EC Journals.ASM.org

Unraveling the Function of the Response Regulator BcSkn7 in the Stress Signaling Network of *Botrytis cinerea*

Anne Viefhues,[a] Ina Schlathoelter,[a] Adeline Simon,[b] Muriel Viaud,[b] Paul Tudzynski[a]

Institut fuer Biologie und Biotechnologie der Pflanzen, Westfaelische Wilhelms Universitaet Muenster, Muenster, Germany[a]; UMR BIOGER, INRA, Thiverval-Grignon, France[b]

Important for the lifestyle and survival of every organism is the ability to respond to changing environmental conditions. The necrotrophic plant pathogen *Botrytis cinerea* triggers an oxidative burst in the course of plant infection and therefore needs efficient signal transduction to cope with this stress. The factors involved in this process and their precise roles are still not well known. Here, we show that the transcription factor Bap1 and the response regulator (RR) *B. cinerea* Skn7 (BcSkn7) are two key players in the oxidative stress response (OSR) of *B. cinerea*; both have a major influence on the regulation of classical OSR genes. A yeast-one-hybrid (Y1H) approach proved direct binding to the promoters of *gsh1* and *grx1* by Bap1 and of *glr1* by BcSkn7. While the function of Bap1 is restricted to the regulation of oxidative stress, analyses of Δ*bcskn7* mutants revealed functions beyond the OSR. Involvement of BcSkn7 in development and virulence could be demonstrated, indicated by reduced vegetative growth, impaired formation of reproductive structures, and reduced infection cushion-mediated penetration of the host by the mutants. Furthermore, Δ*bcskn7* mutants were highly sensitive to oxidative, osmotic, and cell wall stress. Analyses of Δ*bap1 bcskn7* double mutants indicated that loss of BcSkn7 uncovers an underlying phenotype of Bap1. In contrast to *Saccharomyces cerevisiae*, the ortholog of the glutathione peroxidase Gpx3p is not required for nuclear translocation of Bap1. The presented results contribute to the understanding of the OSR in *B. cinerea* and prove that it differs substantially from that of yeast, demonstrating the complexity and versatility of components involved in signaling pathways.

Constantly aggravating crop losses worldwide reinforce the need for research on plant pathogens, such as bacteria, viruses, fungi, and insect pests. According to fungal pathologists, the second most severe plant pathogen is the necrotrophic fungus *Botrytis cinerea* (1). This ascomycete causes rotting of numerous agronomically important crops, including, e.g., strawberries, grapes, and tomatoes (2, 3). Furthermore, serious losses in postharvest storage can be recorded due to its tolerance of low temperatures and quiescent growth phases (4, 5). To enter the plant, *Botrytis* is able to produce phytotoxic metabolites and to secrete a set of cell wall-degrading enzymes. During the decomposition of the host tissue the fungus starts to differentiate melanin-containing conidia for asexual reproduction and dispersal, giving *Botrytis* its common name, the gray mold fungus. In order to protect against the potential invader, the plant reacts with diverse defense mechanisms, e.g., the production of plant hormones, phytoalexins or -anticipins, and the induction of an oxidative burst (6, 7). The latter process was shown to be very effective against biotrophic pathogens that rely on living plant tissue; however *Botrytis* is a necrotroph and benefits from the accelerated plant decay (8, 9).

Like all organisms, *B. cinerea* has to cope with constantly changing environmental conditions, especially during the infection process, and to evoke the appropriate response to a specific stimulus, efficient signaling cascades are necessary. The response to oxidative stress is particularly important in *Botrytis cinerea*. Reactive oxygen species (ROS) are formed as a consequence of normal aerobic metabolism. However, as mentioned above, *Botrytis* has to cope with enhanced amounts of ROS during the pathogen-host interaction produced during the oxidative burst of the plant. Additionally, it was shown that *Botrytis* even contributes to this process by forming its own ROS (10, 11). Therefore, for the tolerance of ROS, efficient signal processing, transmission, and responses are needed.

Particularly well characterized are two-component histidine kinase phosphorelay systems that are involved in the regulation of several phases of life in bacteria and fungi (reviewed in reference 12). These pathways normally comprise a sensor histidine kinase (HK), a His phosphotransfer protein (HPt), and a response regulator (RR). The HK serves as a sensor and responds to a stimulus with the autophosphorylation of a conserved His residue. This phosphoryl group is transferred in several steps via the HPt to an Asp residue in the receiver domain of the RR. A well-characterized two-component signal phosphorelay is the Sln1p pathway in *Saccharomyces cerevisiae*. Hyperosmolarity leads to a reduction of Sln1p phosphorylation and dephosphorylation of the RR Ssk1p via the HPt Ypd1p. The dephosphorylated form of Ssk1p is active and associates with the mitogen-activated protein kinase kinase kinase ([MAPKKK] Ssk2p/Ssk22p) of the high-osmolarity glycerol (Hog1p) pathway, which in turn activates gene expression of osmotic stress-related genes (13–15). Accordingly, phosphorylation of Ssk1 suppresses the Hog1p cascade. The second RR in the Sln1p pathway, Skn7p, is independent of the MAPK pathway and involved in cell wall signaling and cell cycle control. In contrast to

Received 10 March 2015 **Accepted** 24 April 2015

Accepted manuscript posted online 1 May 2015

Citation Viefhues A, Schlathoelter I, Simon A, Viaud M, Tudzynski P. 2015. Unraveling the function of the response regulator BcSkn7 in the stress signaling network of *Botrytis cinerea*. Eukaryot Cell 14:636–651. doi:10.1128/EC.00043-15.

Address correspondence to Paul Tudzynski, tudzyns@uni-muenster.de.

Supplemental material for this article may be found at http://dx.doi.org/10.1128/EC.00043-15.

Copyright © 2015, American Society for Microbiology. All Rights Reserved.

doi:10.1128/EC.00043-15

Downloaded from http://ec.asm.org/ on April 14, 2016 by INRA - old

Ssk1p, Skn7p is activated by phosphorylation. However, not all genes regulated by Skn7p require the aspartyl phosphorylation of the RR. It could be shown that the response to oxidative stress in yeast is independent of the Sln1p phosphorelay (16). For the response to oxidative stress, Skn7p is phosphorylated at a Ser/Thr residue and works in concert with the basic leucine zipper (bZip) transcription factor (TF) Yap1 for gene expression (17, 18). Yap1 is a cytoplasmic protein that translocates to the nucleus under conditions of oxidative stress. In response to hydrogen peroxide, the glutathione (GSH) peroxidase Gpx3p mediates the oxidation of Yap1 by the formation of a disulfide bond between two Cys residues of the protein. Due to this conformational change, the nuclear export signal (NES) becomes masked, causing retention of Yap1 in the nucleus, where it activates gene expression of oxidative stress response (OSR) genes. Many genes encoding key enzymes in the OSR were shown to be regulated by Skn7p and Yap1. Analysis of different promoters showed that the preferred binding sites of both proteins were present in close proximity (19–23).

Potential orthologs of components of the yeast Sln1p pathway are also present in *B. cinerea*. However, the HK with highest homology to Sln1p, *B. cinerea* Hhk5 (BcHhk5), is not involved in the response to osmotic, oxidative, or fungicide stress (Y. Cuesta Arenas and J. van Kan, unpublished data). Although there is only a single HK in *S. cerevisiae*, *B. cinerea* possesses more than 20 HK-encoding genes (24). The evolution of this degree of heterogeneity in the HK family in filamentous ascomycetes may be a consequence of the need to sense different stimuli during pathogenesis and be due to their diverse life styles and a diversity of hosts (reviewed in reference 12). Bos1 (*Botrytis* osmosensing 1) was shown to assume functions to similar to those of the HK Sln1p. Knockout of this HK results in sensitivity to osmotic stress, resistance to fungicides, impaired virulence, and loss of conidiation and also leads to a constitutive activation of the MAPK cascade involving the stress-activated kinase BcSak1 (25, 26). Furthermore, a single HPt (Bhpt1) could be identified that seems to integrate all signals from the different sensor HKs, making this protein central for signal transduction; it is therefore probably essential in numerous fungi, such as *S. cerevisiae*, *Neurospora crassa*, and *Aspergillus nidulans* (27–30). Two RRs are also present in *B. cinerea*. The ortholog of Ssk1p, Brrg1, is necessary for the activation of the MAPK BcSak1. Additionally, *brrg1* deletion mutants are sensitive to osmotic stress and types of oxidative stress and fungicides and are unable to form macroconidia (31). Interestingly, certain phenotypes of the *bos1* deletion mutant are regulated independently of Brrg1 and BcSak1. Therefore, an additional effector pathway for Bos1 is assumed (26). This pathway probably involves the second response regulator BcSkn7, which was recently identified and partly characterized in a parallel investigation (32). It was shown that under osmotic stress BcSkn7 positively regulated phosphorylation of BcSak1; however, phosphorylation and expression analyses showed that regulation of the oxidative stress response probably is independent of BcSak1 phosphorylation, in a similar manner to that observed in yeast.

Therefore, the aim of this work is an extensive characterization of the RR BcSkn7 and the elucidation of possible connections to other known transduction pathways, with the key focus on the oxidative stress response and the interplay with the bZip TF Bap1.

MATERIALS AND METHODS

Strains and growth conditions. Strain B05.10 of *B. cinerea* Pers.:Fr. [*Botryotinia fuckeliana* (de Bary) Whetzel] is an isolate from *Vitis* and is widely used as a standard reference strain (33). The fungus and derived mutant strains were cultivated on different complex media. Synthetic complete medium (CM) was prepared according to Pontecorvo et al. (34). For stress sensitivity assays different supplements were added: H_2O_2 (AppliChem GmbH, Darmstadt, Germany), menadione, SDS, Triton X-100 (AppliChem GmbH, Darmstadt, Germany), calcofluor white, NaCl, sorbitol, fluconazole (Sigma-Aldrich, Steinheim, Germany), and iprodione. Alternatively, the fungus was cultivated on Gamborg's B5 medium (Duchefa Biochemie BV, Haarlem, The Netherlands) supplemented with 2% glucose. Potato dextrose agar (PDA) (Sigma-Aldrich, Steinheim, Germany) was supplemented with 100 g/liter homogenized leaves of French beans (PDAB) (*Phaseolus vulgaris*). For grape agar, undiluted grape juice was supplemented with 0.1% yeast extract (pH of 5), and V8 agar was prepared by adding 0.3% $CaCO_3$ to vegetable juice. As minimal medium Czapek-Dox (CD) (20 g/liter sucrose, 3 g/liter $NaNO_3$, 1 g/liter K_2HPO_4,0.5 g/liter KCl, 0.01 g/liter $FeSO_4 \cdot 7\ H_2O$, 0.5 g/liter $MgSO_4 \cdot 7\ H_2O$, pH 5) was used. Reproduction structures were obtained by incubating the different strains at 20°C either under light conditions (12 h light/12 h darkness) for conidiation for 7 to 10 days or in constant darkness for the formation of sclerotia for 3 weeks. In order to isolate DNA, fungal mycelium was grown for 3 days at 20°C on CM covered with cellophane. For the generation of protoplasts, Glucanex (Novozymes, Denmark) and lyticase (Sigma-Aldrich, Steinheim, Germany) or β-glucanase (InterSpex Products, San Mateo, CA, USA) were added to young mycelia and incubated for 1.5 h. For transformation studies 20 μg of replacement fragments was added according to Schulze Gronover et al. (35). Selection of transformants or single spore isolations were performed using 70 μg/ml of hygromycin B (Invivogen, San Diego, CA) or 70 μg/ml of nourseothricin (Werner-Bioagents, Jena, Germany). *S. cerevisiae* FY834 was grown either in complex YPD medium (0.5% [wt/vol] yeast extract, 2% [wt/vol] glucose, 2% [wt/vol] peptone, pH 5.8) or for selection in minimal dropout synthetic dextrose (SD) medium (20 g/liter glucose, 6.7 g/liter Difco yeast nitrogen base without amino acids [BD, Franklin Lakes, NJ, USA], 0.77 g/liter dropout supplement [Clontech, MountainView, CA, USA], pH 5.8) at 30°C. *E. coli* TOP10F′ (Invitrogen, Groningen, The Netherlands) cells were cultivated in lysogeny broth (LB) (10 g/liter peptone, 5 g/liter yeast extract, 10 g/liter NaCl) (36) at 37°C.

Standard molecular methods. Genomic DNA isolation from fungal mycelium was performed according to Cenis (37). RNA isolation was achieved by phenol-chloroform extraction using TRIzol reagent (Invitrogen) according to the manufacturer's instructions. For Southern blot analyses genomic DNA was restricted and separated via gel electrophoresis. For Northern analysis, 15-μg RNA samples were separated in an agarose gel containing formaldehyde. The respective gel was transferred to a nylon membrane (Nytran SPC Whatman; Schleicher and Schuell, Dassel, Germany) by downward blotting according to Ausubel et al. (88). For blot hybridizations [α-^{32}P]dCTP radioactively labeled probes were incubated in 6× SSPE (1× SSPE is 0.18 M NaCl, 10 mM NaH_2PO_4, and 1 mM EDTA [pH 7.7]), 5× Denhardt's solution, 0.1% sodium dodecyl sulfate (SDS), and 50 mM phosphate buffer, pH 6.6, at 65°C for 16 to 20 h (38). Before blots were exposed to an autoradiographic film, the membranes were washed once or twice.

RT-PCR. To study gene expression, quantitative real-time reverse transcription-PCR (RT-PCR) was performed. Therefore, total RNA was isolated from fungal mycelium, and 2 μg was used as the template for reverse transcription. According to the manufacturer's instructions, reverse transcription-PCR was performed using SuperscriptII (Invitrogen). RT-PCR was carried out using Bio-Rad-iQ SYBR green Supermix and an iCycler thermal cycler (Bio-Rad, Hercules, CA, USA). Programming, data collection, and analyses were performed with iCycler iQ Real-Time Detection System software, version 3.0 (Bio-Rad, Hercules, CA, USA). Gene expression of *bcpks13* (polyketide synthase 13; primers 68 and 69) and

Downloaded from http://ec.asm.org/ on April 14, 2016 by INRA - old

bcscd1 (scytalone dehydratase; primers 70 and 71) was normalized to the expression of the reference genes actin A (*actA*; GenBank accession number XP_001553368; primers 64 and 65) and the elongation factor 1-alpha (*ef1*α; GenBank accession number XP_001551786; primers 66 and 67) according to the $2^{-\Delta\Delta CT}$ (where C_T is threshold cycle) method. Means and standard deviations were calculated from two technical replicates representative of two biological replicates.

Transformation. To create knockout strains the genes *bcgpx3* and *bcskn7* were replaced by use of homologous recombination in the genetic background of the B05.10 wild-type (WT) and/or Δ*bap1* strain (39). For the replacement fragments, the 5′ and 3′ regions of *bcskn7* (1,149 and 986 bp, respectively) and *bcgpx3* (1,006 and 1,003 bp, respectively) were amplified with the primer pairs 8/9 and 10/11 (*bcskn7*) and the pairs 31/32 and 33/34 (*bcgpx3*), respectively (see Table S1 in the supplemental material), which have overlapping regions to the resistance cassette and the shuttle vector pRS426 (40). As a resistance marker, the hygromycin resistance cassette containing the *hph* gene of *Escherichia coli* under the control of the *trpC* promoter of *A. nidulans* was amplified from the vector pCSN44 with the primer pair 12/13. The assembly of the replacement constructs is achieved by the cotransformation of a linearized shuttle vector, resistance cassette, and gene flanks in the uracil-auxotrophic yeast *S. cerevisiae* FY834, where homologous recombination took place (41, 42). Transformants were selected on SD minimal medium without uracil, and for DNA isolation a SpeedPrep yeast plasmid isolation kit (Dualsystems Biotech, Schlieren, Switzerland) was used according to the manufacturer's instructions. For transformation the replacement fragment was amplified with the primers 8/11 (*bcskn7*) and 31/34 (*bcgpx3*). Verification of homologous integration was done by diagnostic PCR (primers 14/15 and 16/17 for *bcskn7* or primers 35/36 and 37/38 for *bcgpx3*) and Southern Blot analysis.

To restore the WT phenotype, the Δ*bcskn7* strain was complemented by targeted integration of *bcskn7* at the native locus, thereby replacing the hygromycin resistance cassette. For the vector construction (pbcskn7-COM$^{\text{in loco}}$; for *in loco* complementation) *bcskn7* was amplified (primers 8/18) and fused to a nourseothricin resistance cassette and the 5′ and 3′ *bcskn7* gene flank by homologous yeast recombination. The nourseothricin cassette (P*oliC*::*nat1*) from the vector pNR1 was amplified with the primer pair 19/20. The complementation vector was cut with PvuI, and the fragment was used for protoplast transformation. Diagnostic PCR (primers 7/14 and 16/21) confirmed targeted integration at the *bcskn7* gene locus.

Complementation of the Δ*bcgpx3* strain was achieved via directed integration of *bcgpx3* with the native promoter and terminator at the *bcniaD* gene locus (primers 39/40) (43). The complementation vector pNDN-OGG was cut with NcoI and NotI, and *bcgpx3* was integrated via yeast recombination. For transformation the vector pNDN-bcgpx3 was cut with ApaI and SacII, and integration was verified via diagnostic PCR (primers 23/28 and 25/26).

For localization and overexpression purposes green fluorescent protein (GFP)-BcSkn7 and GFP-BcGpx3 fusion proteins were constructed, using primers (18/22 or 41/42) with homologous regions to the glucanase terminator and *gfp*. Via yeast recombination the fusion construct was introduced in the NotI-linearized vector pNDN-OGG (42). The vector comprises gene flanks for the *B. cinerea* nitrate reductase, ensuring directed integration at the *bcniaD* locus, a nourseothricin resistance cassette, and *gfp* with the *oliC* promoter for constitutive expression of the fusion protein (43). The vectors pNDN-OG$^{\text{bcskn7}}$G and pNDN-OG$^{\text{bcgpx3}}$G were cut with ApaI and SacI, and the replacement fragments were used for transformation. Diagnostic PCR with the primers 23/24 and 25/26 verified correct integration at the *bcniaD* locus.

For localization of the protein Bap1, a GFP fusion protein was synthesized. For this purpose the vector pNDH-OGG was cut with NcoI, and the amplified gene *bap1* (primers 44/45) with overhangs to *gfp* and the *oliC* promoter were introduced via homologous recombination. The vector contains gene flanks for the *B. cinerea* nitrate reductase, ensuring directed integration at the *bcniaD* locus, a hygromycin resistance cassette, and *gfp* with the *oliC* promoter for constitutive expression of the fusion protein (43). The GFP vector pNDH-O$^{\text{Bap1}}$GG was digested with the enzymes ApaI and SacII and used for transformation. Diagnostic PCR (primers 23/24 and 25/36) proved the integration at the *bcniaD* locus.

Pathogenicity, germination, and penetration. For the analysis of germination, conidiospores were harvested and washed. For sugar-induced germination 1×10^5 spores/ml B5 medium plus 2% glucose was placed on glass slides and incubated overnight. For microscopic studies of penetration either 1×10^5 spores/ml H_2O (appressoria) or mycelium plugs (infection cushions [ICs]) was placed on onion epidermis layers and allowed to germinate overnight. The onion epidermis was washed with H_2O and incubated at 70°C for 1 h in a humid chamber to kill the living cells prior to inoculation, and the surface was stained with lactophenol blue for observation. The pathogenicity assays used *Phaseolus vulgaris* as the host plant according to Schulze Gronover (35) with 10-day-old leaves inoculated with either mycelium plugs or conidium suspension (2×10^5 spores/ml B5 medium plus 2% glucose). For the 1.5-h pregermination step, 10 μl of potassium phosphate (1 M; pH 6.4) was added.

ROS assays. In order to detect exogenous ROS, different assays were used. For a diaminobenzidine (DAB) test, 25 mg of fresh mycelium/strain was harvested from CM plates covered with cellophane and placed in a 24-well plate. The mycelium was overlaid with 1 ml of DAB solution (0.5 mg/ml DAB in 100 mM citric acid pH 3.7) and incubated for 1.5 h in darkness. As a negative control, 1 μl of H_2O_2 (30%) was added to the DAB solution, and for the positive control 2 μl of horseradish peroxidase and 1 μl of H_2O_2 in different dilutions were added to the DAB solution. The evaluation was done visually. For the quantification of produced ROS, a total ROS/superoxide detection kit (Enzo Life Sciences) was used according to Marschall and Tudzynski (44). Conidiospores from 10-day-old CM plates were harvested and adjusted to a spore titer of 1×10^6 spores/ml B5 medium plus 2% glucose. A microtiter plate was filled with 100 μl of the suspension and incubated overnight. The ROS detection mix was prepared according to the manufacturer's instructions, and 100 μl was added to the wells with germinated conidia. Following an incubation time of 5 min, fluorescence was detected using an Infinite 200 Pro (Tecan) microplate reader with standard fluorescein (excitation, 488 nm; emission, 520 nm) and rhodamine (excitation, 550 nm; emission, 610 nm) filter sets. Untreated cells served as negative controls.

Microscopy. Fluorescence and light microscopy was done with an Axio Imager 2 (Zeiss, Jena, Germany) using a 40× objective lens or with an Observer Z.1 (Zeiss, Jena, Germany) using a 20× objective lens. For the visualization of nuclei by Hoechst 33342 and of cell wall structures by calcofluor white (0.1%, in 0.1 M Tris-HCl, pH 8.5) filter set 49, 4′,6′-diamidino-2-phenylindole (DAPI) shift free (excitation G 365, beam splitter FT 395, and emission band pass [BP] 445/50 filters), was used. GFP fluorescence was examined using filter set 38 (excitation BP 470/40, beam splitter FT 495, and emission BP 525/50 filters). For the comparison of conidiation a Discovery V20 stereo microscope (Zeiss, Jena, Germany) was used. The images were captured with an AxioCam MRm camera and analyzed with Axiovision Rel, version 4.8, software.

Y1H assay. For the analysis of physical interactions of a TF to a promoter region of a gene, a direct yeast-one-hybrid (Y1H) assay was performed according to Simon et al. (45). Therefore, promoter regions of the *B. cinerea* genes *gsh1*, *gpx3*, *trx1*, *grx1*, *glr1*, *tsa1*, and *crz1* were cloned upstream of the reporter gene *HIS3* into the vector pINT1_HIS3NB, provided by P. B. Ouwerkerk (46) using primers 46 to 59 (see Table S1 in the supplemental material), and excised fragments were used to transform the yeast strain *S. cerevisiae* CG1945 (Clontech). Selection of homologous integration at the PDC6 locus was done using G418 (47). The transformed yeasts were then tested on SD plates lacking His (SD His$^-$) supplemented with a series of concentrations of 3-aminotriazole (3-AT) to determine the concentration of 3-AT required to reduce the leaky expression of the *HIS* gene. The DNA binding domains (DBD) of analyzed TFs BcSkn7 and Bap1 were cloned into pACTIIst, kindly provided by M. Fromont, using

Downloaded from http://ec.asm.org/ on April 14, 2016 by INRA - old

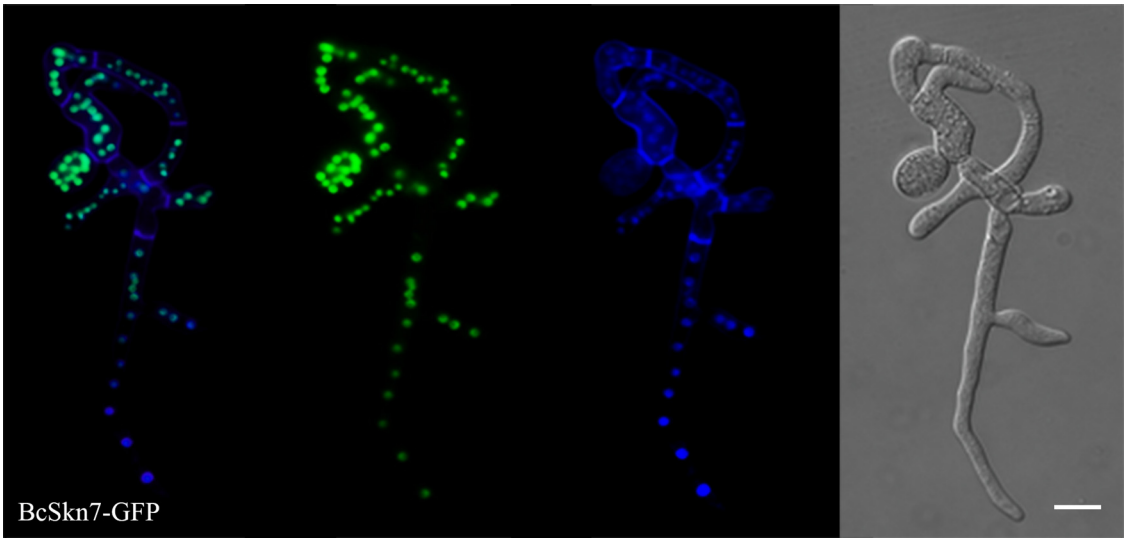

FIG 1 Cellular localization of BcSkn7. Spores of WT::BcSkn7-GFP germinated overnight and were analyzed via fluorescence microscopy. For costaining of nuclei Hoechst 33342 was used. Images from left to right are as follows: overlay, GFP staining, Hoechst staining, and differential interference contrast. Scale bar, 10 μm.

primers 60/61 and 62/63 (see Table S1), where the DBD is fused with the yeast *GAL4* activation domain. For the Y1H assay CG1945 cells containing the respective promoter constructs were transformed with 250 ng of the TF construct. Transformants were selected on SD Leu$^-$ plates as a growth control and on SD Leu$^-$ His$^-$ plates supplemented with 3-AT to show a direct interaction.

RESULTS

BcSkn7 is a putative response regulator in *B. cinerea* and constitutively localizes to the nucleus. Based on the *B. cinerea* genome database, the putative response regulator BcSkn7 was identified. The open reading frame (ORF) of the gene (B0510_6622; Broad Institute *B. cinerea* database [http://www.broadinstitute.org]) has a length of 2,339 bp interrupted by six introns (91, 74, 58, 50, 57, and 65 bp). cDNA analysis confirmed a coding sequence with 1,944 bp. BcSkn7 shows high homology to the yeast Skn7p, in both domain structure and amino acid sequence. On the amino acid level, the protein combines typical domains for regulatory components, such as a C-terminal receiver domain and a DNA binding domain similar to the heat shock factor 1 (Hsf1), in a 648-amino-acid (aa) protein (see Fig. S1A in the supplemental material). Although no nuclear localization sequence (NLS) was found for BcSkn7, it was shown for other fungi that Skn7 constantly localizes to the nucleus (48, 49). To investigate the localization of the RR in *B. cinerea*, BcSkn7 was fused to GFP and expressed in the WT. Germinating spores were used for microscopic analyses. Microscopy showed a constitutive localization of the BcSkn7 protein to the nucleus (Fig. 1), even under nonstress conditions, substantiating its expected role in gene regulation.

To examine the role of BcSkn7, knockout mutants (Δ*bcskn7* transformants T1, T6, and T16) were generated by a gene replacement approach (see Fig. S2B in the supplemental material). Homologous integration and the homokaryotic status of the transformants were verified by diagnostic PCR and Southern blotting (see Fig. S1B and C). Moreover, a complementation vector containing the full-length gene was designed to transform one of the *bcskn7* deletion mutants (see Fig. 6B and C; see also Fig. S2B).

BcSkn7 has an impact on the formation of reproduction structures. The lack of *brrg1*, the gene encoding the alternative response regulator in *B. cinerea*, rendered the mutants unable to produce conidia (31). To find out whether *bcskn7* mutants still form reproduction structures, growth under different light conditions was tested for 14 days. For the wild-type strain B05.10, asexual reproduction structures such as conidia are formed in light, while sclerotia, which represent survival structures and serve as female partners in sexual crossings (fertilization by microconidia leads to formation of apothecia), are formed in constant darkness. Under light/dark conditions (12 h/12 h), the Δ*bcskn7* mutants displayed a delayed and significantly reduced formation of conidiospores (~10 times) in comparison to the level of the WT (Fig. 2A), whereas in constant darkness (24 h) the number of sclerotia was doubled although they were smaller (Fig. 2A and B) than those of the controls. A more detailed examination showed that the Δ*bcskn7* mutant is generally able to form conidiophores, but fewer conidia are produced (Fig. 2C). The developmental processes, such as spore germination and appressorium-mediated penetration of host tissue, were not affected (see Fig. S3A to C in the supplemental material). Therefore, the mutant was generally able to form reproductive organs, but the number and size of these structures differed from those of the WT, and more whitish aerial hyphae were visible (Fig. 2C). As the mycelium of the mutant looked paler than that of the WT, pigmentation defects of the *bcskn7* mutant were assumed. Therefore, the expression of genes involved in melanin biosynthesis (bc*pks13* and *bcscd1*) was examined (50, 51). The strains were grown for 4 days in constant light (Fig. 2D) before being harvested for real-time PCR (RT-PCR) analysis. The mycelium appeared less pigmented on the plates, which could be confirmed by significantly reduced expression of *bcpks13* and *bcscd1* compared to levels in the WT (Fig. 2E). The overexpression (OE) of BcSkn7 (BcSkn7-OE strain) enhanced conidium formation (see Fig. S2A) compared to levels in the controls. While the Δ*bcskn7* mutant showed reduced formation of conidia under light, the BcSkn7-OE strain produced conidia even

Downloaded from http://ec.asm.org/ on April 14, 2016 by INRA - old

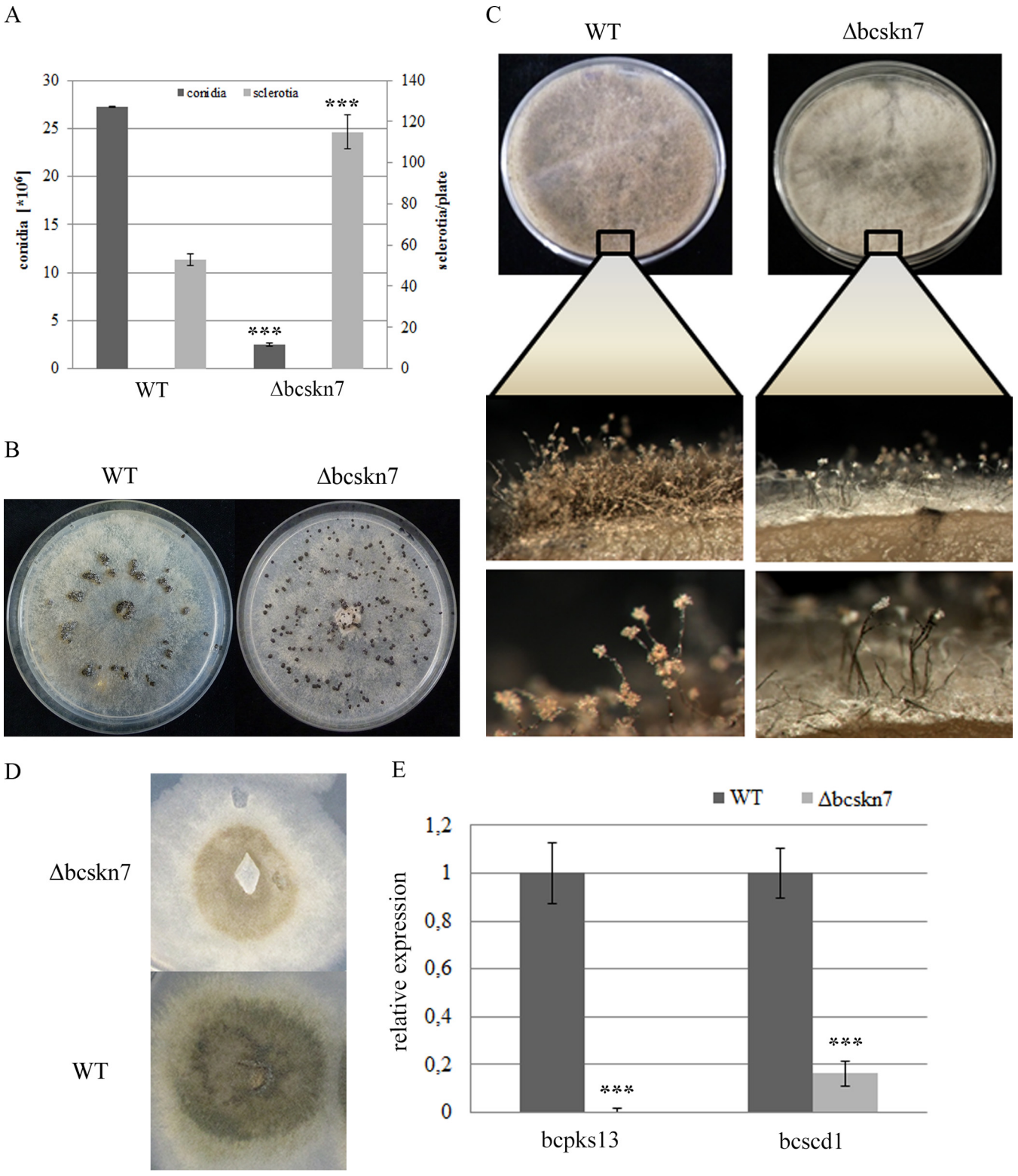

FIG 2 Formation of Δ*bcskn7* reproduction structures in comparison to the WT B05.10. (A) Quantitative evaluation of sclerotium and conidium formation. For sclerotium production strains were grown on CM for 3 weeks in constant darkness, and for conidium formation they were grown for 3 weeks in a 12-h light/dark cycle. Presented are the mean values with standard deviations from three independent experiments (for sclerotia, 5 plates/strain/experiment; for conidia, 3 plates/strain/experiment). Asterisks represent significant differences (*t* test) compared to the WT for each condition (***, $P \leq 0.001$). (B) CM plates with sclerotia of the WT and Δ*bcskn7* strains. The strains were grown for 2 weeks in constant darkness. (C) PDAB plates (2 weeks old) with conidia of the WT and Δ*bcskn7* strains as well as enlarged details of each plate using a stereomicroscope (magnification, ×47 and ×70 for the top and bottom panels, respectively). (D) Growth of the Δ*bcskn7* and WT strains on CM plates covered with cellophane for 4 days in constant light. (E) Expression of two putative melanin biosynthesis genes in the Δ*bcskn7* and WT strains. The strains were grown for 4 days on CM plates under constant light conditions. The expression levels of *bcpks13* and *bcscd1* are relative to those of the reference genes *actA* and *ef1*α and normalized to the level of the WT following RT-PCR. The values are means of two technical replicates and representative of two biological replicates. Asterisks represent significant differences (*t* test) compared to the WT for each condition (***, $P \leq 0.001$). Standard deviations are indicated.

Downloaded from http://ec.asm.org/ on April 14, 2016 by INRA - old

TABLE 1 Influence of different stress conditions on growth rates of the Δ*bcskn7*, Δ*bap1*, Δ*bap1 bcskn7*, and the WT B05.10 strains

Growth condition or type of stress	Stress condition	Colony diam (% of control) for:[a]			
		WT	Δ*bcskn7* strain	Δ*bap1* strain	Δ*bap1 bcskn7* strain
CM control (pH 5, 20°C)		100	100	100	100
Membrane	+ 0.02% SDS	57.0 ± 5.3	23.0 ± 7.0	49.6 ± 1.5	11.6 ± 6.5
	+ 0.02% Triton-X-100	27.5 ± 2.3	0.0 ± 3.2	25.4 ± 4.1	0.0
	+ 5 μg/ml fluconazole	75.0 ± 3.8	6.9 ± 3.2	66.9 ± 5.1	6.5 ± 1.2
Cell wall	+ 2 mg/ml CFW[c]	62.8 ± 5.9	18.4 ± 3.0	66.2 ± 4.8	16.1 ± 3.5
pH	pH 3	87.5 ± 1.8	80.0 ± 9.0	84.0 ± 3.9	80.6 ± 9.1
	pH 9	36.8 ± 3.8	13.8 ± 1.8	27.5 ± 4.1	12.9 ± 2.5
Osmotic	+ 1 M sorbitol	105.0 ± 7.0	87.4 ± 5	100.8 ± 5.9	90.3 ± 7.7
	+ 1 M NaCl	52.5 ± 5.5	40.5 ± 4.1	50.9 ± 8.7	40.6 ± 3.5
Oxidative	+ 5 mM H_2O_2	72.5 ± 7.0	13.8 ± 5.3	8.9 ± 2.3	0.0
	+ 10 mM H_2O_2	52.5 ± 4.0	0.0	0.0	0.0
	+ 20 mM H_2O_2	35.0 ± 1.8	0.0	0.0	0.0
	+ 500 μM menadione	75.0 ± 2.3	18.4 ± 6.2	15.3 ± 3.1	12.9 ± 1.3
Temperature	17°C	105.4 ± 6.3	105.3 ± 10.2	100.3 ± 5.1	103.4 ± 9.6
	25°C	64.2 ± 3.8	27.4 ± 4.1	69.6 ± 3.7	29.7 ± 6.5
	28°C	66.2 ± 2.5	25.3 ± 3.7	68.7 ± 3.8	29.7 ± 5.9
Plant-derived medium	PDAB	142.7 ± 1.0	157.9 ± 8.2	140.9 ± 6.6	167.6 ± 3.5
	Grape agar	69.7 ± 5.5	112.1 ± 11.1	69.5 ± 5.9	134.5 ± 12.3
	V8 agar	101.1 ± 5.0	108.4 ± 12.5	92.0 ± 2.5	113.8 ± 9.1
CD control[b]		100	100	100	100
Ionic	+ 0.1 M CaCl2	103.7 ± 3.5	219.6 ± 9.2	NA[b]	250.0 ± 7.1
	+ 0.2 M $CaCl_2$	88.3 ± 3.4	244.7 ± 6.2	NA	320.8 ± 3.3
	+ 0.4 M $CaCl_2$	63.6 ± 2.8	176.5 ± 7.3	NA	262.5 ± 6.4
	+ $MgCl_2$	107.4 ± 2.6	101.2 ± 13.1	NA	83.3 ± 6.8

[a] The strains were grown for 3 days on the indicated medium under the indicated stress condition. The data are means of three experiments with three plates/strain. Standard deviations (±) are indicated. NA, not analyzed. Percentages are relative to the values for the respective control for the stress group.
[b] CD, Czapek-Dox medium.
[c] CFW, calcofluor white.

in constant darkness. Because of this result, an *in loco* complementation was done using the endogenous promoter to fully restore the WT phenotype (see Fig. 6B and C; see also Fig. S2B in the supplemental material).

BcSkn7 is necessary for cell wall integrity and stress response. As it is reported for other fungi that deletion of *skn7* leads to sensitivity to osmotic or oxidative stress, the *B. cinerea bcskn7* mutant was tested on different stress-inducing media (Table 1) (52–54). The Δ*bcskn7* mutant showed reduced growth on standard complete (CM) and minimal (CD) media (data not shown). The addition of various stressors enhanced this growth defect, especially the influence of oxidative stress: while *bcskn7* mutants grew feebly on 5 mM H_2O_2, at higher concentrations the growth was completely abolished. The addition of menadione also limited growth to 18% (Table 1). Interestingly, the BcSkn7-OE strain seems to be more resistant to oxidative stress, such as H_2O_2 (see Fig. S2C). For other mutants it could be shown that reduced growth on oxidative stress medium is accompanied by enhanced production of ROS (55). Accordingly, the mutants were tested for ROS secretion. In a semiquantitative diaminobenzidine (DAB) assay, the Δ*bcskn7* mutant showed strongly increased H_2O_2 formation in comparison to that of the WT (Fig. 3B). These results were confirmed using the a total ROS/superoxide detection kit, which yields reliable quantitative data in *B. cinerea* (44). Here, a significant increased production of ROS (1.9 to 2.5 times) was confirmed (Fig. 3A).

The role of ROS and also the influence from other stressors were then investigated. An adverse effect caused by osmotic stress was detected for the Δ*bcskn7* mutant, but this effect was less pronounced than that of oxidative stress. Furthermore, sensitivity to temperature changes and to membrane or cell wall destabilizers was observed (Table 1).

The poor growth of the Δ*bcskn7* strain, especially under stressing conditions, suggested defects in cell wall or membrane integrity. Therefore, a protoplast assay with young mycelium was done to examine the effect of cell wall-degrading enzymes (Glucanex and β-glucanase) on the mutant (Fig. 4A). The calculated number of protoplasts showed reduced numbers derived from Δ*bcskn7* strain mycelium (0.6×10^6 to 1×10^6 protoplasts/mg [dry weight] mycelium), indicating a changed configuration of cell wall components, but a change in protoplast formation based on chitinase could not be seen (data not shown), indicating that loss of BcSkn7 modifies the glucan content of the cell wall. Two genes potentially involved in the synthesis of glucan are β-1,3-glucanosyltransferases (B0510_10257 and B0510_3559). The products of these genes have similarities to Gel4 and Gel1 of *Aspergillus fumigatus* that are glucan-elongating enzymes. In Fig. 4B an enhanced expression of these genes can be seen for the Δ*bcskn7* strain, supporting the hypothesis that the glucan content of the cell wall may be enhanced. To test whether changes in the cell wall composition can be detected, calcofluor white staining of germinating conidia was carried out. However, alterations in septation, cell wall thickness, or length of germinated hyphae could not be detected microscopically (data not shown). In contrast, addition of calcofluor white to CM decreased the growth rate of the Δ*bcskn7* strain up to 80%, indicating some destabilization of the

Downloaded from http://ec.asm.org/ on April 14, 2016 by INRA - old

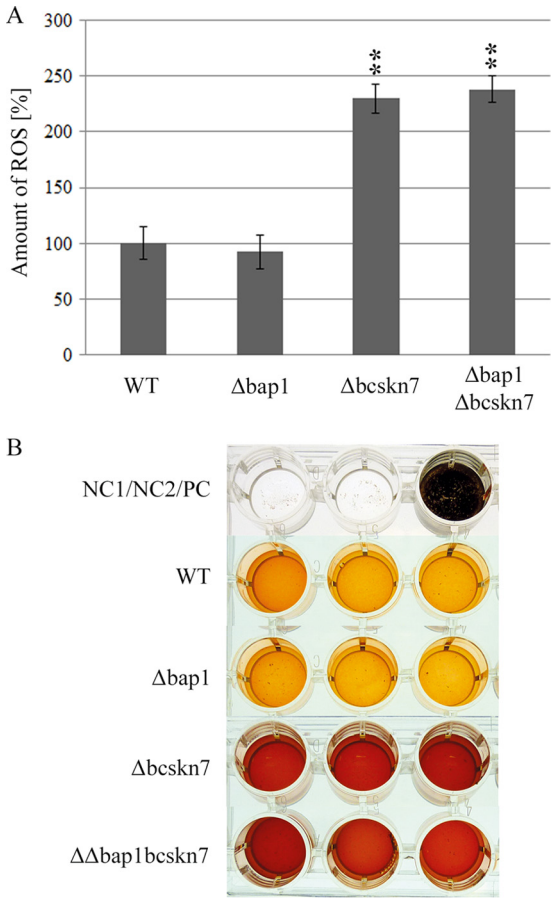

FIG 3 ROS production of Δ*bap1*, Δ*bcskn7*, and Δ*bap1 bcskn7* strains compared to that of the WT B05.10. (A) Determination of ROS amount using a total ROS/superoxide detection kit. The measurements were done in five cycles at nine different positions in each well. The values for the WT were set to 100%; mutants were calculated in relation to that. (B) DAB assay to test H_2O_2 secretion of mutants in comparison to the WT. Fresh mycelium was overlaid with 1 ml of DAB solution and incubated for 1.5 h in darkness. NC, negative control (DAB solution plus 1 μl of H_2O_2); PC, positive control (DAB solution plus 1 μl of H_2O_2 and 1 μl of horseradish peroxidase). The experiment was done in independent triplicates and repeated three times.

cell wall (Fig. 4C and Table 1), and membrane integrity also seems to be influenced. As mentioned before, detergents affecting the membrane, such as SDS and Triton X-100, have a strong influence on the growth of the mutant. Moreover, fluconazole, an inhibitor of ergosterol biosynthesis, reduced the colony diameter of the Δ*bcskn7* mutant by more than 90% (Table 1). These results support the hypothesis that BcSkn7 is either directly or indirectly involved in cell wall integrity and the response to membrane stress.

BcSkn7 is involved in mycelium-derived host infection via infection cushion formation. The role of Skn7 in virulence is species specific. While in *Alternaria alternata* the lack of *skn7* reduces the formation of necrotic lesions, the virulence of *Cochliobolus heterostrophus* and *Gibberella zeae* is not affected (48, 56). Sugar-induced conidium germination of the Δ*bcskn7* strain showed no deviations from the WT in the germination rate and time point (see Fig. S3A and B in the supplemental material). Also the formation of appressoria during penetration of onion epidermis was WT like (see Fig. S3C). It was therefore not surprising that pathogenicity on *Phaseolus vulgaris* primary leaves was unaffected when leaves were inoculated with a spore suspension. Initially, primary lesions formed, and these lesions spread and developed soft rot. However, the ability of the Δ*bcskn7* strain to produce new conidia at the end of the infection cycle was reduced, and more whitish aerial hyphae were present (Fig. 5A). However, infection assays with mycelium plugs of the Δ*bcskn7* strain showed results that differed from those of the WT because the infection pattern was inconsistent, especially on unwounded leaves. Whereas for the WT 100% of the plugs infect, for the Δ*bcskn7* mutant only 53% are able to cause infection (Fig. 5B). On wounded leaves infection rates for the Δ*bcskn7* strain could be enhanced (data not shown). As it is proposed that mycelium-derived infection depends on the formation of infection cushions (ICs) (57), a microscopic analysis was done to examine these structures. As can be seen in Fig. 5C, the number of ICs able to penetrate in the *bcskn7* mutant is decreased up to 43% in comparison to the number for the WT.

Mutation of D424 in BcSkn7 results in osmotic stress sensitivity. For *S. cerevisiae* it is postulated that the osmotic and oxidative stress responses are regulated via different pathways and strategies. While the response to osmotic stress requires the phosphorylation of a conserved aspartic acid residue (Asp426) of Skn7p, the oxidative stress response is regulated via phosphorylation of a serine/threonine (Thr434) (17, 58). To examine this assumption for *B. cinerea*, point mutations for the anticipated amino acids were generated, and the mutants were tested under different stress conditions (Fig. 6A to C). To examine the impact of these amino acids in the receiver domain of BcSkn7, the corresponding aspartic acid residue was replaced by an asparagine (D424N), and to examine changes under oxidative stress, the respective serine was changed to an alanine (S434A) in a complementation approach, using the native gene locus. As can be seen in from the experiment shown in Fig. 6B, Asp424 is obviously essential for the response to salt stress in *B. cinerea*. Additionally, the effect of sorbitol on the mutants' growth was tested (data not shown), and similar growth defects of BcSkn7 (D424N) could be seen. This indicates that Asp424 is crucial for the general osmotic stress response. In contrast, the oxidative stress response was not regulated via Ser434 because the mutants were still able to cope with H_2O_2 (Fig. 6B and C). Other tested stressors, such as menadione, SDS, or calcofluor white, did not affect growth of any mutated strain. These results for BcSkn7 indicate that possibly the mode of phosphorylation determines its effect on gene expression. However, the regulation of the OSR in *B. cinerea* seems to differ from the known mechanism in yeast. Mutation of this amino acid is at least insufficient to prevent the OSR, and therefore other phosphorylation sites are probably involved in the regulation of this process.

BcSkn7 gene regulation is linked to the TF Bap1. To gain more information about the possible interplay of BcSkn7 with other transcription factors involved in the stress response, a *bap1* knockout (39) and a double deletion of *bcskn7* and *bap1* (Δ*bap1 bcskn7* mutants T2, T3, and T17) were included in the phenotypic characterizations. Homokaryosis of Δ*bap1 bcskn7* deletion mutants was verified by diagnostic PCR and Southern blotting (see Fig. S1B and C in the supplemental material). As described previously (39) the data showed an involvement of Bap1 in the response to oxidative stress (Table 1; see also Fig. S4) and excluded an effect on pathogenicity (Fig. 5). The Δ*bap1 bcskn7* mutants

Downloaded from http://ec.asm.org/ on April 14, 2016 by INRA - old

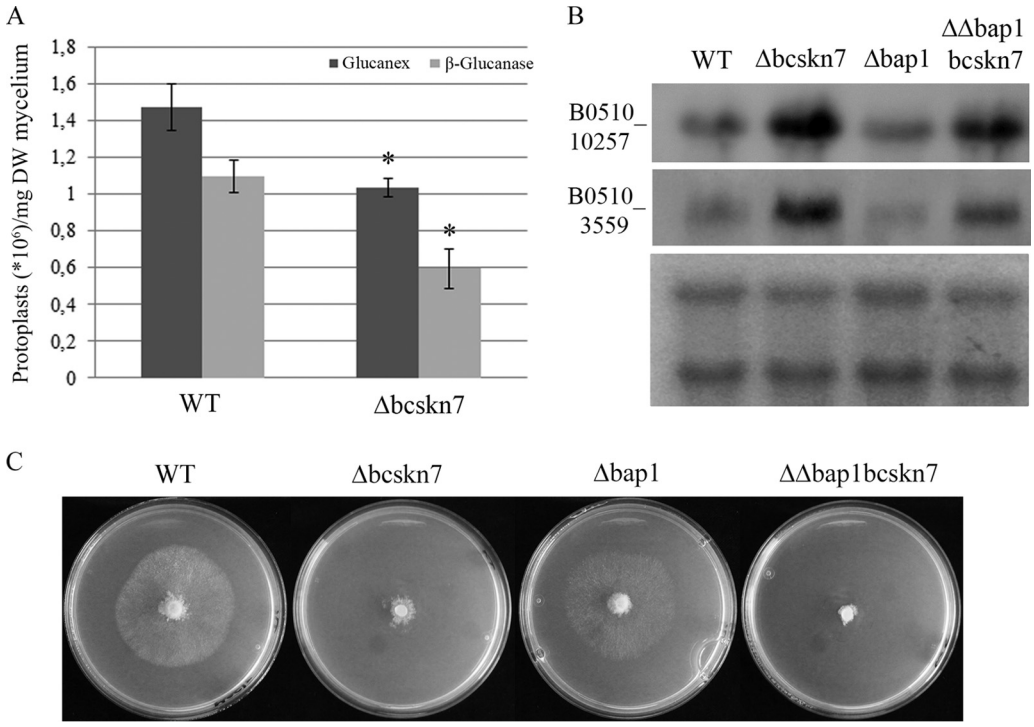

FIG 4 Influence of *bcskn7* deletion on cell wall and membrane integrity. (A) Treatment of the Δ*bcskn7* strain with cell wall-degrading enzymes. Conidia were cultured for 36 h in liquid medium. The harvested culture was incubated for 1.5 h in osmotically stabilized solution containing Glucanex or β-glucanase. The number of protoplasts formed was calculated per mg dry weight (DW). Represented are the mean values, including standard deviations, from three independent experiments. Asterisks denote significant differences (*t* test) compared to the WT for each condition (*, $P \leq 0.05$). (B) Transcription of genes involved in cell wall biosynthesis. Prior to RNA isolation the WT, Δ*bcskn7*, Δ*bap1*, and Δ*bap1 bcskn7* strains were cultivated for 3 days in liquid CD medium. As probes two putative β-1,3-glucanosyltransferase-encoding genes were used (B0510_10257 and B0510_3559). (C) Growth rates of the WT, Δ*bcskn7*, Δ*bap1*, and Δ*bap1 bcskn7* strains in the presence of calcofluor white. Strains were grown for 3 days on CM containing 2 mg/ml calcofluor white.

exhibited defects similar to those of the Δ*bcskn7* mutant, but they were more severe. The growth of the Δ*bap1 bcskn7* mutant was reduced, it was highly sensitive to different types of stress, ROS production was enhanced, and the formation of infection cushions was reduced (Table 1 and Fig. 3 and 5). The defects of the double gene deletion are stronger than those for the Δ*bcskn7* mutant, indicating that loss of Skn7 uncovers an underlying phenotype of Bap1.

Because it has been shown in some fungi that genes that are regulated by Skn7 are also regulated by Ap1 (19, 21, 36, 59), the regulatory function of both proteins was analyzed in *B. cinerea*. To gain a first impression, a set of OSR genes was selected for expression analyses via Northern blotting (thioredoxin reductase, *trr1* [B0510_1511]; glutaredoxin 1, *grx1* [B0510_5784]; γ-glutamylcysteine synthetase, *gsh1* [B0510_3554]; GSH peroxidase, *gpx3* [B0510_553] GSH reductase 1, *glr1* [B0510_2036]; thioredoxin, *trx1* [B0510_2802]; peroxiredoxin, *tsa1* [B0510_4954]). For this purpose the Δ*bap1*, Δ*bcskn7*, Δ*bap1 bcskn7*, and WT B05.10 strains were grown in axenic culture supplemented with or without 10 mM H_2O_2 for 30 min (see Fig. S4 in the supplemental material). All tested genes were induced in the WT under oxidative stress triggered by H_2O_2. The transcription of *trr1*, *gpx3*, *glr1*, and *grx1* was clearly coregulated by both BcSkn7 and Bap1 as there was no expression of these genes under H_2O_2 conditions in all mutants. Also the expression of *trx1* was reduced though not totally abolished. Moreover, Bap1 seems to be important for the regulation of *tsa1* and *gsh1* because induction was reduced or absent in the Δ*bap1* and Δ*bap1 bcskn7* strains. The lack of *bcskn7* resulted in only slightly reduced expression of *tsa1* in the presence of H_2O_2, indicating that further TFs are involved in the regulation of this gene.

To analyze whether the influence of the TFs on these genes is direct or indirect, a yeast one-hybrid (Y1H) assay was performed. In this approach direct physical interactions of proteins with specific DNA regions are detected by reporter gene expression in *S. cerevisiae*. For that purpose the respective DNA binding domains (DBDs) of Bap1 and BcSkn7 were identified *in silico* by pairwise protein sequence analyses with the yeast orthologs Yap1 and Skn7p (see Fig. S1A in the supplemental material). To select a putative target DNA, the two common binding motifs for each TF (Yap1 response element [YRE] and OSR element [OSRE]), known from verified interactions in *S. cerevisiae* (19–21, 60, 61), were used for promoter analyses of OSR genes in *B. cinerea*. By means of bioinformatic tools (FindPattern and MatInspector), the potential TF binding sites in the promoter regions of 48 analyzed genes were predicted, and finally, seven candidates were selected for Y1H analyses (see Table S2 in the supplemental material). Promoter sequences of around 200 bp were selected, containing the most interesting putative binding sites (see Fig. S5B). The interaction assays showed that Bap1 binds to the promoters of *grx1* and *gsh1*. In the case of BcSkn7 an interaction solely with *glr1* was detected (Fig. 7). In some cases the leaky gene expression of *HIS3* was not inhibited by the addition of 3-AT (see Fig. S5A). There-

Downloaded from http://ec.asm.org/ on April 14, 2016 by INRA - old

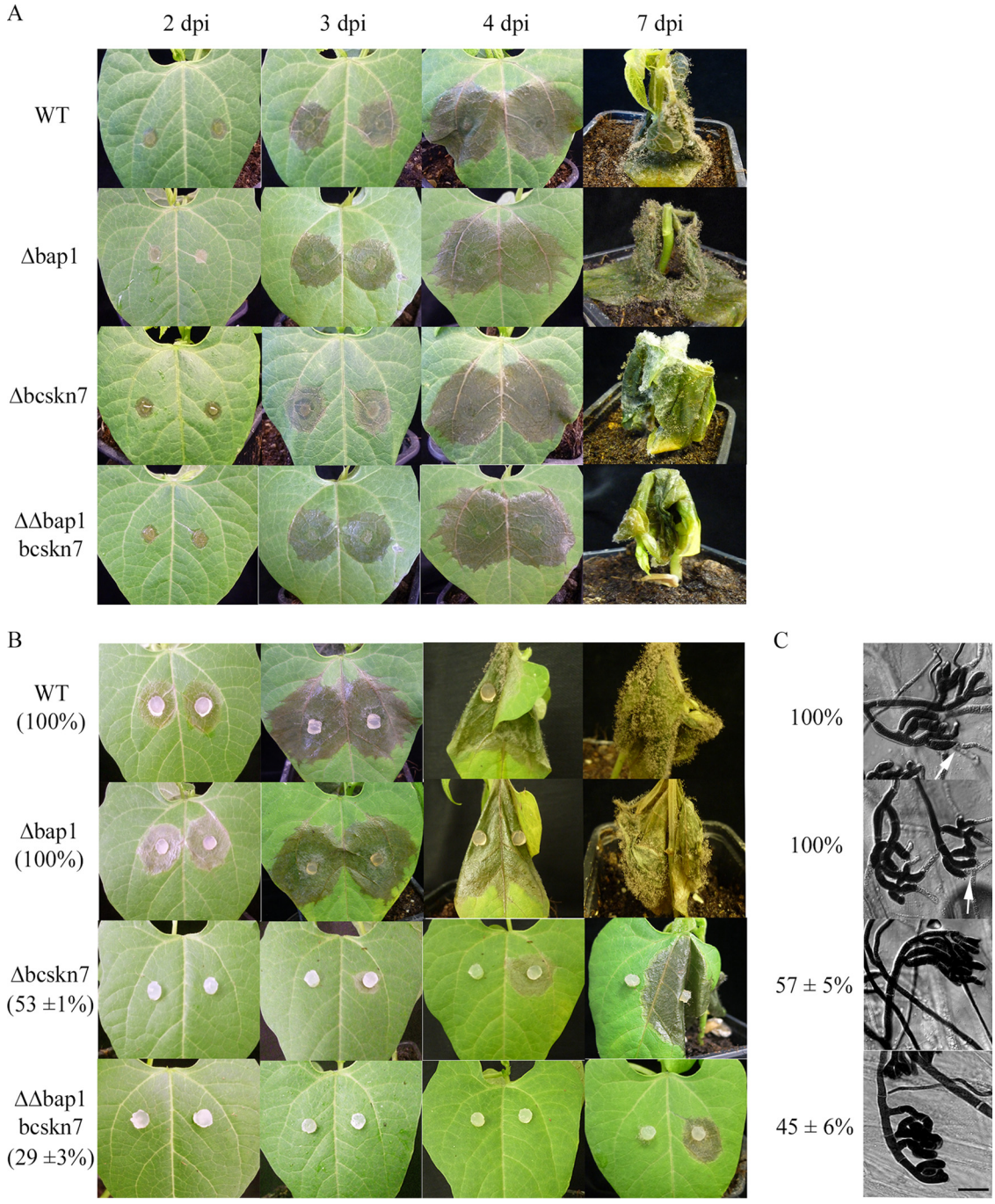

FIG 5 Infection ability of different mutants and the WT. (A) Infection assay on bean leaves of *Phaseolus vulgaris* inoculated with conidial suspensions of the Δ*bap1*, Δ*bcskn7*, Δ*bap1 bcskn7*, and WT B05.10 strains. For infection 2×10^5 spores/ml B5 medium plus 2% glucose were used, and samples were incubated for 7 days. (B) For the pathogenicity assay primary leaves were infected with mycelium of the Δ*bap1*, Δ*bcskn7*, Δ*bap1 bcskn7*, and WT B05.10 strains and incubated for 7 days. Percentages indicate successful infection. Calculations are based on three independent experiments; standard deviations are included. (C) Visualization of infection cushions. Onion epidermal layers were inoculated with mycelia and incubated for 36 to 40 h. By staining with lactophenol blue, hyphae on the surface were stained, while invading hyphae remained colorless (arrow). Percentages indicate successful penetration. Calculations are based on three independent experiments with 100 ICs analyzed per mutant; standard deviations are included. Scale bar, 20 μm. dpi, days postinfection.

fore, the results for the promoters of *gpx3* and *tsa1* could not be evaluated and were omitted.

Northern and Y1H analyses demonstrated that Bap1 and BcSkn7 are both direct and indirect regulators of oxidative stress-responsive genes in *B. cinerea*. Indeed, considering a list containing nearly all promoters of annotated genes in *B. cinerea*, 44.3% contain at least one of the four binding motifs from Bap1 and BcSkn7 (data not shown).

BcGpx3 is not essential for the activation of Bap1 upon oxidative stress. From yeast studies it is known that the glutathione

Downloaded from http://ec.asm.org/ on April 14, 2016 by INRA - old

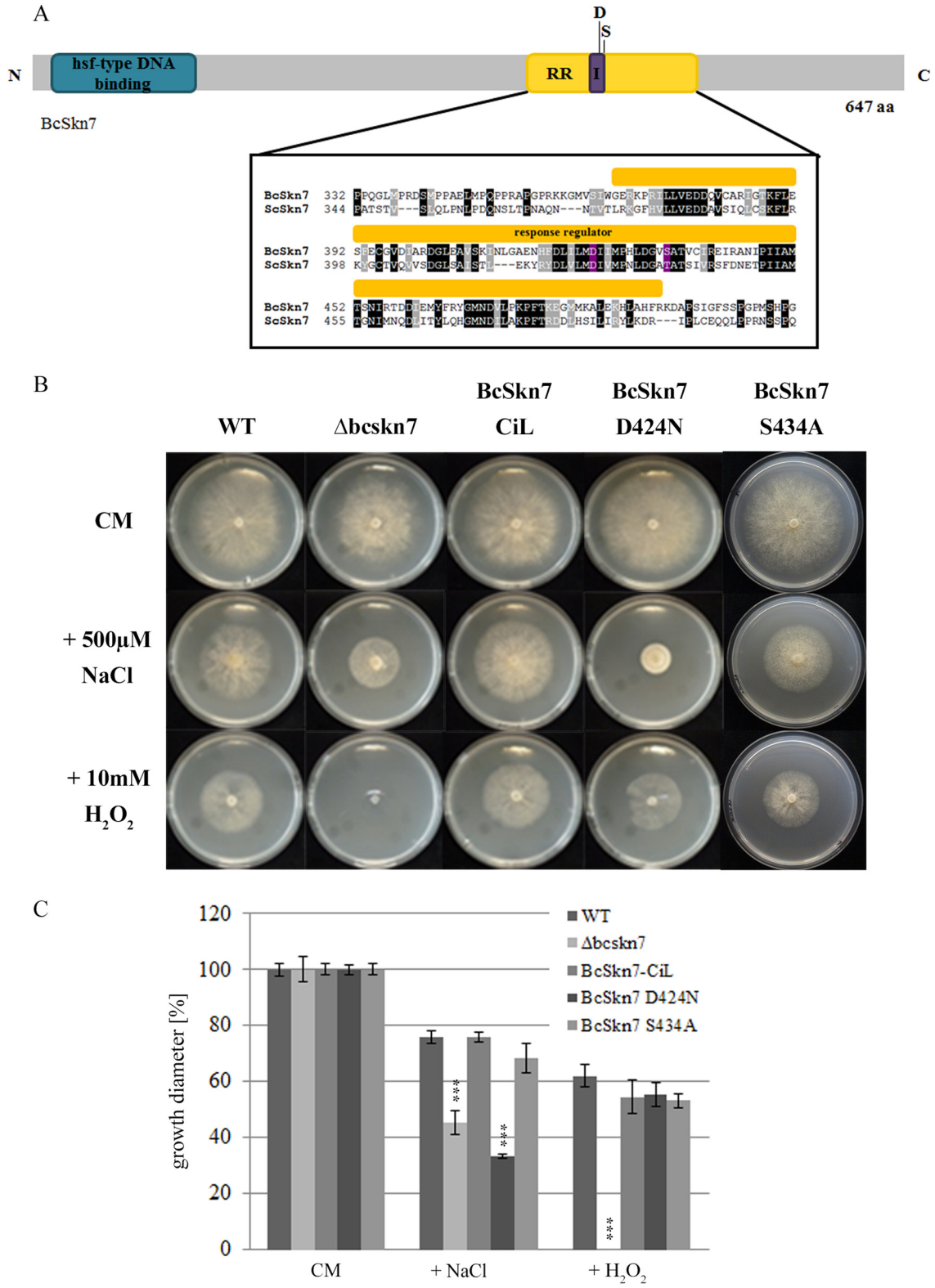

FIG 6 Complementation of the Δ*bcskn7*. (A) Amino acid sequence of the BcSkn7 response regulator (RR) domain in comparison to *S. cerevisiae* Skn7p. Mutated amino acids are shown in purple, identical amino acids are shaded in black, and similar amino acids are shown in gray. I, putative interaction site; Hsf, Heat shock factor. (B) Plate assay with complemented Δ*bcskn7* strain (integration of *bcskn7* into the native gene locus [CiL]), BcSkn7-CiL, and the BcSkn7 (D424N), BcSkn7 (S434A), Δ*bcskn7*, and WT B05.10 strains. Mutants were grown for 3 days on CM and CM supplemented with 10 mM H_2O_2 or 500 µM NaCl. (C) Relative growth diameter of analyzed mutants under different conditions. The strains were grown for 3 days on CM and CM supplemented with 10 mM H_2O_2 or 500 µM NaCl. Growth on CM was set as 100%, and growth on other media was calculated in relation to growth on CM. Asterisks represent significant differences (*t* test) compared to values for the WT for each condition (***, $P \leq 0.001$).

Downloaded from http://ec.asm.org/ on April 14, 2016 by INRA - old

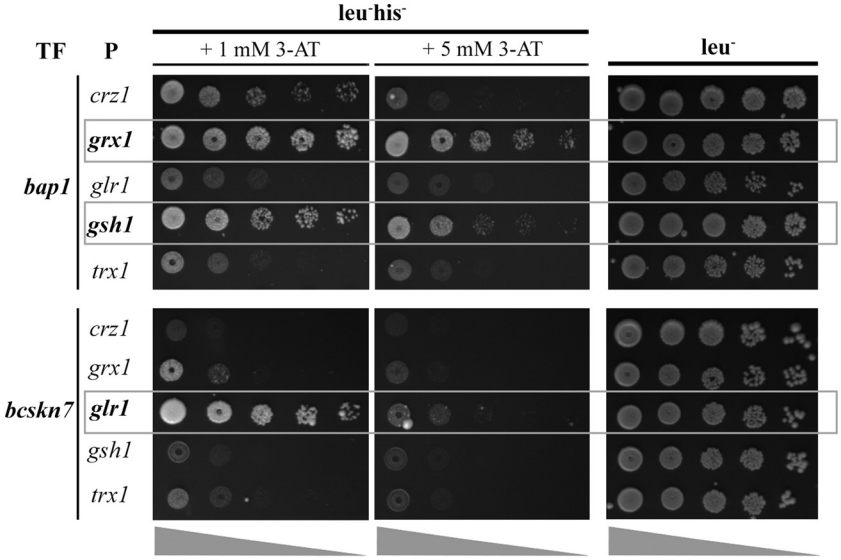

FIG 7 Y1H interaction assay of Bap1 and BcSkn7 DNA binding domains with different promoters of oxidative stress-related genes. Dilutions of the transformants (from 1:1 to 1:10,000) were dropped on SD −Leu medium as growth control and on SD Leu$^-$ His$^-$ medium supplemented with 1 or 5 mM 3-AT for the detection of physical interaction. Promoters written in bold indicate positive interactions. *crz1*, calcineurin-responsive zinc finger transcription factor; *grx1*, glutaredoxin 1; *glr1*, glutathione reductase 1; *gsh1*, γ-glutamyl-cysteine synthetase; *trx1*: thioredoxin; TF, transcription factor; P, promoter.

peroxidase (Gpx), as part of the ROS detoxification complex, has a key function in the activation of Yap1 upon H_2O_2 induction (62). While there are three Gpx proteins present in *S. cerevisiae*, just one Gpx3 homolog was found in *B. cinerea* with high amino acid sequence homology (~72%) (data not shown). The cysteine residue Cys36, which is involved in the interaction with Yap1 in *S. cerevisiae* (62), was conserved in the *B. cinerea* BcGpx3. Analysis of exon/intron organization of the respective gene (B0510_553) predicted an ORF of 783 bp including two introns of 174 bp and 90 bp. For detailed analysis, *bcgpx3* knockout mutants were generated (U. Siegmund, unpublished data) and screened by diagnostic PCR and Southern blotting (Δ*bcgpx3* mutants T1, T4, and T15) (see Fig. S6A in the supplemental material for the Southern blot).

Phenotypic characterization of Δ*bcgpx3* mutant T1 included plate assays under oxidative stress conditions, secretion of H_2O_2, and pathogenicity assays. However, no changes in the behavior of the mutant in comparison to that of the WT could be seen (see Fig. S6B to D).

To learn more about the localization of BcGpx3 and verify whether there is a connection to Bap1, microscopic analyses were performed. By fusing BcGpx3 to GFP, a more or less diffuse distribution of the protein throughout the whole hypha was visible (see Fig. S7A in the supplemental material). The addition of H_2O_2 had no influence on the localization (data not shown). To explore if BcGpx3 is necessary for the activation and therewith translocation of Bap1, Bap1-GFP was expressed in the WT and Δ*bcgpx3* genetic backgrounds. Microscopic analyses confirmed the translocation of the TF Bap1 under inducing conditions in the WT (see Fig. S7B). The same was observed for the Δ*bcgpx3*::*bap1-gfp* strain, indicating that there is no direct or exclusive dependency of Bap1 translocation on BcGpx3 in *B. cinerea*. Additionally, the alternative hypothesis was tested that thioredoxin is required to retranslocate Bap1 to the cytoplasm (62, 63). Expression of Bap1-GFP in the Δ*bctrx1* strain (55) showed normal movement of Bap1 into and out of the nucleus. Therefore, the presence of BcTrx1 is not essential for the inactivation and retranslocation of Bap1 in *B. cinerea* following oxidative stress treatment. In summary, and in contrast to experiments with *S. cerevisiae* (62), localization experiments here proved that BcGpx3 and BcTrx1 are not essential for the translocation of Bap1 under conditions of stress.

DISCUSSION

In this work we investigated the various biochemical and molecular consequences resulting from the loss of *bcskn7* in *B. cinerea*. The presented results not only underlined the importance of BcSkn7 as a transcriptional regulator in the OSR but also revealed a role in cell wall integrity, development, and virulence.

A recent report dealing with BcSkn7 was published while we were working on this paper. A comparison of the results shows that there are several differences between the phenotypes of the Δ*bcskn7* mutants, starting with a divergent gene annotation. In contrast to six introns confirmed by cDNA sequencing in the ORF of *bcskn7* in this study, only four introns are described in Yang et al. (32). Moreover, the results concerning formation of reproductive structures, sensitivity to cell wall stress, and virulence differ. However, one has to keep in mind that Yang et al. (32) worked with the *B. cinerea* strain 38B1, while B05.10 was used in this study. There are some reports that gene deletions can have diverse impacts on growth and virulence in *B. cinerea* in distinct strain backgrounds. For example, deletion of the pectin methylesterase *bcpme1* caused reduced virulence in strain Bd90 (64) but not in the B05.10 background (65). Another example involves the loss of the phytotoxin botrydial, which leads to reduced virulence in T4 but did not affect virulence in SAS56 (66).

Bioinformatic analysis of the BcSkn7 protein in *B. cinerea* B05.10 revealed typical regulatory components such as the C-terminal receiver domain and a DNA binding domain similar to those of heat shock factor 1 (Hsf1). The similarity to Hsf1 possibly explains the sensitivity of the Δ*bcskn7* mutant to elevated temperatures. BcSkn7 might be responsible for the regulation of heat

Downloaded from http://ec.asm.org/ on April 14, 2016 by INRA - old

shock-responsive genes or general stress genes. Fusion of the protein to GFP confirmed the expected localization of BcSkn7 in the nucleus, strengthening its putative role as a TF.

Impact of BcSkn7 and Bap1 on stress response. Signaling cascades have long been known not to be linear cascades but, rather, networks of many interacting components. In the OSR the two yeast components Yap1 and Skn7p are described to be functionally closely related, particularly for the activation of ROS-detoxifying genes (61, 67, 68). In *B. cinerea* a role of these components in redox homeostasis could be confirmed. Deletion of these two major regulators resulted in mutants sensitive to hydrogen peroxide and menadione (Table 1). This hypersensitivity to oxidative stress was also shown among others for *C. heterostrophus*, *Candida albicans*, and *A. fumigatus* (56, 69, 70). We have shown before that Bap1 controls the induction of many classical OSR genes (39). Furthermore, an altered redox status was observed for the Δ*bap1* strain by measuring the glutathione pool (71). Similarly, BcSkn7 is necessary for the induction of detoxifying genes, and deletion therefore probably leads to an imbalanced redox environment. Concomitant with this hypothesis, the Δ*bap1 bcskn7* and Δ*bcskn7* mutants displayed a significantly increased secretion of ROS (Fig. 3). As ROS are constantly formed as by-products of metabolic processes, missing or reduced detoxification due to the lack of *bcskn7* could result in an accumulation of ROS. A coherence between ROS overproduction and sensitivity to external ROS could also be observed for other knockout mutants involved in the oxidative stress response, such as deletion of the thioredoxin reductase *bctrr1* (55).

In addition to the OSR, an influence of Skn7 orthologs on cell wall remodeling was shown for different fungi. *A. nidulans skn7* mutants are sensitive to calcofluor white, and *S. cerevisiae skn7p* knockouts have changes in cell wall composition (58, 72). Also deletion of *bcskn7* resulted in altered growth and sensitivity to cell wall stress-inducing agents such as calcofluor white, indicating a restructuring of the fungal cell wall (Fig. 4). Calcofluor white binds nascent chitin chains and prevents the connection of chitin to β-1,3-glucan and β-1,6-glucan, resulting in a destabilization of the cell wall (73). Increased sensitivity to calcofluor white indicates modification of the glucan-chitin network of the mutant. This is substantiated by the observed increase in resistance to protoplast-forming enzymes. The increased expression of genes encoding enzymes involved in glucan synthesis suggests a primary effect on the glucan backbone. Also, membrane integrity seems to be influenced. We confirmed that the Δ*bcskn7* strain is sensitive to the membrane stressors SDS, Triton X-100, and fluconazole (Table 1). Substantiating observations from Yang et al. (32) showed downregulation of ergosterol biosynthetic genes and increased sensitivity to ergosterol biosynthesis inhibitors in their Δ*bcskn7* mutant, indicating a modified composition of the cell membrane. The Δ*bap1* strain behaved like the WT upon exposure to SDS, but the double deletion mutant was more sensitive than the Δ*bcskn7* strain. In general, the Δ*bap1 bcskn7* mutant showed a stronger phenotype than the single deletion mutants. This indicates that loss of Skn7 uncovers an underlying phenotype of Bap1 in *B. cinerea*, which diverges from observations in *S. cerevisiae* (19). In summary, the absence of BcSkn7 seems more severe than deletion of *bap1*. This demonstrates the extensive role of BcSkn7 beyond the OSR, such as cell wall and membrane integrity, whereas Bap1 function is limited to the OSR (39).

Influence of BcSkn7 on development and pathogenicity. Loss of *bcskn7* revealed functions in fungal development. Spore morphology and germination of the deletion mutants was WT like (see Fig. S3); however, the mutants formed significantly fewer conidiospores and more but smaller sclerotia than the WT (Fig. 2). These results indicate a perturbation of both asexual and sexual differentiation and are in contrast to results observed for *B. cinerea* 38B1. There, a total loss of conidiation was observed, and significantly fewer sclerotia were produced (32). Furthermore, an influence of BcSkn7 on mycelial pigmentation could be observed (Fig. 2D to F), and the overexpression of BcSkn7 in B05.10 resulted in enhanced sporulation, even in constant darkness. Normally, the induction of macroconidia is produced by light and involves the function of light-dependent TFs, such as BcLtf1 (51). It follows, therefore, that overexpression of BcSkn7 leads to an altered light response, maybe due to deregulation of photoreceptor genes.

The phenomenon of altered differentiation in *skn7* mutants has been described in other fungi, e.g., *A. alternata* and *A. nidulans* (48, 72). Additionally, changed melanization was observed in *A. alternata*, *Magnaporthe oryzae*, and *Cryptococcus neoformans* (48, 52, 74). A reduction in melanin biosynthesis is also possible for the Δ*bcskn7* strain because the pigmentation of the mycelium appeared more whitish than that of the WT, and the expression of melanin biosynthesis genes was reduced. (Fig. 2C to E and 5A). Liu et al. (75) also studied the expression of putative melanin biosynthesis genes and demonstrated that *bcsak1* deletion mutants showed delayed pigmentation, whereas pigmentation of the Δ*bos1* mutant was increased (75). Analyses of the mutant's pathogenicity showed a WT-like infection upon inoculation with spores, confirming the results of Yang et al. (32) (Fig. 5A). These observations are in accordance with our previous results for the Δ*bap1* strain and support the hypothesis that ROS detoxification might not be required for successful plant infection though the fungus is exposed to high ROS concentrations. It has already been demonstrated that Bap1-dependent ROS detoxification genes are not even upregulated during fungal attack although H_2O_2 is present around the penetration sites (39). Interestingly, however, plant infection by the *skn7* mutants with mycelium plugs was impaired. This observation goes beyond analyses from Yang et al. (32) and revealed an influence of BcSkn7 on virulence. Only about half of the Δ*bcskn7* mutant plugs were able to cause infection in contrast to those of the Δ*bap1* strain and the WT (Fig. 5B). Additionally, again the effect for the Δ*bap1 bcskn7* strain was more severe as the number of unsuccessful infections increased to 70% in the double deletion mutant. It is generally accepted that mycelium-derived infection in *B. cinerea* depends on the ability to form infection cushions, specialized dense and highly branched structures (76). Analysis of these infection structures showed significantly reduced numbers and penetration for the Δ*bcskn7* and Δ*bap1 bcskn7* strains (Fig. 5C). Defects in mycelium-derived virulence have been shown for *bccrz1*, *bcsak1*, and *bmp1* deletion strains (77, 78). The penetration defect based on mycelium may in part be due to an inability to remodel the cell wall correctly, leading to impaired cell wall integrity. The wall composition may be important for virulence as it could influence the responsiveness of membrane-bound receptors in the cell response (79).

The effects of Ap1 and Skn7 on virulence in other fungi are species specific. Whereas in *A. alternata* significantly fewer necrotic lesions were observed for *skn7* deletion mutants on citrus cultivars, no changes in virulence were detected for *M. oryzae* or *C. heterostrophus* (48, 56, 74).

Downloaded from http://ec.asm.org/ on April 14, 2016 by INRA - old

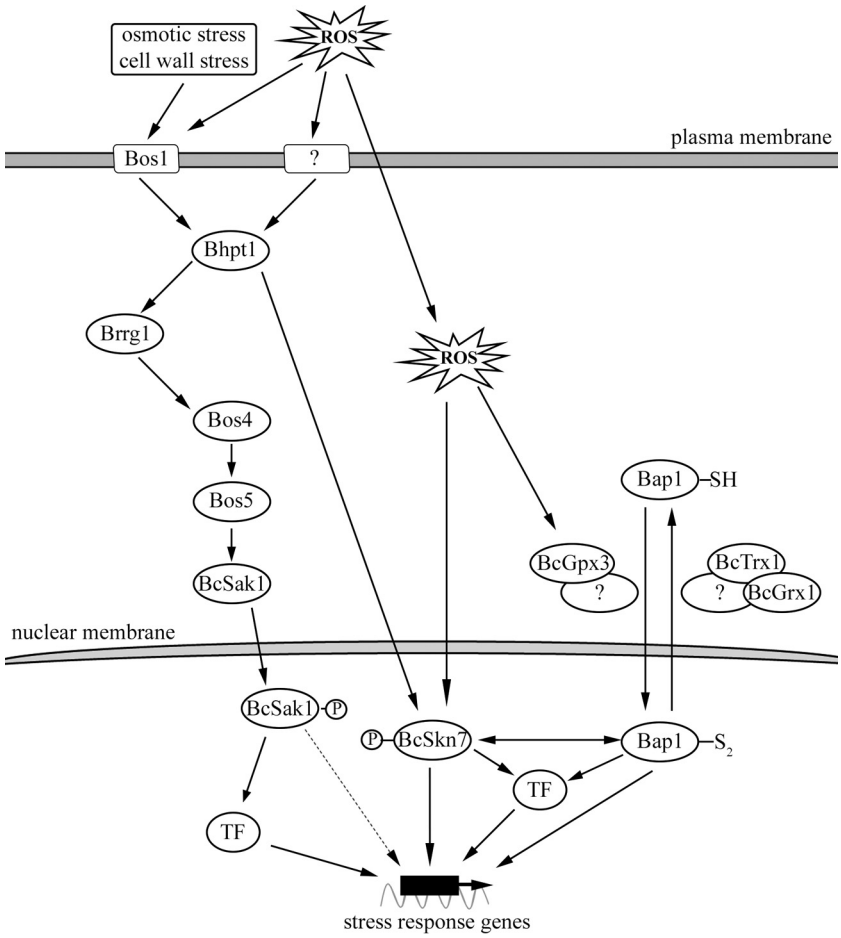

FIG 8 Model showing the putative regulatory network of BcSak1, BcSkn7, and Bap1 in *B. cinerea*. For further details see the text. TF, unidentified transcription factor.

Regulatory functions of Bap1 and BcSkn7. An important function of BcSkn7 and Bap1 could be shown in the regulation of oxidative stress-responsive genes. Most of the investigated genes involved in the OSR were not expressed or were expressed less strongly in strains lacking either *bcskn7* or *bap1* (see Fig. S4 in the supplemental material). However, from these results it is not clear whether the regulation of these genes is direct or indirect. In *S. cerevisiae* DNA binding sites for Yap1 and Skn7p mediating direct interactions with promoters of OSR genes could be identified. These DBD sites are highly conserved among fungi so that similar functions of these motifs are assumed. So far, however, the functionality could not be proved in direct interaction assays in filamentous fungi. In the Y1H approach used here, the binding abilities of Bap1 and BcSkn7 were tested for seven genes for which the putative respective binding motifs could be identified by *in silico* promoter analyses (see Fig. S5).

For Bap1 an interaction with the promoter of *gsh* could be shown (Fig. 7), concomitant with binding studies in yeast (80). Moreover, expression of *gsh* was solely dependent on Bap1, but not BcSkn7, which is confirmed by the absence of an OSRE motif in the promoter of *gsh*. Next to *gsh*, also *grx* seems to be bound and thus directly regulated by Bap1. The Y1H test with BcSkn7 confirmed an interaction with the promoter of *glr1*. Therefore, binding to the OSRE2 motif is likely, but this motif is also present in *crz1*, where no interaction could be observed. The influence of BcSkn7 on *glr1* differs from results in yeast, where *glr1* expression was shown to be solely dependent on Yap1 (21).

The results show that conclusions regarding specific binding motifs of BcSkn7 and Bap1 are difficult to draw. The quantity and composition of binding motifs in each promoter are quite diverse, so specific consensus sequences cannot be assigned for Bap1 and BcSkn7, respectively. However, interpretation of the data should take into account the technical limits of the Y1H technique. Apart from the sequence, the localization of the binding motifs with respect to each other might be important. Close proximity of two binding motifs such as in *glr1* and *grx1* might enhance binding, which would hint toward a codependency of both TFs. In *S. cerevisiae* phosphorylation of Skn7p is postulated to be important for stabilization of the Skn7p/Yap1 protein complex, thereby allowing activation of OSR genes (17). Some of the known Bap1 and BcSkn7 target genes, such as *cat5* and *trr1*, do not contain a common binding motif. Accordingly, the influence is indirect, and other TFs are needed to activate gene expression. Other TFs known to be involved in OSR are Msn2/4p and Hsf1p (81). For example H_2O_2-induced expression of *CTT1* requires Msn2/4p and the stress response element (STRE) binding site. In addition Yap1 and Skn7p are required even though YRE or OSRE binding sites are lacking in these promoters (67, 82, 83). This emphasizes

Downloaded from http://ec.asm.org/ on April 14, 2016 by INRA - old

the complexity of ROS detoxification and regulation as well as signaling in general.

Role of BcGpx3 in the OSR. In yeast the glutathione peroxidase Gpx3p acts as sensor for the activation of Yap1 upon oxidative stress (62). Also the glutathione peroxidase Hyr1 of *M. oryzae* has already been shown to be involved in the oxidative stress response (84). However, our data show that the *B. cinerea* BcGpx3 is involved in neither pathogenicity nor the OSR (see Fig. S6C and D in the supplemental material). Localization studies showed that BcGpx3 is not essential for nuclear retention of Bap1 (see Fig. S7B) though the Ap1 binding site of the yeast enzyme is highly conserved. However, participation of BcGpx3 in the activation of Bap1 cannot be excluded. In *S. cerevisiae*, next to Gpx3p, the Yap1 binding protein 1 (Ybp1) also contributes to Yap1 activation. Still, double deletion of these genes leads to residual peroxide-induced oxidation. Accordingly, other factors such as peroxiredoxins may also serve as activators (21, 85). As there are homologs of these candidate genes present in *B. cinerea*, a similar interplay can be presumed for the activation of Bap1.

Integration of BcSkn7 in the signaling network of *B. cinerea*. BcSkn7 was shown to be involved in the response to different kinds of stress. Based on the presented results, BcSkn7 works as an RR in the two-component signal transduction (TCS) pathway. Via the phosphorylation of D424 the response to osmotic stress is regulated. Furthermore, cell wall integrity and development are clearly regulated via the TCS. In contrast, it is not clear whether BcSkn7 is responding directly to oxidative stress. At least it has been established that the phosphorylation of S434 is insufficient for the regulation of OSR.

One of the few investigated HKs involved in the TCS in *B. cinerea* is Bos1. Deletion of this gene constitutively activates the BcSak1 module and leads to sensitivity to hydrogen peroxide and osmotic stress, fungicide tolerance, reduced production of macroconidia, increased pigmentation, and impaired virulence (25, 26). As expected, the lack of *bcsak1* (and likewise the upstream kinases Bos4 and Bos5) affects the same parameters as deletion of *bos1*, apart from the resistance against fungicides (26, 30, 75, 77, 86, 87). The response regulator Brrg1 seems to be necessary for the activation of BcSak1 as the MAPK is not phosphorylated in mutants lacking *brrg1*. Deletion of *brrg1* resulted in loss of conidiation and increased sensitivity to fungicides, osmotic stress, and H_2O_2-generated oxidative stress (31). These comparative analyses indicate that Bos1 regulates the resistance to fungicides and superoxides and adaptation to polyols independently of Brrg1 and BcSak1. BcSkn7 seems to be a second RR downstream of Bos1. It could already be shown that BcSak1 phosphorylation is drastically reduced under osmotic stress conditions in the Δ*bcskn7* strain (32). The lack of *bcsak1* affects the composition and integrity of the fungal cell wall, indicated by increased tolerance to cell wall-degrading enzymes and increased susceptibility toward cell wall-challenging agents (75). This result agrees with observations made for the Δ*bcskn7* strain, suggesting a response to cell wall stress via this signaling branch. Furthermore, penetration by both mutants based on mycelia is affected. In contrast to the Δ*bos1* mutant but similar to the Δ*bcsak1* mutant, the Δ*bcskn7* strain shows reduced pigmentation. Therefore, there may be a promoting function of BcSkn7 on the regulation of melanin biosynthesis.

In summary, this study helped to further unravel the signaling network of *B. cinerea* (Fig. 8). Characterization of the Δ*bap1 bcskn7* and Δ*bcskn7* mutants revealed that BcSkn7 is involved in several transcriptional programs in response to different types of stress. BcSkn7 and Bap1 are key players in the oxidative stress response and work in concert in gene regulation; however, this pathway seems to be independent of the Bos1 phosphorelay. But cell wall integrity and development, as well as mycelium-derived penetration defects, correspond to the Bos1-BcSak1 pathway and therefore make BcSkn7 an important RR in this TCS.

ACKNOWLEDGMENTS

We thank Brian Williamson for critical reading of the manuscript, Julia Schumacher for discussion, Ulrike Siegmund for help in the generation of *gpx3* deletion mutants, and Jan Kassens for technical assistance.

Financial support by the Deutsche Forschungsgemeinschaft ([DFG] Tu 50/17; A.V. was funded by DFG Graduate School 1409) is gratefully acknowledged.

REFERENCES

1. **Dean R, Van Kan JA, Pretorius ZA, Hammond-Kosack KE, Di Pietro A, Spanu PD, Rudd JJ, Dickman M, Kahmann R, Ellis J, Foster GD.** 2012. The top 10 fungal pathogens in molecular plant pathology. Mol Plant Pathol **13:**414–430. http://dx.doi.org/10.1111/j.1364-3703.2011.00783.x.
2. **Jarvis WR.** 1977. *Botryotinia* and *Botrytis species*: taxonomy, physiology and pathogenicity. A guide to the literature. Canada Department of Agriculture, Ottawa, Canada.
3. **van Kan JA.** 2006. Licensed to kill: the lifestyle of a necrotrophic plant pathogen. Trends Plant Sci **11:**247–253. http://dx.doi.org/10.1016/j.tplants.2006.03.005.
4. **Elad Y, Evenses K.** 1995. Physiological aspects of resistance to *Botrytis cinerea*. Phytopathology **85:**637–643.
5. **Vicedo B, Mde la Leyva O, Flors V, Finiti I, Del Amo G, Walters D, Real MD, Garcia-Agustin P, Gonzalez-Bosch C.** 2006. Control of the phytopathogen *Botrytis cinerea* using adipic acid monoethyl ester. Arch Microbiol **184:**316–326. http://dx.doi.org/10.1007/s00203-005-0048-6.
6. **Jones JD, Dangl JL.** 2006. The plant immune system. Nature **444:**323–329. http://dx.doi.org/10.1038/nature05286.
7. **VanEtten HD, Sandrock RW, Wasmann CC, Soby SD, McCluskey K, Wang P.** 1995. Detoxification of phytoanticipins and phytoalexins by phytopathogenic fungi. Can J Bot **73:**(Suppl 1):518–525.
8. **Govrin EM, Levine A.** 2000. The hypersensitive response facilitates plant infection by the necrotrophic pathogen *Botrytis cinerea*. Curr Biol **10:**751–757. http://dx.doi.org/10.1016/S0960-9822(00)00560-1.
9. **Williamson B, Tudzynski B, Tudzynski P, van Kan JA.** 2007. *Botrytis cinerea*: the cause of grey mould disease. Mol Plant Pathol **8:**561–580. http://dx.doi.org/10.1111/j.1364-3703.2007.00417.x.
10. **Tenberge KB, Beckedorf M, Hoppe B, Schouten A, Solf M, von den Driesch M.** 2002. In situ localization of AOS in host-pathogen interactions. Microsc Microanal **8**(Suppl 2):250–251.
11. **Tudzynski P, Kokkelink L.** 2009. *Botrytis cinerea*: molecular aspects of a necrotrophic life style, p 29–50. *In* Deising H (ed), The mycota: plant relationships, 2nd ed, vol V. Springer-Verlag, Berlin, Germany.
12. **Fassler JS, West AH.** 2013. Histidine phosphotransfer proteins in fungal two-component signal transduction pathways. Eukaryot Cell **12:**1052–1060. http://dx.doi.org/10.1128/EC.00083-13.
13. **Maeda T, Wurgler-Murphy SM, Saito H.** 1994. A two-component system that regulates an osmosensing MAP kinase cascade in yeast. Nature **369:**242–245. http://dx.doi.org/10.1038/369242a0.
14. **Horie T, Tatebayashi K, Yamada R, Saito H.** 2008. Phosphorylated Ssk1 prevents unphosphorylated Ssk1 from activating the Ssk2 mitogen-activated protein kinase kinase kinase in the yeast high-osmolarity glycerol osmoregulatory pathway. Mol Cell Biol **28:**5172–5183. http://dx.doi.org/10.1128/MCB.00589-08.
15. **Lu JM, Deschenes RJ, Fassler JS.** 2003. Saccharomyces cerevisiae histidine phosphotransferase Ypd1p shuttles between the nucleus and cytoplasm for SLN1-dependent phosphorylation of Ssk1p and Skn7p. Eukaryot Cell **2:**1304–1314. http://dx.doi.org/10.1128/EC.2.6.1304-1314.2003.
16. **Hohmann S.** 2002. Osmotic stress signaling and osmoadaptation in yeasts. Microbiol Mol Biol Rev **66:**300–372. http://dx.doi.org/10.1128/MMBR.66.2.300-372.2002.
17. **He XJ, Mulford KE, Fassler JS.** 2009. Oxidative stress function of the

Downloaded from http://ec.asm.org/ on April 14, 2016 by INRA - old

Saccharomyces cerevisiae Skn7 receiver domain. Eukaryot Cell **8:**768–778. http://dx.doi.org/10.1128/EC.00021-09.
18. **Mulford KE, Fassler JS.** 2011. Association of the Skn7 and Yap1 transcription factors in the *Saccharomyces cerevisiae* oxidative stress response. Eukaryot Cell **10:**761–769. http://dx.doi.org/10.1128/EC.00328-10.
19. **Morgan BA, Banks GR, Toone WM, Raitt D, Kuge S, Johnston LH.** 1997. The Skn7 response regulator controls gene expression in the oxidative stress response of the budding yeast *Saccharomyces cerevisiae*. EMBO J **16:**1035–1044. http://dx.doi.org/10.1093/emboj/16.5.1035.
20. **He XJ, Fassler JS.** 2005. Identification of novel Yap1p and Skn7p binding sites involved in the oxidative stress response of *Saccharomyces cerevisiae*. Mol Microbiol **58:**1454–1467. http://dx.doi.org/10.1111/j.1365-2958.2005.04917.x.
21. **Lee J, Godon C, Lagniel G, Spector D, Garin J, Labarre J, Toledano MB.** 1999. Yap1 and Skn7 control two specialized oxidative stress response regulons in yeast. J Biol Chem **274:**16040–16046. http://dx.doi.org/10.1074/jbc.274.23.16040.
22. **Fernandes L, Rodrigues-Pousada C, Struhl K.** 1997. Yap, a novel family of eight bZIP proteins in *Saccharomyces cerevisiae* with distinct biological functions. Mol Cell Biol **17:**6982–6993.
23. **Toone WM, Jones N.** 1999. AP-1 transcription factors in yeast. Curr Opin Genet Dev **9:**55–61. http://dx.doi.org/10.1016/S0959-437X(99)80008-2.
24. **Catlett NL, Yoder OC, Turgeon BG.** 2003. Whole-genome analysis of two-component signal transduction genes in fungal pathogens. Eukaryot Cell **2:**1151–1161. http://dx.doi.org/10.1128/EC.2.6.1151-1161.2003.
25. **Viaud M, Fillinger S, Liu W, Polepalli JS, Le Pecheur P, Kunduru AR, Leroux P, Legendre L.** 2006. A class III histidine kinase acts as a novel virulence factor in *Botrytis cinerea*. Mol Plant Microbe Interact **19:**1042–1050. http://dx.doi.org/10.1094/MPMI-19-1042.
26. **Liu W, Leroux P, Fillinger S.** 2008. The HOG1-like MAP kinase Sak1 of *Botrytis cinerea* is negatively regulated by the upstream histidine kinase Bos1 and is not involved in dicarboximide- and phenylpyrrole-resistance. Fungal Genet Biol **45:**1062–1074. http://dx.doi.org/10.1016/j.fgb.2008.04.003.
27. **Posas F, Wurgler-Murphy SM, Maeda T, Witten EA, Thai TC, Saito H.** 1996. Yeast HOG1 MAP kinase cascade is regulated by a multistep phosphorelay mechanism in the SLN1-YPD1-SSK1 "two-component" osmosensor. Cell **86:**865–875. http://dx.doi.org/10.1016/S0092-8674(00)80162-2.
28. **Banno S, Noguchi R, Yamashita K, Fukumori F, Kimura M, Yamaguchi I, Fujimura M.** 2007. Roles of putative His-to-Asp signaling modules HPT-1 and RRG-2, on viability and sensitivity to osmotic and oxidative stresses in Neurospora crassa. Curr Genet **51:**197–208. http://dx.doi.org/10.1007/s00294-006-0116-8.
29. **Furukawa K, Hoshi Y, Maeda T, Nakajima T, Abe K.** 2005. *Aspergillus nidulans* HOG pathway is activated only by two-component signalling pathway in response to osmotic stress. Mol Microbiol **56:**1246–1261. http://dx.doi.org/10.1111/j.1365-2958.2005.04605.x.
30. **Yan L, Yang Q, Sundin GW, Li H, Ma Z.** 2010. The mitogen-activated protein kinase kinase BOS5 is involved in regulating vegetative differentiation and virulence in *Botrytis cinerea*. Fungal Genet Biol **47:**753–760. http://dx.doi.org/10.1016/j.fgb.2010.06.002.
31. **Yan L, Yang Q, Jiang J, Michailides TJ, Ma Z.** 2011. Involvement of a putative response regulator Brrg-1 in the regulation of sporulation, sensitivity to fungicides, and osmotic stress in *Botrytis cinerea*. Appl Microbiol Biotechnol **90:**215–226. http://dx.doi.org/10.1007/s00253-010-3027-z.
32. **Yang Q, Yin D, Yin Y, Cao Y, Ma Z.** 2014. The response regulator BcSkn7 is required for vegetative differentiation and adaptation to oxidative and osmotic stresses in *Botrytis cinerea*. Mol Plant Pathol **16:**276–287. http://dx.doi.org/10.1111/mpp.12181.
33. **Quidde T, Büttner P, Tudzynski P.** 1999. Evidence for three different specific saponin-detoxifying activities in *Botrytis cinerea* and cloning of a gene coding for a putative avenacinase. Eur J Plant Pathol **105:**273–283. http://dx.doi.org/10.1023/A:1008796006051.
34. **Pontecorvo G, Roper JA, Hemmons LM, MacDonalds KD, Bufton AW.** 1953. The genetics of *Aspergillus nidulans*. Adv Genet **5:**141–238. http://dx.doi.org/10.1016/S0065-2660(08)60408-3.
35. **Schulze Gronover C, Schorn C, Tudzynski B.** 2004. Identification of *Botrytis cinerea* genes up-regulated during infection and controlled by the Gα subunit BCG1 using suppression subtractive hybridization (SSH). Mol Plant Microbe Interact **17:**537–546. http://dx.doi.org/10.1094/MPMI.2004.17.5.537.
36. **Shalaby S, Larkov O, Lamdan NL, Horwitz BA.** 2014. Genetic interaction of the stress response factors ChAP1 and Skn7 in the maize pathogen *Cochliobolus heterostrophus*. FEMS Microbiol Lett **350:**83–89. http://dx.doi.org/10.1111/1574-6968.12314.
37. **Cenis JL.** 1992. Rapid extraction of fungal DNA for PCR amplification. Nucleic Acids Res **20:**2380. http://dx.doi.org/10.1093/nar/20.9.2380.
38. **Sambrook J, Fritsch EF, Maniatis T.** 1989. Molecular cloning: a laboratory manual, 2nd ed. Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY.
39. **Temme N, Tudzynski P.** 2009. Does *Botrytis cinerea* ignore H_2O_2-induced oxidative stress during infection? Characterization of *Botrytis* activator protein 1. Mol Plant Microbe Interact **22:**987–998. http://dx.doi.org/10.1094/MPMI-22-8-0987.
40. **Christianson TW, Sikorski RS, Dante M, Shero JH, Hieter P.** 1992. Multifunctional yeast high-copy-number shuttle vectors. Gene **110:**119–122. http://dx.doi.org/10.1016/0378-1119(92)90454-W.
41. **Winston F, Dollard C, Ricupero-Hovasse SL.** 1995. Construction of a set of convenient *Saccharomyces cerevisiae* strains that are isogenic to S288C. Yeast **11:**53–55. http://dx.doi.org/10.1002/yea.320110107.
42. **Colot HV, Park G, Turner GE, Ringelberg C, Crew CM, Litvinkova L, Weiss RL, Borkovich KA, Dunlap JC.** 2006. A high-throughput gene knockout procedure for Neurospora reveals functions for multiple transcription factors. Proc Natl Acad Sci U S A **103:**10352–10357. http://dx.doi.org/10.1073/pnas.0601456103.
43. **Schumacher J,** 2012. Tools for Botrytis cinerea: New expression vectors make the gray mold fungus more accessible to cell biology approaches. Fungal Genet Biol. **49:**483–497. http://dx.doi.org/10.1016/j.fgb.2012.03.005.
44. **Marschall R, Tudzynski P.** 2014. A new and reliable method for live imaging and quantification of reactive oxygen species in *Botrytis cinerea*: technological advancement. Fungal Genet Biol **71:**68–75. http://dx.doi.org/10.1016/j.fgb.2014.08.009.
45. **Simon A, Dalmais B, Morgant G, Viaud M.** 2013. Screening of a *Botrytis cinerea* one-hybrid library reveals a Cys2His2 transcription factor involved in the regulation of secondary metabolism gene clusters. Fungal Genet Biol **52:**9–19. http://dx.doi.org/10.1016/j.fgb.2013.01.006.
46. **Meijer AH, Ouwerkerk PB, Hoge JH.** 1998. Vectors for transcription factor cloning and target site identification by means of genetic selection in yeast. Yeast **14:**1407–1415.
47. **Ouwerkerk PB, Meijer AH.** 2001. Yeast one-hybrid screening for DNA-protein interactions. Curr Protoc Mol Biol. Chapter 12:Unit 12.12. http://dx.doi.org/10.1002/0471142727.mb1212s55.
48. **Chen LH, Lin CH, Chung KR.** 2012. Roles for SKN7 response regulator in stress resistance, conidiation and virulence in the citrus pathogen Alternaria alternata. Fungal Genet Biol **49:**802–813. http://dx.doi.org/10.1016/j.fgb.2012.07.006.
49. **Raitt DC, Johnson AL, Erkine AM, Makino K, Morgan B, Gross DS, Johnston LH.** 2000. The Skn7 response regulator of *Saccharomyces cerevisiae* interacts with Hsf1 in vivo and is required for the induction of heat shock genes by oxidative stress. Mol Biol Cell **11:**2335–2347. http://dx.doi.org/10.1091/mbc.11.7.2335.
50. **Schumacher J, Simon A, Cohrs KC, Traeger S, Porquier A, Dalmais B, Viaud M, Tudzynski B.** 2015. The VELVET complex in the gray mold fungus *Botrytis cinerea*: impact of BcLAE1 on differentiation, secondary metabolism and virulence. Mol Plant Microbe Interact http://dx.doi.org/10.1094/MPMI-12-14-0411-R.
51. **Schumacher J, Simon A, Cohrs KC, Viaud M, Tudzynski P.** 2014. The transcription factor BcLTF1 regulates virulence and light responses in the necrotrophic plant pathogen *Botrytis cinerea*. PLoS Genet **10:**e1004040. http://dx.doi.org/10.1371/journal.pgen.1004040.
52. **Bahn YS, Kojima K, Cox GM, Heitman J.** 2006. A unique fungal two-component system regulates stress responses, drug sensitivity, sexual development, and virulence of *Cryptococcus neoformans*. Mol Biol Cell **17:**3122–3135. http://dx.doi.org/10.1091/mbc.E06-02-0113.
53. **Hagiwara D, Mizuno T, Abe K.** 2011. Characterization of the conserved phosphorylation site in the *Aspergillus nidulans* response regulator SrrA. Curr Genet **57:**103–114. http://dx.doi.org/10.1007/s00294-010-0330-2.
54. **Saijo T, Miyazaki T, Izumikawa K, Mihara T, Takazono T, Kosai K, Imamura Y, Seki M, Kakeya H, Yamamoto Y, Yanagihara K, Kohno S.** 2010. Skn7p is involved in oxidative stress response and virulence of *Candida glabrata*. Mycopathologia **169:**81–90. http://dx.doi.org/10.1007/s11046-009-9233-5.
55. **Viefhues A, Heller J, Temme N, Tudzynski P.** 2014. Redox systems in

Downloaded from http://ec.asm.org/ on April 14, 2016 by INRA - old

Botrytis cinerea: impact on development and virulence. Mol Plant Microbe Interact **27:**858–874. http://dx.doi.org/10.1094/MPMI-01-14-0012-R.

56. **Oide S, Liu J, Yun SH, Wu D, Michev A, Choi MY, Horwitz BA, Turgeon BG.** 2010. Histidine kinase two-component response regulator proteins regulate reproductive development, virulence, and stress responses of the fungal cereal pathogens *Cochliobolus heterostrophus* and *Gibberella zeae*. Eukaryot Cell **9:**1867–1880. http://dx.doi.org/10.1128/EC.00150-10.
57. **Garcia-Arenal F, Sagasta EM.** 1980. Scanning electron microscopy of *Botrytis cinerea* penetration of bean (*Phaseolus vulgaris*) hypocotyls. J Phytopathol **99:**37–42. http://dx.doi.org/10.1111/j.1439-0434.1980.tb03757.x.
58. **Brown JL, Bussey H, Stewart RC.** 1994. Yeast Skn7p functions in a eukaryotic two-component regulatory pathway. EMBO J **13:**5186–5194.
59. **Cuellar-Cruz M, Briones-Martin-del-Campo M, Canas-Villamar I, Montalvo-Arredondo J, Riego-Ruiz L, Castano I, De Las Penas A.** 2008. High resistance to oxidative stress in the fungal pathogen *Candida glabrata* is mediated by a single catalase, Cta1p, and is controlled by the transcription factors Yap1p, Skn7p, Msn2p, and Msn4p. Eukaryot Cell **7:**814–825. http://dx.doi.org/10.1128/EC.00011-08.
60. **Kuge S, Jones N.** 1994. YAP1 dependent activation of TRX2 is essential for the response of *Saccharomyces cerevisiae* to oxidative stress by hydroperoxides. EMBO J **13:**655–664.
61. **Tsuzi D, Maeta K, Takatsume Y, Izawa S, Inoue Y.** 2004. Regulation of the yeast phospholipid hydroperoxide glutathione peroxidase GPX2 by oxidative stress is mediated by Yap1 and Skn7. FEBS Lett **565:**148–154. http://dx.doi.org/10.1016/j.febslet.2004.03.091.
62. **Delaunay A, Pflieger D, Barrault MB, Vinh J, Toledano MB.** 2002. A thiol peroxidase is an H_2O_2 receptor and redox-transducer in gene activation. Cell **111:**471–481. http://dx.doi.org/10.1016/S0092-8674(02)01048-6.
63. **Vivancos AP, Castillo EA, Jones N, Ayte J, Hidalgo E.** 2004. Activation of the redox sensor Pap1 by hydrogen peroxide requires modulation of the intracellular oxidant concentration. Mol Microbiol **52:**1427–1435. http://dx.doi.org/10.1111/j.1365-2958.2004.04065.x.
64. **Valette-Collet O, Cimerman A, Reignault P, Levis C, Boccara M.** 2003. Disruption of *Botrytis cinerea* pectin methylesterase gene Bcpme1 reduces virulence on several host plants. Mol Plant Microbe Interact **16:**360–367. http://dx.doi.org/10.1094/MPMI.2003.16.4.360.
65. **Kars I, McCalman M, Wagemakers L, Van Kan JAL.** 2005. Functional analysis of *Botrytis cinerea* pectin methylesterase genes by PCR-based targeted mutagenesis: Bcpme1 and Bcpme2 are dispensable for virulence of strain B05.10. Mol Plant Pathol **6:**641–652. http://dx.doi.org/10.1111/j.1364-3703.2005.00312.x.
66. **Siewers V, Viaud M, Jimenez-Teja D, Collado IG, Gronover CS, Pradier JM, Tudzynski B, Tudzynski P.** 2005. Functional analysis of the cytochrome P450 monooxygenase gene *bcbot1* of *Botrytis cinerea* indicates that botrydial is a strain-specific virulence factor. Mol Plant Microbe Interact **18:**602–612. http://dx.doi.org/10.1094/MPMI-18-0602.
67. **Lee J, Spector D, Godon C, Labarre J, Toledano MB.** 1999. A new antioxidant with alkyl hydroperoxide defense properties in yeast. J Biol Chem **274:**4537–4544. http://dx.doi.org/10.1074/jbc.274.8.4537.
68. **Calvo IA, Garcia P, Ayte J, Hidalgo E.** 2012. The transcription factors Pap1 and Prr1 collaborate to activate antioxidant, but not drug tolerance, genes in response to H_2O_2. Nucleic Acids Res **40:**4816–4824. http://dx.doi.org/10.1093/nar/gks141.
69. **Singh P, Chauhan N, Ghosh A, Dixon F, Calderone R.** 2004. SKN7 of *Candida albicans*: mutant construction and phenotype analysis. Infect Immun **72:**2390–2394. http://dx.doi.org/10.1128/IAI.72.4.2390-2394.2004.
70. **Lamarre C, Ibrahim-Granet O, Du C, Calderone R, Latge JP.** 2007. Characterization of the SKN7 ortholog of *Aspergillus fumigatus*. Fungal Genet Biol **44:**682–690. http://dx.doi.org/10.1016/j.fgb.2007.01.009.
71. **Heller J, Meyer AJ, Tudzynski P.** 2012. Redox-sensitive GFP2: use of the genetically encoded biosensor of the redox status in the filamentous fungus *Botrytis cinerea*. Mol Plant Pathol **13:**935–947. http://dx.doi.org/10.1111/j.1364-3703.2012.00802.x.
72. **Vargas-Perez I, Sanchez O, Kawasaki L, Georgellis D, Aguirre J.** 2007. Response regulators SrrA and SskA are central components of a phosphorelay system involved in stress signal transduction and asexual sporulation in *Aspergillus nidulans*. Eukaryot Cell **6:**1570–1583. http://dx.doi.org/10.1128/EC.00085-07.
73. **Levin DE.** 2005. Cell wall integrity signaling in *Saccharomyces cerevisiae*. Microbiol Mol Biol Rev **69:**262–291. http://dx.doi.org/10.1128/MMBR.69.2.262-291.2005.
74. **Motoyama T, Ochiai N, Morita M, Iida Y, Usami R, Kudo T.** 2008. Involvement of putative response regulator genes of the rice blast fungus *Magnaporthe oryzae* in osmotic stress response, fungicide action, and pathogenicity. Curr Genet **54:**185–195. http://dx.doi.org/10.1007/s00294-008-0211-0.
75. **Liu W, Soulie MC, Perrino C, Fillinger S.** 2011. The osmosensing signal transduction pathway from *Botrytis cinerea* regulates cell wall integrity and MAP kinase pathways control melanin biosynthesis with influence of light. Fungal Genet Biol **48:**377–387. http://dx.doi.org/10.1016/j.fgb.2010.12.004.
76. **Tenberge KB.** 2007. Morphology and cellular organisation in *Botrytis* interactions with plants, p 67–84. *In* Elad Y, Williamson B, Tudzynski P, Delen N (ed), Botrytis: biology, pathology and control. Springer, Dordrecht, The Netherlands.
77. **Segmuller N, Ellendorf U, Tudzynski B, Tudzynski P.** 2007. BcSAK1, a stress-activated mitogen-activated protein kinase, is involved in vegetative differentiation and pathogenicity in *Botrytis cinerea*. Eukaryot Cell **6:**211–221. http://dx.doi.org/10.1128/EC.00153-06.
78. **Schumacher J, Kokkelink L, Huesmann C, Jimenez-Teja D, Collado IG, Barakat R, Tudzynski P, Tudzynski B.** 2008. The cAMP-dependent signaling pathway and its role in conidial germination, growth, and virulence of the gray mold *Botrytis cinerea*. Mol Plant Microbe Interact **21:**1443–1459. http://dx.doi.org/10.1094/MPMI-21-11-1443.
79. **Fassler JS, West AH.** 2011. Fungal Skn7 stress responses and their relationship to virulence. Eukaryot Cell **10:**156–167. http://dx.doi.org/10.1128/EC.00245-10.
80. **Wu AL, Moye-Rowley WS.** 1994. GSH1, which encodes gamma-glutamylcysteine synthetase, is a target gene for yAP-1 transcriptional regulation. Mol Cell Biol **14:**5832–5839. http://dx.doi.org/10.1128/MCB.14.9.5832.
81. **Martinez-Pastor MT, Marchler G, Schuller C, Marchler-Bauer A, Ruis H, Estruch F.** 1996. The *Saccharomyces cerevisiae* zinc finger proteins Msn2p and Msn4p are required for transcriptional induction through the stress response element (STRE). EMBO J **15:**2227–2235.
82. **Winderickx J, de Winde JH, Crauwels M, Hino A, Hohmann S, Van Dijck P, Thevelein JM.** 1996. Regulation of genes encoding subunits of the trehalose synthase complex in *Saccharomyces cerevisiae*: novel variations of STRE-mediated transcription control? Mol Gen Genet **252:**470–482. http://dx.doi.org/10.1007/BF02173013.
83. **Rep M, Proft M, Remize F, Tamas M, Serrano R, Thevelein JM, Hohmann S.** 2001. The *Saccharomyces cerevisiae* Sko1p transcription factor mediates HOG pathway-dependent osmotic regulation of a set of genes encoding enzymes implicated in protection from oxidative damage. Mol Microbiol **40:**1067–1083. http://dx.doi.org/10.1046/j.1365-2958.2001.02384.x.
84. **Huang K, Czymmek KJ, Caplan JL, Sweigard JA, Donofrio NM.** 2011. HYR1-mediated detoxification of reactive oxygen species is required for full virulence in the rice blast fungus. PLoS Pathog **7:**e1001335. http://dx.doi.org/10.1371/journal.ppat.1001335.
85. **Veal EA, Ross SJ, Malakasi P, Peacock E, Morgan BA.** 2003. Ybp1 is required for the hydrogen peroxide-induced oxidation of the Yap1 transcription factor. J Biol Chem **278:**30896–30904. http://dx.doi.org/10.1074/jbc.M303542200.
86. **Heller J, Ruhnke N, Espino JJ, Massaroli M, Collado IG, Tudzynski P.** 2012. The mitogen-activated protein kinase BcSak1 of Botrytis cinerea is required for pathogenic development and has broad regulatory functions beyond stress response. Mol Plant Microbe Interact **25:**802–816. http://dx.doi.org/10.1094/MPMI-11-11-0299.
87. **Yang Q, Yan L, Gu Q, Ma Z.** 2012. The mitogen-activated protein kinase kinase kinase BcOs4 is required for vegetative differentiation and pathogenicity in *Botrytis cinerea*. Appl Microbiol Biotechnol **96:**481–492. http://dx.doi.org/10.1007/s00253-012-4029-9.
88. **Ausubel FM, Brent R, Kingston RE, Moore DD, Seidman JG, Smith JA, Struhl K.** 1987. Current protocols in molecular biology. John Wiley and Sons, New York, NY.

Downloaded from http://ec.asm.org/ on April 14, 2016 by INRA - old