mutated for the plp1 and ieb1 loci simultaneously. If the same ieb1-gRNA was used in both transformations, the authors should discuss the possible reasons for these different efficiencies for the same sgRNA.
- The efficiency of targeted gene deletion decreased as the number of mutated genes in the genome increased. The deletion of the spl1 gene (the first knockout mutant generated using the Crispr/Cas protocol) occurred in 68% of the transformants tested. However, this percentage decreased to 3 or 9% for targeting new genes in the 10x strain. Please, discuss this accumulative inefficiency.
- Please add in table 2 the percentages of homokaryons generated in each transformation round when the two targeted genes were simultaneously mutated.
- The final transformation round of the 10x strain to generate the pg1 and pg2 double mutant also affected the bcin14g00600 gene, coding for a polyketide synthetase. The authors claimed that this gene is not expressed in planta, and its putative role in virulence is discarded. However, it is well known that the virulence factors are differentially expressed depending on the host and the different stages of the infection process. Therefore, please, show the bcin14g00600 gene expression in the hosts used to study fungal virulence.
- One of the objectives of this work is to compare the virulence of the knockout strains sequentially as the different genes are mutated. However, the authors used strains with completely different genomic backgrounds. For example, the spl1-single mutant or the quadruple mutant $4x^R$ (deleted for the spl1, nep1, nep2 and xyn11A genes) strains generated by homologous recombination of different resistance cassettes were phenotypically compared with the 6x,8x,10x, 11x, and 12x strains, all of them obtained with the Crispr/Cas method. However, the free-selective marker spl1-single mutant and 4x strains were also generated. Please, discuss/precise the reasons for that choice.
- Overall, please clarify the statistical analysis of the data. Please, include the number of repetitions of each assay. The way used to define the maximum and minimum lengths of the whiskers is missed, and the whiskers themselves are omitted in some graphs. The authors performed a Dunnett test to compare

the differences between the necrotic areas produced by the mutant strains or their secretomes on different host leaves. Since this test compares means from experimental groups against a control group mean, and the authors established differences between mutant strains (lines 267-269), the Tukey test should be a better option.

- Concerning the phenotypic characterization of the multiple mutant strains, the authors established that the production of sclerotia and conidia are similar in all strains (Figure 2). Please, include the quantification of these asexual reproductive structures.
- The authors studied the necrotic area produced by the multiple mutant strains generated by sequential deletion of 4 up to 12 genes (the 4xR and 12x strains, respectively) on different hosts (Figure 3). The deleted genes in the 4xR strain were the spl1, xyn11a, nep1 and nep2 genes. Since the role of the death-plant inducing proteins and toxins in pathogenesis is analysed, some conclusions related to the virulence tests should be further discussed.
  -
  - The sentence "On all tested tissues, infection efficiency of the mutants decreased with the increasing number of deleted genes" (Lines 216-217) should be reconsidered as describes only the results on bean leaves (figure 3A).
  - The data of tomato leaves infection showed that only four strains were significantly different from the wild type: the 4xR strain that only produced a slight reduction of the necrotic area; and the 11x, 12xpg and 12xbb strains that showed a further decrease of the lesion sizes and were statistically similar. Therefore, the infection efficiencies of the 4x, 6x, 8x, 10x strains were similar, although six more genes were deleted in the 10x strain than in the 4x strain. Similarly, the mutation of the pg2 gene in the 11x strain did not produce any change. These results suggest that PG1, BOT2 and BOA6 and, to a lesser extent, SPL1, XYN11a, NEP1 or NEP2 are involved in the infection of this host.
  - Based on a one-sample t-test, all strains were similar but statistically different from the wild type on detached maize leaves. Therefore, there is a contradiction between these results and the interpretation of the infection efficiency of the 10x strain (lines 219-220). Also, Tukey's multiple comparison test confirmed that the significant reduction of the necrotic area in maize leaves was similar in all tested strains. These results suggest that only the deleted genes in the 4xR strain are involved in the infection of this host. Since NEP1 and NEP2 are only active on dicot, SPl1 and XYN11A should be relevant in this process.
  - The statistical analysis of the results of the virulence assays on apple fruits also showed that the 4x, 6x, 8x, and 10x strains were statistically similar and, therefore the significant reduction of the necrotic area observed in these mutants is related to the mutation of the spl1, xyn11a, nep1 or nep2 genes.
  - The analysis of the virulence tests on tomato leaves suggests a slight effect of the mutation of the spl1, xyn11a, nep1 and nep2 genes (strains 4x, 6x,8x). However, the 10x strain produced smaller necrotic areas than the 4x, 6x, and 8x strains. Therefore, XYL1 or GS1 should also be involved in the infection process.

- NEP1 and NEP2 have already been discarded as virulence factors. Altogether, data suggest that SPL1, XYN11a, XYL1 or GS1 should be involved in the infection process, although the effect of their mutation depends on the host. These results are not consistent with the phenotypic analysis of the corresponding single mutant strains that are shown in the manuscript.

- Bean leaves were infected with the 12x and wild type strains to detect and measure the necrotic area by microscopic analysis (Figure 4), and results are shown as a percentage. Please, clarify what was considered 100%. Also, precise the meaning of the following sentence: “These data show that the CDIPs deleted in these mutants are involved in the early stages of lesion formation on unwounded leaf tissue” (Lines 245-246). The 12xpp and 12xbb strains are mutated in 12 genes. Does the sentence suggest that the twelve genes mutated in each 12x strain are involved in the early stages of the infection process? No other mutants than 12x strains were assayed.
- Please, clarify in figure 5 what was considered 100%. A Dunnett test was used to analyse these data that only allows comparing the lesions produced by each mutant with the wild type. Therefore, please, check the following statement: “Compared to 10x, the secretomes of 12xpg and 12xbb showed decreased activity on V. faba and N. benthamiana, respectively, indicating differential effects of the loss of PG1/PG2 (in 12xpg) and botrydial/botcinin (in 12xbb) on the different plant species (Fig. 5A-D)”. Tukey test should be performed to compare the mutant strains. The secretomes were diluted in GB5 medium before infiltration. Please, include the infiltration controls with just the GB5 medium at the same final concentration used in the secretomes assays.
- Table S2 summarizes the proteomic analysis of the on planta produced secretomes of the multiple mutant strains. Please, explain the high standard deviation values, greater than the mean values in many assays. Was any statistical analysis performed? Please, a more detailed discussion is needed to understand the low but detectable values found for SPL1, PG1 and PG2 in the secretomes of strain mutated for these genes.
- Table S2 summarizes the proteomic analysis of the on planta produced secretomes of the multiple mutant strains. Please, explain the high standard deviation values, greater than the mean values in many assays. Was any statistical analysis performed? Please, a more detailed discussion is needed to understand the low but detectable values found for SPL1, PG1 and PG2 in the secretomes of strain mutated for these genes.