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1 **Comparative quantitative proteomics of osmotic signal transduction mutants in *Botrytis***
2 ***cinerea* explain mutant phenotypes and highlight interaction with cAMP and Ca²⁺**
3 **signalling pathways**

4
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18
19 **AUTHOR CONTRIBUTIONS STATEMENT**

20 JK designed and performed all experiments, assisted by MD for the proteomics workflow.
21 MZ performed proteomic data analysis and corresponding statistics. AS performed *in silico*
22 cluster analysis and functional enrichment studies. SF and MZ conceived the initial project.
23 All authors participated to manuscript writing under the coordination of SF.

24
25 The authors disclose any conflict of interest.

26
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29
30 **Data accession:**

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32

33 **SUMMARY**

34 Signal transduction (ST) is essential for rapid adaptive responses to changing environmental
35 conditions. It acts through rapid post-translational modifications of signalling proteins and
36 downstream effectors that regulate the activity and/or subcellular localisation of target
37 proteins, or the expression of downstream genes. We have performed a quantitative,
38 comparative proteomics study of ST mutants in the phytopathogenic fungus *Botrytis cinerea*
39 during axenic growth under non-stressed conditions to decipher the roles of two kinases of the
40 hyper-osmolarity pathway in *B. cinerea* physiology. We studied the mutants of the sensor
41 histidine kinase Bos1 and of the MAP kinase Sak1.

42 Label-free shotgun proteomics detected 2425 proteins, 628 differentially abundant between
43 mutants and wild-type, 270 common to both mutants, indicating independent and shared
44 regulatory functions for both kinases. Gene ontology analysis showed significant changes in
45 functional categories that may explain *in vitro* growth and virulence defects of both mutants
46 (secondary metabolism enzymes, lytic enzymes, proteins linked to osmotic, oxidative and cell
47 wall stress). The proteome data also highlight a new link between Sak1 MAPK, cAMP and
48 Ca²⁺ signalling.

49 This study reveals the potential of proteomic analyses of signal transduction mutants to
50 decipher their biological functions.

51

52

53

54 INTRODUCTION

55 *Botrytis cinerea* is a necrotrophic, polyphageous plant pathogenic fungus responsible for grey
56 mold disease of nearly 600 plant genera [1], many of which have significant agronomic
57 importance. *B. cinerea* can infect different plant organs such as leaves, flowers or fruits and
58 causes extensive pre- and post-harvest damage leading to yield losses. The resulting economic
59 impact may then be considerable [2, 3].

60 The infection process of *B. cinerea* involves the secretion of lytic enzymes, production of
61 toxins, reactive oxygen species and small RNAs [4]. In return, *B. cinerea* has to face to many
62 stresses caused by its environment and in particular by the infected plant such as osmotic,
63 oxidative and cell wall stress, phytoalexins and other plant defence compounds [5]. Response
64 and adaptation to these stresses is therefore crucial for the survival of the fungus. To be able
65 to persist in this hostile environment and to counter the plant defence, the fungus develops
66 rapid adaptive responses. It perceives these stresses and transmits them to the cell in order to
67 give an adequate response *via* signalling pathways [6].

68 One of the main signalling pathways in eukaryotes involves MAPKs (Mitogen Activated
69 Protein Kinase), evolutionary conserved signalling pathways. Depending on their
70 phosphorylation status, the activated MAPKs act by regulating numerous cellular processes,
71 notably by activating transcription factors [7]. MAPK cascades are preserved in ascomycetes,
72 but their roles differ according to the fungal species [8]. In *Saccharomyces cerevisiae*, five
73 MAPK pathways have been described, controlled respectively by Fus3, Kss1, Slt2, Hog1 and
74 Smk1 [9]. The functionally redundant MAPKs Fus3 and Kss1 are involved in sexual and
75 asexual reproduction. Slt2 regulates cell cycle progression and cell wall integrity, while Hog1
76 regulates the response to osmotic and oxidative stresses. The less studied MAPK Smk1 is
77 involved in the assembly of the ascospore wall. Some MAPK pathways have partially
78 overlapping functions or are interconnected [10]. With the exception of Smk1, homologues of
79 these MAPKs are found in filamentous fungi [8]. Thus, the *B. cinerea* MAPKs Bmp1, Bmp3
80 and Sak1 are respectively the homologues of the yeast proteins Fus3/Kss1, Slt2 and Hog1
81 (reviewed in [6]).

82 In *B. cinerea*, the MAPK Bmp1 is involved in vegetative growth, hydrophobic surface
83 perception, pathogenicity, spore and sclerotium formation [11-13]. The MAPK Bmp3 is
84 involved in adaptation to hypo-osmotic and oxidative stress and to the fungicide fludioxonil
85 [14].

86 The Sak1 MAPK pathway controls fungal development such as growth and macroconidium
87 formation. It is involved in the adaptation to various stresses such as osmotic (ionic),

88 oxidative and cell wall stress but also in infection [15, 16]. Indeed, the $\Delta sak1$ mutant is unable
89 to form infection structures and thus to penetrate its host unless the latter is injured [15].
90 Cross-talk between the Bmp3 and Sak1 pathways has been detected during oxidative stress
91 [16].

92 Sak1 targets transcription factors such as BcReg1 and BcAtf1 [17, 18]. BcReg1 is involved in
93 the regulation of secondary metabolite synthesis, sporulation and host tissue colonization [17]
94 while BcAtf1 is involved in sporulation and the regulation of genes involved in stress
95 response [18].

96 Upstream of the osmoregulatory MAPK pathway, the perception and transmission of signals
97 pass through a network of signaling proteins referred to as two-component systems. In fungi,
98 the histidine kinase sensor is a hybrid protein (HHK) that contains both a sensor domain and a
99 response regulatory (RR) domain [19]. Thus, following the perception of a signal, the
100 histidine kinase catalyses its autophosphorylation on the conserved histidine residue, then the
101 transfer of the phosphate group to a conserved aspartate residue of the RR domain. The signal
102 is then transmitted *via* a phosphotransfer protein (PTH) to a RR protein [19]. In *B. cinerea*,
103 there are two histidine kinases upstream of the Sak1 pathway, Bos1 and BcHK5. The class VI
104 histidine kinase, BcHK5, is the homologue of the unique HHK in *S. cerevisiae* Sln1.
105 Indispensable in yeast because of its role in regulating stress response, this histidine kinase is
106 not essential for development and pathogenicity in *B. cinerea* (reviewed in [6]). In several
107 fungi, the deletion of class III histidine kinase leads to resistance to phenylpyrroles and
108 dicarboximides but also to sensitivity to osmotic, oxidative and cell wall stresses [20-22]. This
109 holds true also in *B. cinerea* [23]. An original finding was the the constitutive phosphorylation
110 of the MAPK Sak1 in the $\Delta bos1$ mutant, indicating that the two-component system negatively
111 controls the activation of the osmoregulatory pathway [24].

112 Heller et al [25] conducted a small scale transcriptomic analysis of the *B. cinerea* $\Delta sak1$
113 mutant compared to the wild type. Albeit performed on a limited set of genes, this microarray
114 analysis revealed Sak1 as central element in the overall response to stress and in the infectious
115 process at the transcriptional level.

116 Another level in systematic analysis is achieved through proteomics. The analysis of the
117 accumulation of intracellular proteins takes into account all regulations during protein
118 synthesis (*i.e.*, transcription and translation) and degradation (mRNA and protein turn-over)
119 [reviewed in 26]. Although shotgun proteomics have in principle democratized global
120 proteome analyses, such as subcellular proteomics, dynamic proteomic changes and post-
121 translational modification, the required technical equipment and know-how or access to

122 proteomic platforms, as well as costs of analysis are still important limiting factors orientating
123 functional genomics in favour of transcriptomics [27]. Proteomic analyses in *Botrytis cinerea*
124 have mainly focused on sub-proteomes (e.g., secreted or membrane proteins) or
125 phosphoproteomes [28-37].

126 In this study, we analysed the role of the osmotic ST pathway in the biology of *B. cinerea* and
127 particularly the role of the histidine kinase Bos1 and the MAPK Sak1. In order to highlight
128 proteins whose abundance depends on the osmoregulatory pathway, we performed a
129 comparative proteomic analysis of the $\Delta sak1$ and $\Delta bos1$ mutants compared to the wild type
130 strain during exponential growth under axenic growth conditions.

131 Protein abundance variations corroborated the implication of both protein kinases in
132 biological functions related to the infection process. Moreover, this study highlighted cross-
133 talk between the Sak1 and the cAMP pathway which, on its turn, is involved in pathogenicity
134 as well. Finally, our study revealed different sets of regulation patterns among the detected
135 proteins.

136

137 **MATERIAL AND METHODS**

138 **Strains, medium and culture conditions**

139 *B. cinerea* wild-type strain, B05.10 [38], as well as the mutants $\Delta sak1$ and $\Delta bos1$ [15, 24]
140 were cultivated on solid Sisler medium (KH₂PO₄ 2 g l⁻¹, K₂HPO₄ 1.5 g l⁻¹, (NH₄)₂SO₄ 1 g l⁻¹,
141 MgSO₄ 7H₂O 0.5 g l⁻¹, glucose 10 g l⁻¹, yeast extract 2 g l⁻¹, agar 15 g l⁻¹).

142 Pre-cultures were made from calibrated explants (5 mm) deposited on plate with solid Sisler
143 medium previously covered with a sterile cellophane membrane (Bio-Rad, USA). The
144 cultures were maintained 4 days at 20°C, in darkness. The mycelium was recovered and
145 crushed for 1 minute in 100 mL of Sisler liquid medium using an Ultra Turax. 500 µl of this
146 shred was spread on solid Sisler cellophane coated medium. The cultures were incubated in
147 the dark at 20°C for 2 days. After these 2 days, the samples were collected and frozen in
148 liquid nitrogen and freeze-dried. Four replicates were made for each strain.

149

150 **Protein extraction and tryptic digestion**

151 Proteins of the various genotypes were extracted from lyophilized mycelia of exponential
152 growth phase after grinding mycelium in liquid nitrogen. Proteins were precipitated using a
153 precipitation solution (10% trichloroacetic acid (TCA) and 0.07% β-mercaptoethanol, in
154 acetone). After vortex and centrifugation at 9838 g at -20°C, the pellet was rinsed three times
155 in acetone with 0.07% β-mercaptoethanol to remove TCA. After drying, the pellet was

156 weighed and proteins were solubilized at 20 μ l per mg in ZUT buffer (Urea 6 M, Thiourea 2
157 M, DTT 10 mM, Tris-HCl 30 mM pH 8.8, 0.1% of ZALS (ProgentaTM Zwitterionic Acid
158 Labile Surfactants - Protea Bioscience). After 5 min of centrifugation at 14000 g and 25°C,
159 the supernatant was collected.

160 Protein concentration was measured with the "2D Quant kit" (GE Healthcare Life Sciences),
161 according to the supplier's instructions. 40 μ g of extracted proteins were sampled and the
162 volume adjusted with ZUT buffer to adjust the concentration to 4 μ g μ l⁻¹. Proteins were
163 alkylated with iodoacetamide (final concentration 50 mM) for 45 min at room temperature in
164 the dark. Samples were then diluted with 50 mM ammonium bicarbonate solution to reduce
165 the urea concentration to 1 M. Proteins were digested overnight at 37°C with 800 ng trypsin
166 (Promega). Digestion was stopped by adding 5 μ l TFA (Trifluoroacetic acid) at 20%, then
167 incubated 30 min at room temperature to cleave ZALS. The peptide extracts were desalted on
168 C18 column (StrataTMXL 100 μ m Polymeric Reversed Phase - Phenomenex).

169

170 **LC-MS/MS analysis**

171 For each sample 4 μ l of the peptide mixture obtained by trypsin digestion were injected and
172 separated by HPLC (High Pressure Liquid Chromatography) Nano LC-Ultra system
173 (Eksigent, United Kingdom). Chromatography uses a 30 cm C18 column (Nanoseparations,
174 Netherlands) and a 99.9% CH₃CN + 0.1% formic acid buffer gradient, according to the
175 following steps: 5% to 35% buffer for 110 min; 35% to 95% buffer for 3 min; 95% buffer for
176 10 min. The injection into the QexactivePLUS mass spectrometer (Thermo Fisher Scientific,
177 USA) was performed from a nano-electrospray source (New Objective, USA).

178 The whole system was controlled by Xcalibur 4.0 with the following acquisition method: the
179 acquisition of MS spectra was performed over a range $m/z = 400 - 1400$ with a resolution of
180 70000. The eight most intense ions (Top 8) underwent HCD (Higher energy collisional
181 dissociation) fragmentation with collision energy of 27%, thus obtaining an MS/MS spectrum
182 acquired at a resolution of 17500. A dynamic exclusion of 40 sec was used.

183 Peptide identification was performed using X!Tandem software [39] (PILEDRIIVER
184 01/04/2015 version) where mass changes were indicated. The mass change corresponding to
185 carbamidomethylation of cysteines (57.02146 Da) was reported as a systematic change.
186 Potential modifications have been added such as oxidation of methionines (15.99491 Da) and
187 acetylation to N-terminal (+42.01056 Da). The mass tolerance of the precursor was 10 ppm
188 while that of the fragment was 0.02 Da. Only one missed cleavage was allowed.

189 The *B. cinerea* database provided to the software was retrieved from Ensembl
190 (http://fungi.ensembl.org/Botrytis_cinerea/, release 34) and previously functionally re-
191 annotated from published data [40] and automatic annotations obtained with Pfam [41]. A
192 database of standard contaminants has also been added. The identified proteins were filtered
193 and grouped with X!Tandem Pipeline v3.4.0 [42]. Data with a peptide E-value < 0.01, at least
194 two peptides per protein and a protein E-value of 10^{-4} were retained. The FDR (False
195 Discovery Rate) was estimated at 0.02% for peptides and 0% for proteins.

196 Identified proteins, peptides and their corresponding spectra were deposited in PROTiCdb
197 database [43] at the following URL http://moulon.inra.fr/protic/botrytis_signalling using the
198 following login: “review” and password (required during the review process only): “review”.
199 After acceptance of the manuscript, data can be accessed by doi
200 10.15454/1.5506737620833718E12.

201 **Quantification, statistical analysis and annotation**

202 Quantification was based on the analysis of the MS1 XIC (eXtracted Ion Current) of each of
203 the identified peptides, by using the MassChroQ software v2.2 [44]. XIC data (peak intensity
204 integration) was used to quantify proteins for which at least two specific peptides were
205 quantified in at least 95% of the samples. For proteins that did not reach this criterion,
206 detected peaks were counted (peak counting, PC) instead of being integrated.

207 For XIC quantification, normalization was performed according to the median
208 sample/reference ratio of peptide ion XIC values, the reference being a WT sample. Peptide
209 ion missing XIC data were imputed according to the correlation between peptides of the same
210 protein. The relative abundance of each protein was computed by summing the intensities of
211 its specific peptides. An analysis of variance (linear model) on \log_{10} -transformed abundances
212 was performed to study the variations according to the strain. Proteins with adjusted p-value <
213 0.01 and a fold-change (mutant/WT) above 1.5 or below 0.66 were considered significantly
214 affected by the mutant.

215 For PC, an analysis of variance was performed by using a general linear model and the same
216 criteria as those employed for XIC quantification were used to consider a protein significantly
217 affected by the mutation. The R software (version 3.4.4 [45]) was used for all statistical
218 analyses.

219 Proteins were grouped into regulatory groups and run through gene ontology (GO) functional
220 enrichment analysis using FungiFun2 (<https://sbi.hki-jena.de/fungifun/fungifun.php>)[46].
221 Categories with adjusted exact p-values < 0.05 were considered as enriched. Lists of expert

222 annotated genes [6, 40, 47-49] were also used to perform enrichment analyses via Fisher's
223 exact tests. Significance thresholds were set as follows: number of proteins ≥ 4 (except for
224 alternative respiration with $n \geq 2$); oddsratio > 2 ; p-value < 0.05 .

225 The same regulatory groups were analysed through the STRING database v11 [50] using
226 default parameters and subsequent MCL cluster analysis [51].

227

228 **Intracellular cAMP measurements**

229 The different strains were grown under conditions identical to those for the proteomic
230 analyses. Intracellular cAMP concentration was measured using the Amersham cAMP
231 Biotrak Enzyme-immunoassay System kit (GE Healthcare). 4 mg of lyophilized mycelium
232 was crushed in a Fast-Prep machine (MP Biomedicals) using Garnet Matrix A (MP
233 Biomedicals) and Lysis Buffer 1B (supplied in the assay kit). After centrifugation, the
234 supernatant was collected and used for the enzyme test. The calculation of the cAMP
235 concentration was performed against a standard curve plotted using a four-parameter logistic
236 regression method. The cAMP concentrations were normalized to the nucleic acid
237 concentration in the extract, measured at $\lambda = 260$ nm and expressed as fmoles ng^{-1} of nucleic
238 acids.

239

240 **RESULTS**

241 **Quantitative proteomic analysis of osmotic signal transduction mutants**

242 $\Delta bos1$ and $\Delta sak1$ mutants' protein contents were compared to the parental wild-type strain
243 B05.10 exponentially grown under axenic *in vitro* conditions. After tryptic digestion and
244 peptide analysis by LC-MS/MS, protein identification and quantification were performed.
245 67% of the detected spectra could be assigned to peptides. 2425 proteins out of the 11701
246 predicted *B. cinerea* proteins (20.7% of the theoretical proteome) were detected in the total
247 dataset. XIC analysis allowed to obtain quantitative profiles of 1263 proteins out of the
248 previously mentioned 2425 (52%). The others were analysed by peak counting (PC). All
249 quantitative data are provided in Table S1.

250

251 **Identification of differentially abundant proteins between wild-type and osmotic ST** 252 **mutants**

253 In the $\Delta bos1$ mutant, the abundance of 417 proteins (377 in XIC and 40 in PC) differed
254 significantly from the wild-type (applied thresholds: adjusted p-value < 0.01 ; fold-change
255 (mutant/WT) > 1.5 or < 0.66), while in the $\Delta sak1$ mutant the abundance of 481 proteins (419

256 in XIC and 62 in PC) differed significantly from the wild-type. 270 proteins were common to
257 both mutants (Figure 1).

258 Fig. 1 recapitulates the number of proteins whose abundance varied significantly in both
259 mutants jointly or independently. Among the 270 proteins differentially produced in both
260 mutants, 243 were affected in the same sense (100 up-regulated vs. 143 down-regulated
261 proteins reflecting common regulation). The number of proteins whose abundance variations
262 were contrasting in both mutants, and consequently following potentially opposing regulation
263 patterns, was considerably lower (n=27). We further detected proteins controlled
264 independently by either Sak1 (88 positively and 123 negatively) or Bos1 (89 positively, 58
265 negatively). Lists of corresponding proteins are provided in Table S2.

266

267 **Enrichment study network analysis of functional categories**

268 In order to better understand the biological functions affected in both signal transduction
269 mutants we conducted functional enrichment analyses. The first enrichment study of
270 differential accumulating proteins in the $\Delta bos1$ and $\Delta sak1$ mutants was based on gene-
271 ontology (GO) terms of biological processes for the automatically annotated proteins of the
272 reference genome sequence [40, 52]. Fig. 2 illustrates the major findings of this study. 10%
273 (18/172) of the upregulated proteins in the $\Delta bos1$ mutant are linked to significantly enriched
274 categories, principally related to purine nucleotide and several amino acid biosynthetic
275 processes. In the same mutant 5% of the down-regulated proteins (13/245) are linked to the
276 enriched glycosylation and glycolytic processes. Only 3% of the upregulated proteins in the
277 $\Delta sak1$ mutant (7/236) are linked to significantly enriched processes, *i.e.* amino-acid
278 biosynthetic processes. Interestingly a high proportion of the downregulated proteins in the
279 $\Delta sak1$ mutant (92/245; 38%) are part of significantly enriched biological processes,
280 principally oxido-reduction processes, carbohydrate metabolism and proteolysis. The details
281 of GO functional enrichment analyses are available in Table S3.

282 In addition to the enrichment study of automatically annotated proteins, we performed a
283 network-analysis of the up- and down-regulated datasets in both ST mutants on the STRING-
284 web interface (string-db.org). The STRING database integrates known and predicted protein-
285 protein interactions (physical and functional interactions) and generates protein linkage maps
286 [50, 53]. Database search and MCL-clustering [51] allowed to draw the protein networks of
287 up- or down-regulated proteins in the $\Delta bos1$ and the $\Delta sak1$ mutants (Fig. 3, suppl. Table S4).

288 Proteins up-regulated in the $\Delta bos1$ mutant could be linked to amino-acid and purine
289 metabolism as by the GO enrichment analysis. In addition, proteins linked to ribosome

290 biogenesis and translocation and folding of mitochondrial proteins were upregulated. For the
291 down-regulated proteins in the $\Delta bos1$ mutant (Fig. 3B), the STRING network analysis
292 precised the cellular functions detected by GO-enrichment to central C-metabolism, glycogen-
293 mobilization and cell-wall synthesis. Similar cellular functions were detected among the
294 proteins down-regulated in the $\Delta sak1$ mutant (Fig. 3D; central C-metabolism, glycogen
295 metabolism) in addition to oxidative stress response and nucleotide metabolism. In the case of
296 up-regulated proteins of the $\Delta sak1$ mutant (Fig. 3C), the STRING network analysis was far
297 more informative than GO enrichment and strikingly different from $\Delta bos1$ up-regulated
298 proteins. In addition to amino-acid and carboxylic acid metabolism, cellular functions linked
299 to the cytoskeleton (including endo- & exocytosis) and to stress response were detected.

300

301 As both studies described above were based only on automatic annotations, we also
302 performed Fisher's exact test using expert annotations of *B. cinerea* genes [6, 40, 47, 49]
303 (suppl. Table S5).

304 Fig. 4A presents a summarized view of results obtained for the data sets of up/down-regulated
305 proteins in each mutant. Globally, down-regulated proteins in $\Delta bos1$ and $\Delta sak1$ are enriched
306 in secondary metabolism proteins, CAZYmes (Carbohydrate-Active enZYmes), early
307 secreted proteins, proteases and proteins involved in oxidative stress response. The same
308 categories were found enriched in the $\Delta bos1$ up-regulated data-set - with the exception of
309 CaZYmes and the addition of alternative respiration proteins. A quite different profile was
310 observed for the $\Delta sak1$ upregulated class of proteins, which was found enriched only in
311 secondary metabolism proteins, proteases and alternative respiration proteins. These data
312 highlight some important functions affected by both mutations under axenic, unstressed
313 growth conditions, such as secondary metabolism, the production of lytic enzymes and
314 secreted proteins as well as the response to oxydative stress. Some of these functions may
315 reflect the *in vitro* growth phenotypes of both mutants, e.g. sensitivity to oxidative stress or
316 loss of pathogenicity.

317 The concurrent enrichment of the same functional categories in different data sets hints to
318 complex regulatory patterns. We conducted the same enrichment study in the different
319 regulatory classes outlined in Fig. 1 in order to evaluate if Bos1 and Sak1 may preferentially
320 regulate positively or negatively certain functional categories. As outlined in Fig. 4B
321 secondary metabolism proteins were found enriched in five regulatory classes, proteins of the
322 early secretome in four, and proteases in three. CAZYmes were found enriched in the class
323 under positive control of Bos1 alone and under combined positive control of Bos1 and Sak1,

324 while oxidative stress response proteins were found enriched in the classes under positive
325 control of either Sak1 alone or combined with Bos1. Three functional categories were
326 enriched in only one regulatory class: predicted secreted proteins (under negative control of
327 Bos1 and positive control of Sak1); alternative respiration (negatively controlled by Bos1 and
328 Sak1) and signal pathway components (negative control of Sak1).

329

330 **Quantitative proteomic analysis of specific cellular functions**

331 The above described global comparative and statistical analysis of the proteomic data allowed
332 to draw first conclusions on the affected functions in both mutants and revealed more or less
333 complex regulatory patterns. As these analyses are based on the simple presence of
334 differentially regulated proteins in each data set, as next step, we used the quantitative
335 proteomic data to go deeper in the analysis of both ST mutants and to precise the regulation of
336 specific cellular functions in *B. cinerea* by the Bos1 and Sak1 protein kinases.

337

338 *Regulation of secondary metabolism*

339 Secondary metabolites are important players during the infection of plants by *B. cinerea* [54].
340 Although this study did not focus on secondary metabolism and growth conditions were not
341 designed for, astonishingly, our proteomic analysis revealed contrasting regulatory classes for
342 secondary metabolism enzymes (Fig. 4B).

343 A focus on the Bot proteins (Table 1) involved in botrydial synthesis [55] revealed an increase
344 in the abundance of BcBot4 (cytochrome P450 monooxygenase), BcBot2 (sesquiterpen
345 cyclase), and BcBot3 (cytochrome P450 monooxygenase) in the $\Delta sak1$ mutant, while these
346 proteins were less abundant in the $\Delta bos1$ mutant. The abundance of BcBot1 (cytochrome
347 P450 monooxygenase) did not vary significantly in the $\Delta sak1$ mutant but decreased in the
348 $\Delta bos1$ mutant. The abundance of proteins of the *Bcbot* gene cluster is positively regulated by
349 the histidine kinase Bos1 while MAPK Sak1 negatively regulates several of them, indicating
350 the regulatory roles of both proteins in botrydial biosynthesis.

351 Analysing the *Bcboa* cluster (Table 1) involved in botcinic acid synthesis [56], only BcBoa1
352 (putative NmrA-like regulator) and BcBoa17 (putative dehydrogenase) were detected as
353 significantly different compared to the wild-type. BcBoa1 was more abundant in the $\Delta bos1$
354 mutant compared to the wild-type, while its accumulation was reduced in the $\Delta sak1$ mutant.
355 On the opposite, BcBoa17 was found more abundant only in the $\Delta sak1$ mutant than in the
356 wild-type.

357 Among the other secondary metabolism proteins (Table 1), nine proteins of the BcNRPS7-
358 BcPKS5 cluster [57] were detected in our proteomic analysis: the non-ribosomal peptide
359 synthetase BcNRPS7, calycin, O-methyltransferase, alpha/beta hydrolase, methyltransferase,
360 enoyl-reductase, aldolase and the polyketide synthase BcPKS5. All nine proteins were
361 identified as being more abundant in the $\Delta sak1$ mutant compared to the wild-type. In the
362 $\Delta bos1$ mutant the abundance of three of these proteins also varied, that of enoyl-reductase and
363 aldolase was found increased but to a lesser extent than in the $\Delta sak1$ mutant. These results
364 highlighted negative regulation of the BcNRPS7-BcPKS5 cluster by MAPK Sak1 and mostly
365 independent of Bos1.

366 Proteins involved in oxalic acid or melanin biosynthesis (Table 1) were affected in the
367 osmosensing mutants. Especially in $\Delta sak1$, the abundance of oxaloacetate acetylhydrolase
368 decreased considerably, as did the abundance of three key enzymes of melanin biosynthesis:
369 the tetrahydroxynaphthalene reductases BcBrn1 and BcBrn2, scytalone dehydratase BcScd1
370 [58]. Only BcBrn2 abundance was reduced in the $\Delta bos1$ mutant.

371

372 *Proteins involved in osmotic, oxidative and cell wall stress response*

373 Considering the proteome of osmosensing signal transduction mutants, we were particularly
374 interested in functions related to osmotic stress (Table 2). Glycerol is involved in the osmotic
375 stress response [59] while mannitol is involved in both osmotic and oxidative stress response
376 [60, 61]. Previous studies showed the requirement for Sak1 in glycerol biosynthesis [24].
377 Analysis of our proteomic data with respect to polyol metabolism revealed a reduction in
378 glycerol dehydrogenase and glycerol-3-phosphate dehydrogenase content in both mutants.
379 Regarding mannitol metabolism, we observed a significant decrease in mannitol
380 dehydrogenase and mannitol-1-phosphate 5-dehydrogenase in the $\Delta sak1$ mutant.

381 The Bos1-Sak1 signal transduction pathway was shown to be involved in oxidative stress
382 response [24] and were corroborated by functional enrichment analyses (Figs 2-4). We based
383 our quantitative analysis of proteins linked to oxidative stress (Table 2) on a recent review of
384 Siegmund and Viefhues [49]. Superoxide dismutase is involved in the detoxification process
385 of superoxide ions. The deletion of *sak1* causes a decrease in the abundance of BcSod1
386 protein compared to the wild-type. Hydrogen peroxide is detoxified by the action of catalases.
387 Among the eight catalases of the *B. cinerea* genome, three were found differentially produced
388 in $\Delta sak1$ and/or $\Delta bos1$. The Cat7 protein showed reduced abundance in the $\Delta sak1$ mutant,
389 while CatA was more abundant in the $\Delta bos1$ mutant, compared to the wild-type. Catalase
390 Cat5 is less present at both in $\Delta sak1$ and $\Delta bos1$.

391 Detoxification of ROS also requires the action of thioredoxins and disulfide-isomerases or
392 peroxiredoxins (PRX). We observed a decrease in the biosynthesis of thioredoxin BcTRX1
393 and three disulfide isomerases in the $\Delta sak1$ mutant. The abundance of only one disulfide
394 isomerase was impacted in both $\Delta sak1$ and $\Delta bos1$ mutants. Out of nine PRX of the *B. cinerea*
395 genome, three were identified as differentially abundant in $\Delta bos1$ and $\Delta sak1$ compared to the
396 wild strain. PRX1 and PRX9 are less abundant in both $\Delta bos1$ and $\Delta sak1$. In contrast, PRX8 is
397 more abundant in both mutants.

398 Non-enzymatic mechanisms like the glutathione system are also involved in ROS
399 detoxification. We found that four glutathione S-transferases (GST) were differentially
400 abundant in $\Delta sak1$ compared to the wild-type. GST 2 and 9 were less abundant while GST 5
401 and 14 were more abundant than the wild-type. Glutathione reductase BcGlr1 is important for
402 the reduction of glutathione disulfide (GSSG) and the regeneration of glutathione (GSH). The
403 abundance of this protein was decreased in the $\Delta bos1$ mutant as well as in $\Delta sak1$.
404 Glutoredoxin (Grx) is an oxidoreductase that is reduced via glutathione. Glutathione is then
405 oxidized to GSSG. In the $\Delta sak1$ mutant, the amount of glutoredoxin is lower compared to the
406 wild-type.

407 Altogether, these data reveal important modifications in the content of proteins that play
408 essential roles in ROS detoxification function in both ST mutants.

409 Fungal cell walls are mainly composed of chitin and β -1,3-glucans [62]. $\Delta bos1$ and $\Delta sak1$
410 mutants were shown to be affected in cell wall integrity [16]. Our proteomic study showed
411 that in the $\Delta sak1$ mutant, the abundance of both chitin synthases BcCHSIIIa and BcCHSV
412 was increased compared to the wild type, while in the mutant $\Delta bos1$ a decrease in the
413 abundance of β -1,3-glucan synthase was observed.

414

415 *Crosslink between Sak1 and other signalling pathway proteins*

416 Signal pathway components were found enriched in the regulatory class under negative Sak1
417 control (Fig. 4B). In particular, an accumulation of proteins involved in the G protein and
418 cAMP pathways was observed in the $\Delta sak1$ mutant (Table 3). One of the three $G\alpha$ subunits
419 (BCG1) of the heterotrimeric complex $G\alpha\beta\gamma$ was found overproduced in the $\Delta sak1$ mutant. To
420 a lesser extent, the CAP1 protein associated with adenylate cyclase and the regulatory subunit
421 of protein kinase A (PKAR) were found more abundant. Thus, Sak1 negatively regulates the
422 abundance of these proteins. In the $\Delta bos1$ mutant, no difference in abundance these proteins
423 compared to the wild-type was detected.

424 In order to confirm the detected link of Sak1 in cAMP pathway, we measured the cAMP
425 concentration in both osmosensing mutants, $\Delta bos1$ and $\Delta sak1$. In the $\Delta sak1$ mutant, cAMP
426 concentration was two to three-times higher compared to the wild-type strain (Figure 5). In
427 contrast, the cAMP concentration in the $\Delta bos1$ mutant was comparable to the wild-type
428 confirming the negative regulation of the cAMP pathway by Sak1 independently of Bos1.

429

430 In the $\Delta sak1$ mutant, several proteins involved in the calcium signalling pathway such as two
431 calcium calmodulin-dependent kinases and one calcineurin-like phosphoesterase also
432 emerged from our analysis (Table 3). Both calcium calmodulin-dependent kinases were found
433 regulated in opposite ways by Sak1: BcCMK1, was more abundant while the abundance of
434 the second one was decreased. In the same strain, the abundance of a calcineurin-like
435 phosphoesterase was slightly increased. Considering the $\Delta bos1$ mutant, the above cited
436 proteins did not vary, but a SERCA (Sarco/Endoplasmic Reticulum Ca^{2+} APTase) type
437 calcium transporter was detected as less abundant than in the wild type.

438 In conclusion, our data clearly show an interconnection between the osmoregulating pathway
439 and other signalling pathways, notably with G protein, cAMP and calcium signal
440 transduction.

441

442 *Regulation of secreted proteases and cell wall degrading enzymes*

443 Functional enrichment analyses indicated important impacts of *bos1* and *sak1* mutations on
444 CAZYme, secreted protein and protease production. Quantitative proteomic analysis of strains
445 grown in axenic conditions revealed 15 glycoside hydrolases, out of which 10 were less
446 abundant in both $\Delta bos1$ and $\Delta sak1$ mutants (Table 4). Moreover, two peptidases, one
447 carboxypeptidase and two α/β -hydrolases were also less abundant in both mutants. Other
448 enzymes, like one pectine lyase and one glycoside hydrolases were more abundant in $\Delta bos1$
449 than in the wild-type while less abundant in $\Delta sak1$.

450 Proteomic analysis also revealed an intracellular accumulation of cell wall degrading enzymes
451 (CWDEs), annotated as secreted enzymes, especially in the $\Delta bos1$ mutant. Among these
452 proteins, glycoside hydrolases, pectin lyase A, pectate lyase A, endo-beta-1,4-glucanase
453 Cel5A, and α/β -hydrolase were identified. We also noted the accumulation of
454 endopolygalacturonase 1 BcPGA1 and a putative pectin methyltransferase. All these secreted
455 proteins, generally induced by plant cell wall extracts, are involved in the modification and
456 degradation of the plant wall and, consequently, in plant infection [31, 35-37].

457

458 **DISCUSSION**

459 *Comparative proteomics of signal transduction mutants in Botrytis cinerea*

460 In this work, we compared the steady-state protein levels of protein kinase mutants involved
461 in osmosensing in the plant pathogenic fungus *Botrytis cinerea*. Analysing three different
462 strains grown under axenic conditions led to the identification of roughly 1/5 of the predicted
463 *B. cinerea* proteome. The abundance of 628 proteins (26% of the detected proteins) differed
464 significantly in either or both mutants (>400 proteins per genotype). These results highlight
465 the strong impact of protein kinase inactivation on protein production and turnover even in the
466 absence of external stress and is in agreement with other proteomic studies of fungal ST
467 mutants [63-69] and, in the case of $\Delta sak1$, with the transcriptional results obtained by Heller
468 and colleagues [25].

469

470 *Regulation of protein abundance by Bos1 and/or Sak1*

471 The protein kinases of this study are part of the same ST pathway. The histidine kinase Bos1
472 is a sensor-kinase of hyper-osmotic conditions [23] and negatively regulates the basal
473 phosphorylation status of the Hog1-like MAPK Sak1 [24]. Comparing the proteomes of both
474 mutants, we found that i/ the major part of the differential proteins (>60%) corresponds to
475 proteins regulated independently by either Bos1 or Sak1, ii/ 43% of the differentially
476 accumulating proteins are regulated by both protein kinases, but that iii/ only a small fraction
477 (27 proteins) is controlled in opposing fashion by Bos1 and Sak1 which seems to be in
478 contradiction to the negative regulation. Considering protein abundance – not the
479 phosphorylation status – our results show that Bos1 and Sak1 act in a common direction under
480 non-stressing conditions. Their opposing activities as suggested by [24] may impact on the
481 phosphorylation status of target proteins and/or on protein abundance under stress conditions
482 principally. Validation of either of both hypotheses requires additional proteomic and
483 phosphoproteomic studies. However, the results obtained in this study also highlight a
484 considerable part of independent functions for both kinases, in agreement with previous
485 phenotypic studies [15, 16, 24].

486

487 *Differentially abundant proteins are enriched in functional categories linked to infection*

488 Functional enrichment studies revealed important biological functions affected by both
489 mutants under axenic, unstressed growth conditions. In addition, they pinpointed to complex
490 regulatory patterns of several functional categories (proteins co-regulated or independently
491 regulated by Bos1 and Sak1). Enzymes or proteins involved in secondary metabolism were

492 enriched in most regulatory categories, revealing differential implications of the Bos1 and
493 Sak1 kinases in their biosynthesis. Among the SM pathways detected, one can mention those
494 of the toxins botrydial and botcinic acid, both involved in *B. cinerea* virulence [55, 56], but
495 also oxalic acid. Although not essential to *B. cinerea* virulence, this organic acid contributes
496 to lesion expansion through acidification essential for lytic enzymes [66, 70, 71]. The strong
497 reduction (100x) of oxaloacetate hydrolase (OAH) in the $\Delta sak1$ mutant may contribute to its
498 reduced aggressiveness.

499

500 Among the proteins involved in stress resistance, our proteomic data revealed important
501 changes for proteins involved in ROS production or detoxification. Especially, the $\Delta sak1$
502 mutant had strongly reduced quantities of several ROS detoxifying enzymes (SOD, disulfide-
503 isomerase, catalases). These are rather intriguing results. Even though both mutants are hyper-
504 sensitive to hydrogen-peroxide and paraquat, sensitivity is even stronger in the $\Delta bos1$ mutant.
505 But this latter mutant also shows increased tolerance to menadione compared to the wild-type
506 and the $\Delta sak1$ mutant [24]. These phenotypes are not reflected by the differential steady-state
507 levels of ROS detoxifying enzymes. We hypothesize that the constitutive production of the
508 corresponding enzymes is not sufficient to prime the fungus against oxidative stress, but
509 requires induced production under oxidative stress conditions, controlled not only by the
510 MAPK Sak1, but also by the MAPK Bmp3 and other yet unknown kinases, as highlighted by
511 transcription analyses [25].

512 A previous study showed the implication of Bos1 and Sak1 in cell wall integrity in *B. cinerea*
513 without precising the respective cell wall modifications. Both $\Delta bos1$ and $\Delta sak1$ mutant are
514 more sensitive than wild-type in regard to the two dyes, Congo red and Calcofluor White, but
515 display differential sensitivity to other cell wall inhibitors [16]. The observed increase of
516 chitin-synthase proteins (CHS3, CHS5) in the $\Delta sak1$ mutant correlates nicely with its
517 increased tolerance to nikkomycin Z, a specific inhibitor of *Saccharomyces cerevisiae* Chs3
518 [72], and the related compound polyoxin B. The $\Delta bos1$ mutant displays increased sensitivity
519 to congo-red [16], a component interacting with the glucan network of the fungal cell wall
520 [73]. This phenotype can be correlated to the decreased amount of beta-glucan-synthase
521 observed for this mutant. Still, precise determination of cell wall composition is required to
522 understand the mutants' cell wall defects.

523 Among the enriched category of secreted lytic enzymes, we observed reductions in the large
524 family of glycosyl-hydrolases in both mutants. In contrast, other lytic enzymes, especially
525 plant cell wall degrading enzymes (CWDE) were found accumulating in the $\Delta bos1$ mutant.

526 According to the applied protocol, we did not expect to detect many CWDEs as the growth
527 conditions did not induce their production. Moreover, our proteomic data include principally
528 cytoplasmic and membrane bound proteins, since secreted proteins may have diffused into the
529 extracellular growth medium through the cellophane sheets [74, 75]. Therefore, the
530 underlying question is if these enzymes are produced in higher amounts in this mutant or if
531 they accumulate in the cytoplasm due to an eventual secretion defect? One may speculate on a
532 potential secretion defect in the $\Delta bos1$ mutant as several proteins involved in secretion and
533 vesicle transport are less abundant in the $\Delta bos1$ mutant (Table S1; *i.e.*, the Rab-GTPase Sas1,
534 myosin, Sec18, Sey1, Yop1, Surf4, dynamin [76-80]).

535

536 *Link to other signaling pathways*

537 Functional enrichment and quantitative, comparative proteomic data indicate a negative
538 regulation of the cAMP pathway by the MAPK Sak1, reflected by the overproduction of one
539 G α sub-unit, Cap and PkaR proteins as well as of higher intracellular cAMP levels measured
540 in the $\Delta sak1$ mutant. An additional effect of the *sak1*-deletion was observed on proteins linked
541 to Ca²⁺ signalling. This fits to the observation made by Schumacher and collaborators that the
542 G α sub-unit BCG1 is linked to the Ca²⁺ signalling pathway *via* PLC1 [81], corroborating the
543 links between the ST pathways identified by our proteomic data. These results also highlight
544 that signalling proteins are not only regulated through phosphorylation and de-
545 phosphorylation events, but also at the level of protein abundance. The relationship between
546 the MAPK Sak1 the cAMP or Ca²⁺ signalling pathways may act at different levels: *via*
547 phosphorylations and *via* the control of protein production and turnover.

548 Signalling pathways interact in all living organisms. In particular the hyperosmolarity and cell
549 wall integrity pathways are known to interact in fungi [8, 10], as this was shown also for *B.*
550 *cinerea* [16, 25]. Here we provide additional evidence for the involvement of the
551 hyperosmolarity pathway in cell wall integrity, oxidative stress and its interaction with other
552 signaling pathways. In addition, the observed changes in secondary metabolism proteins,
553 notably those involved in botrydial and botcinic acid biosynthesis corroborate interaction of
554 Sak1 with the G-protein, cAMP and Ca²⁺ signaling. Indeed, all these pathways were shown to
555 be involved in the regulation of transcription of BOT and BOA gene clusters and production
556 of these SM (reviewed in [82]). The finding that OAH production is under Sak1 control is
557 interesting as well. A recent publication revealed that OAH expression and the production of
558 oxalate is controlled by the MAPKK of the CWI pathway *via* the protein kinase Sch9 without
559 involvement of the MAPK Bmp3 [70]. It would now be interesting to analyse the respective

560 roles of Sch9 and Sak1 in addition to their connection with respect to the regulation of OAH
561 production.

562

563 In conclusion, steady state proteomic analyses of ST mutants are complementary to
564 phenotypic and transcriptomic analyses to characterize or to confirm the biological functions
565 of ST pathways, e.g., [66]. As the proteome reflects gene expression (*i.e.* transcription and
566 RNA turn-over), protein production and turn-over, when combined with transcriptomics it
567 may unravel additional regulations (e.g. protein degradation or secretion problems) of
568 important function. Only few comparative proteomic studies of intracellular proteins of fungal
569 ST mutants have been published so far [63-65, 67-69], although their potential to reveal
570 regulations of unsuspected biological functions cannot be denied. In the plant pathogenic
571 fungus *Magnaporthe oryzae* the regulators of G-protein signalling proteins were shown to
572 collectively regulate amino-acid metabolism [65]. In baker's yeast multiplex comparative
573 proteomics highlighted the implication of deubiquitylating proteins in mitochondrial
574 regulation and phosphate metabolism [63]. The role of protein kinase A in fungal biology was
575 established by a quantitative proteomic study in the opportunistic human pathogen
576 *Cryptococcus neoformans* using a conditionally expressed *PKA1* gene allowing to identify a
577 large spectrum of biological functions regulated by Pka1 [68]. Ultimately, time-lapse
578 experiments including dynamic transcriptomic, proteomic and phosphoproteomic studies may
579 allow to decipher the complete regulatory scheme of ST pathways and their downstream
580 biological effects.

581

582

583

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589

590

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857
858 Table 1: Differentially produced secondary metabolism proteins in $\Delta bos1$ and $\Delta sak1$ mutants.
859 Bold numbers indicate over threshold values.

	Accession number	Name	Description	$\Delta bos1$ /WT	$\Delta sak1$ /WT
Melanin	Bcin04g04800	BcBRN1	Tetrahydroxynaphthalene reductase	1,434	0,088
	Bcin03g08100	BcBRN2	Tetrahydroxynaphthalene reductase	0,622	0,007
	Bcin03g08110*	BcSCD1	Conidial pigment biosynthesis scytalone dehydratase Arp1	0,826	0,043
Oxalate	Bcin12g01020	Bcoah	Oxaloacetate partial	0,263	0,010
BoA cluster	Bcin01g00010	BcBoA1	NmrA-like	3,381	0,525
	Bcin01g00160	BcBoA17	Putative Dehydrogenase	0,965	3,202
BcNRPS7 - BcPKS5 cluster	Bcin01g11450	BcNRPS7	Non-ribosomal peptide synthetase	0,535	10,278
	Bcin01g11470	nd	Calycin	1,130	12,699
	Bcin01g11480*	nd	Bifunctional P450: NADPH-P450 reductase	1,306	4,000
	Bcin01g11490	nd	O-methyltransferase	1,286	15,438
	Bcin01g11500	nd	Alpha beta hydrolase	1,229	1,885
	Bcin01g11520	nd	Non-ribosomal peptide synthetase	1,401	9,058
	Bcin01g11530	nd	Zinc-binding dehydrogenase family	1,929	19,547
	Bcin01g11540*	nd	Aldolase	4,500	14,500
BOT cluster	Bcin12g06370	BcBot4	Cytochrome P450	0,055	1,536
	Bcin12g06380	BcBot1	Cytochrome P450	0,152	0,918
	Bcin12g06390	BcBot2	Sesquiterpen cyclase	0,514	1,895
	Bcin12g06400	BcBot3	Cytochrome P450	0,134	1,829

860 * proteins identified by peak counting

861

862

863 Table 2: Differentially produced proteins involved in the response to different stresses in
864 $\Delta bos1$ and $\Delta sak1$ mutants compared to wild-type. Bold numbers indicate over threshold
865 values.

	Accession number	Name	Description	$\Delta bos1/WT$	$\Delta sak1/WT$
Oxidative stress response	Bcin06g01180*	BcCATA	Catalase	3,750	1,125
	Bcin03g01920	BcCAT5	Catalase	0,120	0,039
	Bcin09g04400*	BcCAT7	Catalase	1,040	0,040
	Bcin03g03390	BcSod1	Superoxide dismutase	0,727	0,283
	Bcin12g04280	BcTRX1	Thioredoxin	0,814	0,496
	Bcin06g05730	BcPDI1	Disulfide isomerase	0,753	0,573
	Bcin01g08850	nd	Disulfide isomerase	1,021	0,636
	Bcin05g08370	nd	Disulfide isomerase	0,827	0,653
	Bcin08g02380	nd	Disulfide isomerase erp38	0,580	0,640
	Bcin01g04900*	gstII	Glutathion s-transferase	1,125	0,156
	Bcin14g03160*	BcGST5	Glutathion s-transferase	3,000	15,000
	Bcin06g03060	BcGST9	Glutathion s-transferase	0,520	0,232
	Bcin07g03430	BcGST14	Glutathion s-transferase	0,762	3,980
	Bcin04g05300	BcGLR1	Glutathion-Disulfide reductase	0,473	0,360
	Bcin10g01240	BcGRX1	Glutaredoxin	0,750	0,635
	Bcin04g00570	BcPRX1	Peroxiredoxin	0,647	0,617
Bcin09g03930	BcPRX8	Peroxiredoxin	2,011	7,046	
Bcin10g01030	BcPRX9	Peroxiredoxin	0,265	0,055	
Osmotic stress response and carbon metabolism	Bcin08g06300	nd	6-phosphofruktokinase	0,635	0,591
	Bcin08g04730	nd	Fructose-1,6-bisphosphatase	0,590	0,815
	Bcin02g08340	TPS1	Trehalose-6-phosphate synthase	0,657	0,722
	Bcin11g05700	BcHxk1	Hexokinase	0,581	0,484
	Bcin15g04970	nd	Glucose-6-phosphate isomerase	0,629	0,588
	Bcin11g04710	nd	Glycerol dehydrogenase	0,440	0,562
	Bcin08g03150	nd	Dihydroxyacetone kinase	0,324	0,239
	Bcin04g06400	nd	Glycerol-3-phosphate dehydrogenase	0,589	0,557
	Bcin16g03380	nd	Mannitol dehydrogenase	0,837	0,501

	Bcin15g05600*	BcCND9	Mannitol-1-phosphate 5-dehydrogenase	0,767	0,333
Cell-wall stress	Bcin04g03120	BcCHSIIIa	Chitin synthase	0,697	1,591
	Bcin12g05370	BcCHSV	Chitin synthase	0,736	1,638
	Bcin02g06930	nd	1,3-beta-glucane synthase	0,491	1,309

866 * proteins identified by peak counting

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868

869 Table 3: Signalling proteins of the AMPc, calcium and cell cycle signalling pathways whose
 870 abundance differs significantly in $\Delta bosI$ and/or $\Delta sakI$ mutants compared to wild type. Bold
 871 numbers indicate over threshold values.

	Accession number	Name	Description	$\Delta bosI/WT$	$\Delta sakI/WT$
cAMP signalling	Bcin05g06770	BCG1	Guanine nucleotide-binding subunit alpha	1,391	2,429
	Bcin03g07980	BcCAP1	Adenylyl cyclase-associated	1,484	1,538
	Bcin04g01630	BcPKAR	cAMP-dependent kinase regulatory subunit	1,024	1,708
Calcium signalling	Bcin01g09320	nd	Calcium-translocating P-type SERCA-type	0,574	1,279
	Bcin07g04730	BcCMK1	Calcium calmodulin-dependent kinase	0,994	1,742
	Bcin05g04730	nd	Calcium calmodulin-dependent kinase	0,964	0,422
	Bcin13g05090	nd	Calcineurin-like phosphoesterase	1,099	1,536
Cell cycle signalling	Bcin03g02930	Cla4	p21-activated protein kinase	0,495	0,640

872

873

874 Table 4: Putative secreted lytic enzymes, differentially produced in $\Delta bos1$ and $\Delta sak1$ mutants.

875 Bold numbers indicate over threshold values.

	Accession number	Name	Description	$\Delta bos1/WT$	$\Delta sak1/WT$
Secreted enzymes	Bcin02g01070	nd	Alpha glucosidase subunit	0,421	0,502
	Bcin01g11330	nd	Beta glucosidase subunit	0,527	0,590
	Bcin01g11500	nd	Alpha beta hydrolase	1,229	1,885
	Bcin13g03090	nd	Alpha beta hydrolase	11,676	1,366
	Bcin02g03090	nd	Glycoside hydrolase family 1	0,222	0,084
	Bcin14g00480	nd	Glycoside hydrolase family 3	0,383	0,225
	Bcin03g03480*	nd	Glycoside hydrolase family 10	0,400	0,200
	Bcin02g01420*	nd	Glycoside hydrolase family 13	0,686	0,171
	Bcin04g04190	nd	Glycoside hydrolase family 15	0,169	0,126
	Bcin12g03390	nd	Glycoside hydrolase family 15	0,204	0,053
	Bcin01g06010	nd	Glycoside hydrolase family 16	3,803	3,635
	Bcin05g01520	nd	Glycoside hydrolase family 16	0,159	0,105
	Bcin01g11220	nd	Glycoside hydrolase family 17	2,191	0,519
	Bcin11g06440	nd	Glycoside hydrolase family 31	0,285	0,201
	Bcin14g00650*	nd	Glycoside hydrolase family 31	0,404	0,213
	Bcin10g05710	nd	Glycoside hydrolase family 32	0,954	0,454
	Bcin09g02680	nd	Glycoside hydrolase family 63	0,387	0,254
	Bcin16g01950	nd	Glycoside hydrolase family 63	0,655	0,601
	Bcin02g06940	nd	Glycoside hydrolase family 72	1,632	0,935
	Bcin08g02390	nd	Peptidase S28	0,401	0,151
	Bcin07g04370	nd	Peptidase S41	0,406	0,099
	Bcin10g04320	Bccp4	Carboxypeptidase Y	1,346	2,518
	Bcin08g00280*	nd	Carboxypeptidase S1	0,281	0,125
	Bcin14g02510	BcLCC2	Laccase 2	3,867	0,294
	Bcin14g03430*	nd	Pectine lyase A	1,600	0,200
	Bcin03g04010	cel5A	Endo-beta-1,4-gluconase	2,659	1,484
	Bcin03g05820	nd	Pectate lyase A	7,408	0,773
	Bcin13g03090	nd	Alpha beta hydrolase	11,676	1,366

	Bcin01g11500	nd	Alpha beta hydrolase	1,229	1,885
	Bcin06g02510	nd	Alpha beta hydrolase	0,117	0,108
	Bcin02g05500*	nd	Alpha beta hydrolase	0,154	0,115

876 * proteins identified from peak-counting

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880 **SUPPORTING INFORMATION LEGENDS**

881 **Table S1:** List of differentially produced proteins in $\Delta bos1$ and $\Delta sak1$ mutants and fold-
882 changes relative to wild-type. An asterisk follows the protein accession numbers identified by
883 peak counting.

884 **Table S2:** Protein datasets of different regulatory classes.

885 **Table S3:** Raw output of FungiFun2 GO enrichment.

886 **Table S4:** Raw output of annotated protein functions after MCL cluster analysis of STRING
887 network analysis.

888 **Table S5:** Statistical analysis of enrichment of expert annotated functional categories.

889

890 **FIGURE LEGENDS**

891 **Fig. 1:** Flow-chart of quantitative proteomic data analysis in *Botrytis cinerea* $\Delta bos1$ and
892 $\Delta sak1$ mutants. The numbers indicate proteins whose abundance significantly differ between
893 strains during *in vitro* growth in steady state conditions (adjusted p-value < 0.01).

894 **Fig. 2:** Gene Ontology (GO) enrichment analyses on data sets of up/down-regulated proteins
895 in $\Delta bos1$ and $\Delta sak1$ mutants. Only enriched biological process (BP) categories are displayed.
896 The numbers indicate the numbers of proteins in each enriched category. BP categories can
897 overlap, *i.e.*, a protein can belong to several overlapping categories.

898 **Fig. 3:** STRING network analysis of up- (A, C) and down-regulated (B, D) proteins in $\Delta bos1$
899 (A, B) and $\Delta sak1$ (C, D) mutants. STRING (<http://string-db.org>)[50] was used to visualize
900 predicted protein-protein interactions using the corresponding proteins from *B. cinerea* strain
901 BcDW1[47].

902 **Fig. 4:** Protein enrichment in functional categories according to expert annotation [6, 40, 48,
903 49, 82] of detected proteins according to regulatory clusters. The red bar indicates
904 significantly enriched functional categories, the length of the bars reflects the proportion of
905 enriched proteins relative to the reference genome [40, 52]. Panel A refers to proteins up- or
906 downregulated in the $\Delta bos1$ and $\Delta sak1$ mutants respectively; panel B to the different
907 regulatory classes: (-) indicates negative regulation, (+) positive regulation either by Bos1 or
908 by Sak1 or by both kinases.

909 **Fig. 5:** Intracellular concentration of cAMP in signal transduction mutants and parental strain
910 B05.10. Values and standard deviations of three independent assays are indicated. Different
911 letters indicate significantly different intracellular cAMP accumulation ($P < 0,05$).

912