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ORIGINAL ARTICLE

Identification of genes involved in fungal responses to strigolactones using mutants from fungal pathogens

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Abstract Strigolactones (SLs) as components of root exudates induce hyphal branching of arbuscular mycorrhizal (AM) fungi which is thought to favor the establishment of the beneficial symbiosis. Little is known on how AM fungi respond to SLs. Since AM fungi are poor model systems due to their obligate biotrophism and the lack of genetic transformation protocols, we took advantage of the sensitivity of several phytopathogenic fungi to GR24, a synthetic SLs analog. With the aim to identify the molecular determinants involved in SLs response in AM fungi and assuming conserved mechanisms in the fungal kingdom, we exploited the fungal pathogens *Botrytis cinerea* and *Cryphonectria parasitica,* for which mutant collections are available. Exposure of *B. cinerea* and *C. parasitica* to GR24 embedded in solid medium led to reduction of fungal radial growth. We set up the screening of a set of well-characterized gene deletion mutants to isolate genotypes with altered responses to SLs. Two *B. cinerea* mutants (defective of BcTrr1, a thioredoxin reductase and BcLTF1, a GATA transcription factor) turned out to be less responsive to GR24. One feature shared by the two mutants is the overproduction of reactive oxygen species (ROS). Indeed, an oxidizing effect was observed in a *B. cinerea* strain expressing a redox-sensitive GFP2 in the mitochondrial intermembrane space upon exposure to GR24. ROS and mitochondria are, therefore, emerging as mediators of SLs actions.

Keywords Strigolactones · GR24 · *Botrytis cinerea* · *Cryphonectria parasitica* · Reactive oxygen species · Mutant strains

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Introduction

Strigolactones (SLs) are plant signaling molecules, derived from the carotenoid-synthesis pathway (Al-Babili and Bouwmeester 2015), that contribute to define plant morphology and architecture by controlling several aspects of plant growth (Ruyter-Spira et al. 2013; Rasmussen et al. 2013; Brewer et al. 2013; Liu et al. 2013). They consist of an ABC-ring system connected via an enol ether bridge to a butenolide D ring (Cavar et al. 2014; Suppl. Fig. 1). SLs are a large class of natural compounds consisting of over 20 structural variants, most of which differ only by having one instead of two methyl groups on the cyclohexenyl A-ring or by having various combinations of hydroxyl or acetoxyl substituents on the A- and B-rings (Yoneyama et al. 2009; Zwanenburg et al. 2016a). SLs also occur in two distinct stereochemical configurations and the stereochemistry of some SLs was recently revised (Xie et al. 2013; Scaffidi

et al. 2014). SLs from the orobanchol-like family have an *'ent'* oriented C-ring while in the strigol-like family the C-ring has the opposite chirality of the orobanchol-like family (Xie et al. 2013; Scaffidi et al. 2014; Zwanenburg et al. 2016a, b; Suppl. Fig. 1).

All plant species examined so far produce a mixture of SLs (Cavar et al. 2014), varying in the amounts and ratios depending on growth stages and nutrient conditions (Yoneyama et al. 2012). Interestingly, SLs were first discovered as molecules released by plant roots into the rhizosphere and acting as inducers of seeds germination in parasitic plants of the *Orobancheaceae*, a serious agricultural pest (Parker 2009). In addition, in soil SLs have a positive function for plants since they stimulate the branching of pre-symbiotic hyphae in arbuscular mycorrhizal (AM) fungi (Akiyama et al. 2005; Besserer et al. 2006, 2008) possibly increasing the probability to establish a direct contact between the fungus and the plants roots. AM fungi establish in fact one of the most widespread and ancient symbiotic associations with the roots of most land plants (Bonfante and Genre 2010; Gutjahr and Parniske 2013). This mutualistic interaction is based on nutrients exchange: the fungus transfers to the host plant water and mineral nutrients (i.e., phosphorus, nitrogen) and, in turn, obtains carbohydrates necessary for the completion of its life cycle (van der Heijden et al. 2015). Besides promoting plant growth, through an improved mineral nutrition, AM fungi sustain other ecologically and economically important functions such as soil aggregation and water retention, tolerance to biotic and abiotic stresses and increase in plant biodiversity (Gianinazzi et al. 2010). Despite their clear economic and ecological importance, our knowledge of the functioning of AM fungi remains poor compared to other microorganisms. The AM interaction commences before the partners are in physical contact (Bonfante and Genre 2015). So far few molecules of plant origin have been described as having a stimulating activity towards AM fungi (Schmitz and Harrison 2015). Hydroxy fatty acids were shown to promote multiple lateral branching, although only in *Gigapora* species (Nagahashi et al. 2010; Nagahashi and Douds 2011). More recently on the basis of studies carried out on plant mutants, cutin monomers were shown to promote hyphopodia formation (Gobbato et al. 2012). But SLs were first identified as AM fungi-stimulating factors (Akiyama et al. 2005). Using GR24, a synthetic molecule commonly used as a reference for evaluating SLs bioactivity, Besserer et al. (2008) showed that in AM fungi the mitochondrial metabolism is activated. In particular, the treatment of AM fungus *Gigaspora rosea* with GR24 causes a rapid increase in the NADH concentration, the NADH dehydrogenase activity, and the ATP content of the fungal cell. Stimulation of the fungal mitotic activity was also observed several days after this initial boost. These results suggested that SLs are important plant signals involved in switching AM fungi toward a pre-symbiotic state. Interestingly, it has been shown that, in AM fungi, GR24 stimulates the production of short chitin oligomers whose perception on the root epidermis leads to the activation of calcium (Ca^{2+}) spiking, a key component of the signaling pathway involved in the initial stages of root colonization (Genre et al. 2013).

How AM fungi perceive and respond to SL remains unknown. Very recently, it has been demonstrated that GR24 induces a rapid $[Ca^{2+}]_i$ elevation in *Gigaspora margarita* hyphae suggesting the occurrence of Ca^{2+}-based sensing mechanisms for detecting and responding to SLs (Moscatiello et al. 2014). The use of SLs analogs that allowed structure–activity relationship studies suggested that AM fungi may use distinct modes of perception, since AM fungi turned out to be more demanding in term of structural requirements of the SL molecule to stimulate hyphal branching activity (Akiyama et al. 2010). Indeed, the analysis of the *Rhizophagus irregularis* genome, the only so far available for AM fungi (Tisserant et al. 2013; Lin et al. 2014), has not revealed clear fungal homologs of the SLs receptor complex (d14 and Max2) described in plants (de Saint et al. 2013; Liu et al. 2013).

AM fungi are a rather complex biological system, since they are obligate biotrophs and they are multinucleated throughout their life cycle (Lanfranco and Young 2012; Young 2015). So far, no stable genetic transformation protocols have been established for AM fungi (Requena and Helber 2007).

Dor et al. (2011) demonstrated that, beside AM fungi, a number of pathogenic fungi were sensitive to GR24, although other works using different experimental systems showed contrasting results (Steinkellner et al. 2007; Torres-Vera et al. 2014; Foo et al. 2016). Dor et al. (2011) set up a simple biological assay where GR24 was embedded in the solid medium where the fungi were inoculated: all the tested fungi, including *Botrytis cinerea,* a well-studied plant-interacting fungus for which complete genome sequence (Amselem et al. 2011) and mutants of different signaling pathways are available, showed a reduced radial growth. In addition, depending on the fungal species and concentrations used, an impact on hyphal branching was also observed (Dor et al. 2011).

Assuming that the molecular mechanisms involved in the response to SLs are somehow conserved in the fungal kingdom, we have exploited a number of characterized deletion mutants of *B. cinerea* and some from the other fungal pathogen *Cryphonectria parasitica* to isolate genotypes with altered responses to GR24. We show here that two *B. cinerea* mutants (defective of BcTrr1, a thioredoxin reductase and BcLTF1, a GATA transcription factor) display a lower sensitivity to GR24 compared to the wild type. Both mutants are impaired in reactive oxygen species

(ROS) metabolism. Indeed, exposure to GR24 led to an oxidizing effect in a *B. cinerea* strain expressing a redox-sensitive GFP2 at the level of the mitochondrial intermembrane space. These findings suggest that fungal responses to SLs rely on ROS and mitochondria.

Materials and methods

Fungal strains and cultivation methods

Strain B05.10 of *Botrytis cinerea* Pers.:Fr. [*Botryotinia fuckeliana* (de Bary) Whetzel] is a putative haploid strain obtained after benomyl treatment of an isolate from *Vitis* (Quidde et al. 1999), and was used as a host strain for transformation and as a wild type (wt) control in all experiments.

B. cinerea knock-out mutants were previously generated by replacing the respective gene via homologous integration of a resistance cassette containing the resistance marker genes (hygromycin/nourseothricin) under the control of the *trp*-promoter. The knock-out constructs were generated via the yeast recombination system that was described previously (Colot et al. 2006). All the strains used in this study are listed in Table 1.

Depending on the different experiments, the wild type and mutant strains were grown on several complex media. For conidiation, strains were incubated for 7 days under light conditions (18 °C) on PDAB medium [Potato dextrose agar (PDA-Sigma-Aldrich Chemie, Steinheim, Germany) supplemented with 10 % homogenized bean leaves of *Phaesolus vulgaris*]. For standard cultivation, synthetic complete medium (CM) was prepared according to Pontecorvo et al. (1953). As minimal medium, Gamborg B5 medium (containing macro-, microelements and vitamins; Duchefa—3.17 g/l) was used. Microscopic analyses were accomplished by preparing conidiospores in Gamborg B5 medium supplemented with 2 % of glucose. Short time storage of the strains was done at 4 °C on agar plates. For long time storage of mycelium, agar plugs were transferred to sterile H_2O and stored at 4 °C, while storage of conidia was done at −20 °C in 30 % (v/v) glycerol.

Cryphonectria parasitica Δ*cpku80* (in this work considered a wild type strain) and Δ*cpkk1*, Δ*cpkk2*, Δ*cpkk3* knock-out strains were kindly provided by Dr. Massimo Turina (Institute for Sustainable Plant Protection, CNR, Torino). Mutants were generated by site-directed double homologous recombination with a construct containing a gene cassette conferring hygromycin resistance (*hph*) and flanked by MAP2 K-specific sequences that were introduced into the Δ*cpku80* strain, a *C. parasitica* isolate highly efficient at homologous recombination (Moretti et al. 2014). Wild type and mutant strains were maintained on PDA solid medium and kept at 6 °C. For long storage, strains were conserved under 15 % glycerol at −80 °C. As minimal medium, the Gamborg B5 medium (3.17 g/l) was used.

Growth conditions for the screening

B. cinerea and *C. parasitica* strains were firstly pre-cultivated for 3 days at 20 °C on solid complete medium (CM) and potato dextrose medium (PDA), respectively. All standard cultivations were done under diurnal light conditions. Afterwards, one plug containing mycelia was taken individually with the small end of sterile glass Pasteur pipette from the edge of colonies growing on solid medium and used for inocula on solid B5 medium supplemented with 2 % glucose. The screening was carried out in 3.5 cm microtiter wells (7 ml of medium/well) or 9 cm Petri dishes: wt and mutant strains were analyzed in triplicate on GR24 (from 1×10^{-4} to 1×10^{-8} M) and acetone control in parallel. Petri dishes were kept in a dark room at 20 °C and at 24, 48, 72 and 96 h the diameter of the hyphal radial growth was monitored.

Stock solutions of racemic (±)-GR24, *ent* 2′-epi-GR24 (MW 298.29) or racemic EGO10 (MW 309) were prepared dissolving 3 mg of the specific molecule in 1 ml acetone to get a 10^{-2} M (10 mM) solution. The 10^{-2} M stock solutions were made fresh before all the screening. Once dissolved in acetone the solutions were stored at −20 °C. 2′-epi-GR24 was kindly provided by Dr. Xie and Dr. Yoneyama (Utsunomiya University, Japan).

Measurements of redox-sensitive GFP (roGFP2) fluorescence

For measurements of the redox state, or in more detail the ratio of the glutathione system (GSH/GSSG), conidia were harvested, washed and diluted to a final concentration of 10^5 conidia/ml in GB5 medium supplemented with 2 % glucose as well as 1 mM $(NH_4)_2HPO_4$. Droplets of 20 µl were placed on a slide and incubated in a humid chamber for 16 h. Analysis took place using an inverted microscope (Leica DMIRE2) equipped with a Leica TCS SP2 scan head (Leica Microsystems, Wetzlar, Germany) and a 63× water-immersion lens in multi-track mode with line switching. For measuring the samples, excitation wavelength were set to 405 nm (oxidized) and 488 nm (reduced). Emission was set to 510 nm. For every sample, up to 12 Z stacks of optical sections were taken and projected as average projections. Evaluation was performed using the ImageJ (v. 1.44f; http://rsb.info.nih.gov/ij/) program. For background normalization, a Gaussian blur application was made with a sigma of 2.0. With the RatioPlus feature, a ratio between the pictures of the oxidized and the reduced channel was

Table 1 *B. cinerea* strains used in this study. All strains were obtained in *B. cinerea* B05.10

Name	Strain	Features	Origin
WT:B05.10	*B. cinerea* B05.10	Wild type	Büttner et al. (1994) and Quidde et al. (1999)
Δbcvel1	Knock out of bcvel1	Fungal-specific, regulatory protein	Schumacher et al. (2012)
Δbclae1	Knock out of bclae1	Fungal-specific, regulatory protein	Schumacher (2015)
Δbcltf1	Knock out of bcltf1	Light-responsive transcription factor	Schumacher et al. (2014)
Δbcg1	Knock out of bcg1	Gα subunit of heterotrimeric G protein	Gronover et al. (2001)
Δbcg2	Knock out of bcg2	Gα subunit of heterotrimeric G protein	Gronover et al. (2001)
Δbcg3	Knock out of bcg3	Gα subunit of heterotrimeric G protein	Döhlemann et al. (2006)
Δbop1	Knock out of bop1	G protein-coupled receptor/ opsin-like 1	Heller et al. (2012)
Δbtp1	Knock out of btp1	Transmembran protein with 7 TM	Schulze Gronover et al. (2005)
Δbac	Knock out of bac	Adenylatcyclase BAC	Klimpel et al. (2002)
Δbcpka2	Knock out of bcpka2	PKA catalytic subunit 2 (BcPKA2)	Schumacher et al. (2008b)
Δbpk4	Knock out of bpk4	Ran1-like protein kinase	Schulze Gronover (2004)
Δbcreg1	Knock out of bcreg1	Ryp-like protein; putative transcriptional regulator	Michielse et al. (2011)
Δbcplc1	Knock out of bcplc1	Phospholipase C, calcium signaling	Schumacher et al. (2008a)
Δbcmid1	Knock out of bcmid1	Calcium channel protein	Harren et al. (2012b)
Δbccch1	Knock out of bccch1	Calcium channel protein	Harren et al. (2012b)
Δbccch1/bcmid1	Knock out of bccch1/bcmid1	Calcium channel protein	Harren et al. (2012b)
Δbmp1	Knock out of bcbmp1	MAP kinase	Zheng et al. (2000) and Döhlemann et al. (2006)
Δbcsak1	Knock out of bcsak1	MAP kinase	Segmüller et al. (2007)
Δbos1	Knock out of bos1	Histidin kinase class III - osmosensor	Viaud et al. (2006)
Δbcatf1	Knock out of bcatf1	ATF1 transcription factor	Temme et al. (2012)
Δbap1	Knock out of bap1	AP1-like transcription factor	Temme and Tudzynski (2009)
ΔbcnoxA	Knock out of bcnoxA	NADPH oxidase catalytic subunit A	Segmüller et al. (2008)
ΔbcnoxB	Knock out of bcnoxB	NADPH oxidase catalytic subunit B	Segmüller et al. (2008)
ΔbcnoxAΔbcnoxB	Double knock out mutant	NADPH oxidase catalytic subunits A and B	Segmüller et al. (2008)
ΔbcnoxR	Knock out of bcnoxR	NADPH oxidase regulatory subunit	Segmüller et al. (2008)
Δbcplsl	Knock out of bcpls1	Tetraspanin	Siegmund et al. (2013)
ΔbcnoxD	Knock out of bcnoxD	ER-localized protein	Siegmund et al. (2015)
Δbctrr1	Knock out of bctrr1	Thioredoxin reductase	Viefhues et al. (2014)
Δbctrx1	Knock out of bctrx1	Thioredoxin	Viefhues et al. (2014)
Δbcgr1	Knock out of bcgr1	Glutathione reductase	Viefhues et al. (2014)
Δbcgr2	Knock out of bcgr2	Glutathione reductase	Viefhues et al. (2014)
Δbcskn7	Knock out of bcskn7	Response regulator	Viefhues et al. (2015)
B05.10 + roGFP2_HDEL	Wild type with redox-sensitive roGFP2 retained in the ER	RoGFP2 ER strain	Marschall et al. (2016)
B05.10 + roGFP2_mito	Wild type with redox-sensitive roGFP2 targeted to mitochondria	RoGFP2 mitochondrial strain	Marschall et al. (2016)
B05.10:GRX-roGFP2.12	Wild type with redox-sensitive roGFP2 in cytosolic regions	RoGFP2 cytosolic strain	Heller et al. (2012)
Δbcltf1 + roGFP2_mito	Δbcltf1 with redox-sensitive roGFP2 targeted to mitochondria	RoGFP2 mitochondrial strain	Marschall et al. (2016)

conducted. Mean values were measured (405/488 nm ratio) with a clipping value of 2.0. The grayscale was converted to color using the look-up table "Fire" of the ImageJ program. The threshold was set from 0 to 4 for all intensity ratios.

Statistical analyses

Statistical analyses were performed through one-way analysis of variance (one-way ANOVA) and Tukey's post hoc test, using a probability level of $p < 0.05$. All statistical analyses were performed using the PAST statistical package (version 2.16; Hammer et al. 2001).

Results

Characterization of fungal growth in the presence of SLs analogs

With the long-term aim to identify genes involved in SLs perception and/or signaling in AM fungi, we decided to use an indirect approach exploiting other plant-interacting fungi such as *B. cinerea,* which was previously shown to be sensitive to the synthetic SLs analog GR24 (Dor et al. 2011). We decided to set up the screening of a set of characterized deletion mutants of the *B. cinerea* B05.10 strain (Table 1) to isolate genotypes with altered responses to GR24. In addition to the extensive structure–activity relationship study on AM fungi described by Akiyama et al. (2010), recent findings confirmed that the response to SLs is highly specific depending on the configuration at the C-2′ position (Scaffidi et al. 2014). All natural SLs share the same R configuration. On this basis, we decided to use for our experiments both racemic (+)-GR24 (mixture of two enantiomers, one with R and one with S configuration at C-2′) and the enantiopure (−)-*ent*-2′-epi-GR24 (R configuration at C-2′, Suppl. Fig. 1).

At first, we investigated whether the specific *B. cinerea* B05.10 wild type strain was sensitive to GR24 using the biological assay, described by Dor et al. (2011), where GR24 was embedded in the solid medium containing different GR24 concentrations. The GR24 used was a racemic solution of the two enantiomers (+)-GR24 and (−)-*ent*-GR24 ((±)-GR24). An inhibition of the fungal radial growth was strongly evident at 5×10^{-5} M (±)-GR24 concentration (Fig. 1) at all the time points, while a weaker inhibition was also observed for 1×10^{-5} M (±)-GR24. Almost no effect was observed for acetone, the solvent used to dissolve (±)-GR24, compared to the control.

The fungal growth pattern was observed in detail at the edge of the colony by means of a stereomicroscope. The presence of (±)-GR24 led to a disordered hyphal growth

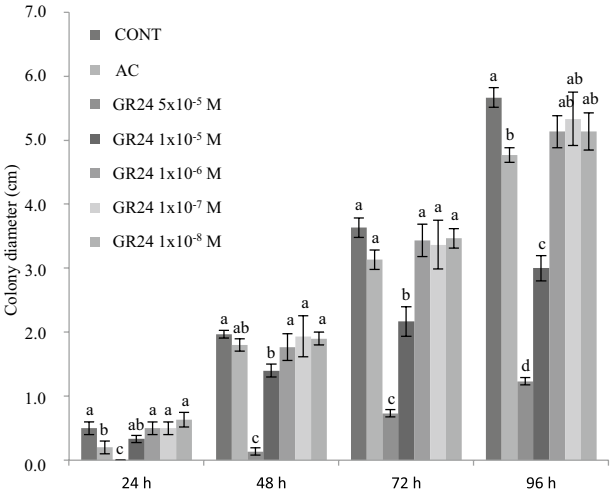

Fig. 1 Effect of (±)-GR24 on *B. cinerea* wt strain growing (colony diameter, cm) on B5 solid medium (supplemented with 2 % glucose) at 20 °C in the dark after 24, 48, 72 and 96 h of inoculation. Data for each condition are presented as mean ± standard deviation. *Different letters* indicate statistically significant difference ($p < 0.05$, ANOVA) within each time point. *CONT* no GR24, *AC* acetone

(Fig. 2). The hyphal network appeared denser and hyphal branching seemed to be increased. Thus, hyphal branching was also monitored in germinating hyphae grown in liquid medium and exposed to 5×10^{-5} M (±)-GR24. Branching in the control and acetone treatment was limited to the 2nd order while exposure to GR24 triggered the formation of branches up to the 4th order (Fig. 3).

The fungal radial growth was also tested in the presence of the active SL enantiomer (−)-*ent*-2′-epi-GR24 (Suppl. Fig. 1). As for racemic GR24, a reduced fungal growth was only evident at 5×10^{-5} M (Fig. 4). This molecule turned out to be slightly less active compared to (±)-GR24.

In addition, an indolyl-based SL analog was also tested, EGO10 (Suppl. Fig. 1), which was specifically designed for large-scale applications since its synthesis requires a simpler protocol and cheaper reagents compared to GR24 (Prandi et al. 2011). Also in this case EGO10, which was previously shown to induce hyphal branching in AM fungi (Prandi et al. 2011), led to an inhibition of *B. cinerea* radial growth when used at 10^{-4} and 10^{-5} M concentrations (Fig. 5). EGO10 was used as a racemic mixture in all the experiments.

Screening of fungal mutants for altered response to GR24

Thirty-two *B. cinerea* mutant strains, defective in genes mainly involved in signaling, were analyzed (Table 1). To optimize the assays, the screening was carried out in 3.5 cm microtiter wells. Each mutant strain was analyzed at least in triplicate on B5 solid medium (supplemented with 2 %

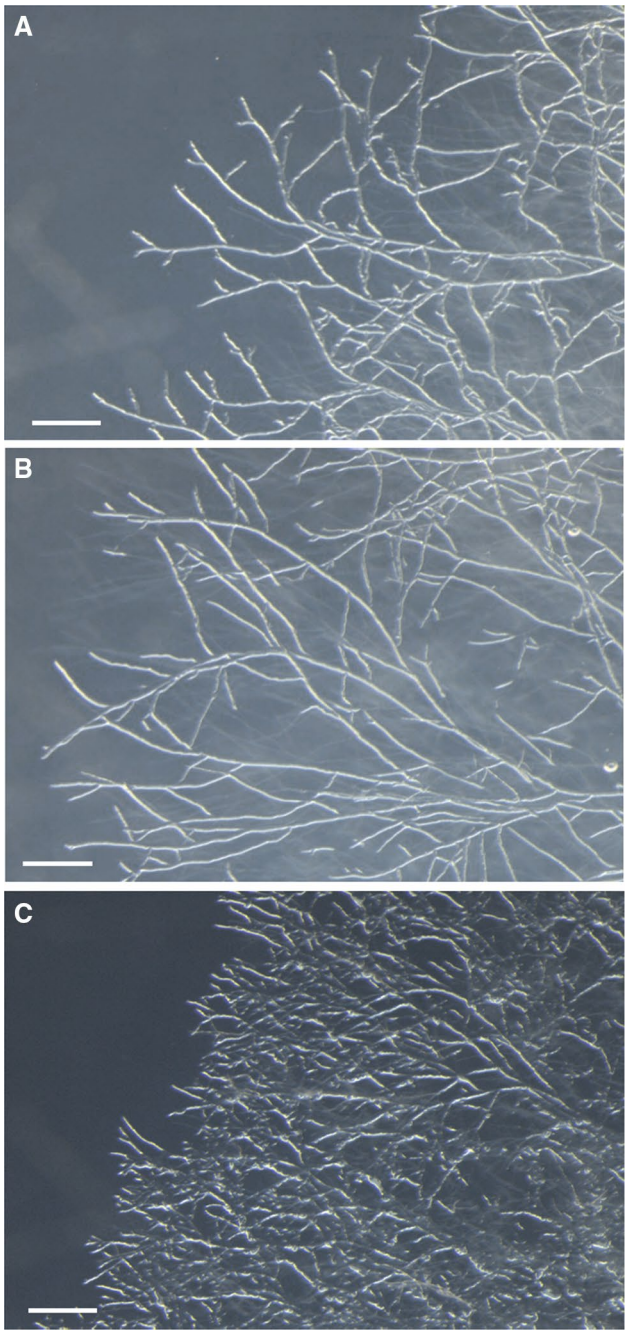

Fig. 2 Hyphal growth pattern of *B. cinerea* wt strain developing in the B5 solid medium (supplemented with 2 % glucose) (**a**) or in the presence of acetone (**b**) or 5×10^{-5} M (±)-GR24 (**c**). *Pictures* taken at the edge of the colony, 48 h post-inoculation. *Bars* 100 µm

glucose) containing (±)-GR24 (5×10^{-5}M as a mixture of the two enantiomers) and acetone in parallel. This concentration was selected since it induced a clear effect on fungal growth (Fig. 1). The hyphal radial growth of the mutants was evaluated and compared to that of the wt strain at 24, 48, 72 and 96 h. Two mutant strains out of 32 turned out to be significantly ($p < 0.05$) less responsive to GR24 (Fig. 6). One mutant is defective in BcLTF1 (Fig. 6b), a light responsive transcription factor belonging to the GATA family, which was recently described in *B. cinerea* as important for virulence and light responses (Schumacher et al. 2014). The second strain is defective in a thioredoxin reductase (Fig. 6b) which has a key role in the control of cell redox homeostasis by forming reduced disulfide bonds (Arnér and Holmgren 2000). In the specific case of *B. cinerea*, it has been shown that the balanced redox status maintained by the thioredoxin system is essential for development and pathogenesis (Viefhues et al. 2014). A reduced inhibition of radial growth was observed for the BcLTF1 and the thioredoxin reductase mutants compared to the wt strain (Fig. 6). The thioredoxin reductase mutant showed a reduced growth compared to the wt strain already in the acetone-containing medium (control condition) and the presence of (±)-GR24 only slightly modified its growth.

Hyphal branching was also monitored in germinating hyphae of the two mutants grown in liquid medium with or without 5×10^{-5} M (±)-GR24. The two mutants showed a hyphal morphology similar to the wt strain in the absence of GR24, while they displayed a less pronounced branching, especially Δ*bcltf1*, compared to the wt in the presence of GR24 (Suppl. Fig. 2).

Mutants defective in different members of calcium signaling pathways, subunits of the ROS producing NADPH oxidase complex and modules of MAPK cascades grew like the wt. To confirm whether mutations in modules of the MAPK signaling do not alter the response to (±)-GR24, a second plant pathogen (*Cryphonectria parasitica*) was investigated. The presence of (±)-GR24 embedded in the B5 solid medium at a concentration of 10^{-4} M reduced the radial growth of *C. parasitica* wt (Fig. 7a). Mutant strains for three mitogen-activated protein kinase kinases (MAP2Ks), Cpkk1, Cpkk2, and Cpkk3 involved in the three main MAP cascades described in fungi were available (Moretti et al. 2014). The radial growth in the presence of (±)-GR24 of the three mutants was similar to that of the wt strain, suggesting that these MAP cascades are not involved in the response to (±)-GR24 (Fig. 7b).

Investigations on reactive oxygen species

Interestingly, one feature shared by the two *B. cinerea* mutants identified in the screening was an unbalanced ROS (reactive oxygen species) homeostasis. Both mutants are hypersensitive to oxidative stress and produce more hydrogen peroxide (Schumacher et al. 2014; Viefhues et al. 2014). This suggests that ROS homeostasis is important to respond to SLs. To investigate this issue, we exploited *B. cinerea* strains expressing a redox-sensitive GFP2 (roGFP2) which allows quantitative ratiometric analysis of redox dynamics in the cytosol (Heller et al. 2012) as well as inside of the mitochondrial intermembrane space (herein

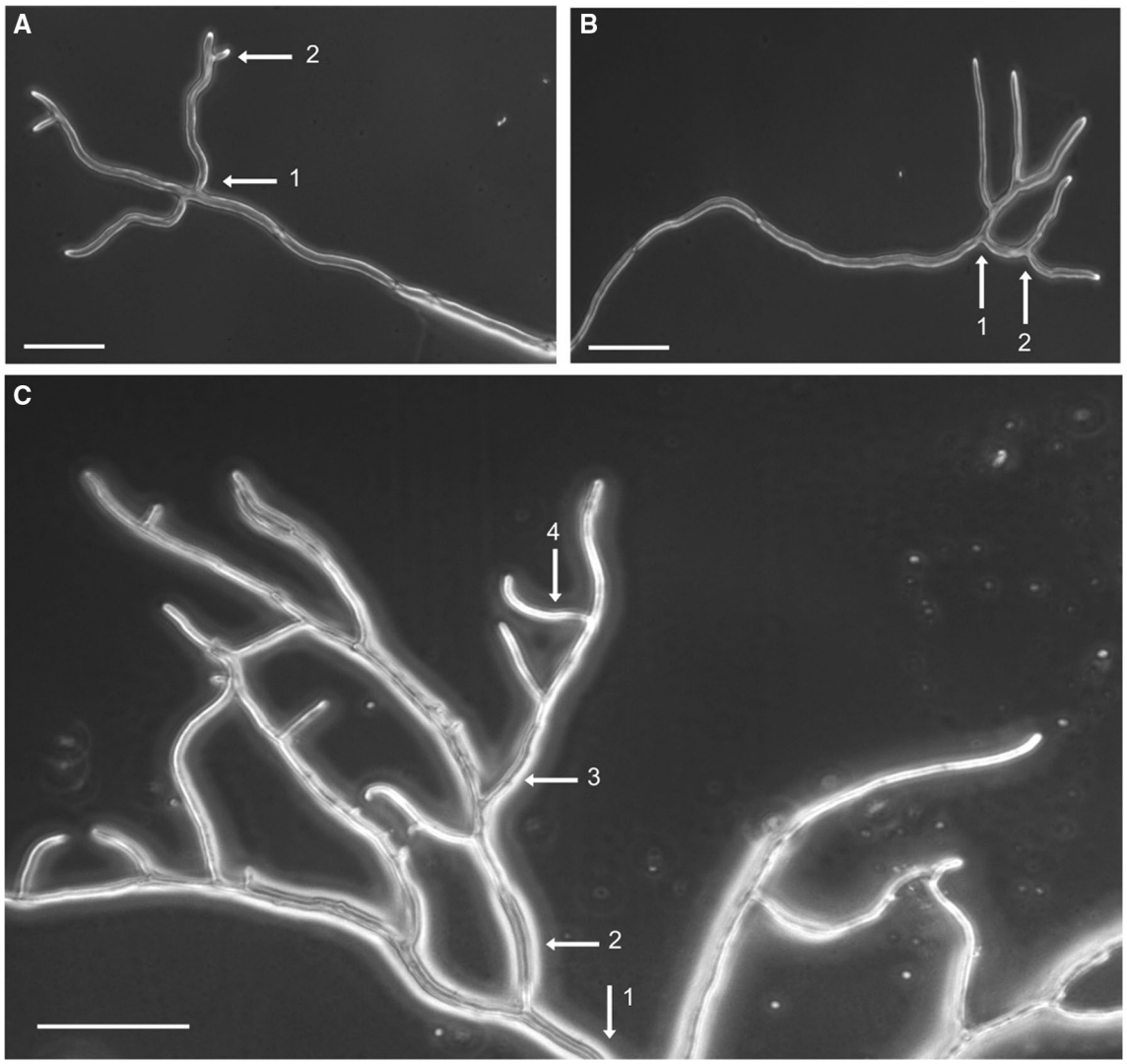

Fig. 3 Hyphal branching of *B. cinerea* wt strain developing in the B5 liquid medium (supplemented with 2 % glucose) (**a**) or in the presence of acetone (**b**) or 5×10^{-5} M (±)-GR24 (**c**). *Bars* 50 µm. Order of branching is indicated by *white arrows* and *numbers*

referred to as mitochondria) and the endoplasmic reticulum (Marschall et al. 2016). The system is based on the measurement of roGFP2 fluorescence at 510 nm after excitation at two different wavelengths: 405 nm, indicating the oxidized state, and at 488 nm indicating the reduced state of the roGFP2. Upon (±)-GR24 exposure, no change in the redox status was observed in *B. cinerea* strains expressing the roGFP2 at the level of cytosol or endoplasmic reticulum (data not shown). By contrast, an oxidizing effect, as revealed by fluorometric measurements, was found in the *B. cinerea* strain expressing a redox-sensitive GFP2 at the level of the mitochondria (Fig. 8a). After the addition of (±)-GR24, the redox state inside the mitochondria (white arrows) changed from a more reduced (blue color) to a more oxidized redox state (red/yellow color). However, the oxidizing effect was not as strong as when induced by the addition of 10 mM hydrogen peroxide (positive control). To elucidate whether the lower sensitivity to (±)-GR24 of the Δ*bcltf1* mutant is related to changes in the redox state, the mutants strain, expressing the different roGFP2 constructs (Marschall et al. 2016), was also investigated in fluorometric measurements. While (±)-GR24 had also no effect on the cytoplasmic and endoplasmic redox state, the mitochondrial 395/488 ratio was enhanced again (Fig. 8b). Interestingly, the effect of (±)-GR24 was not as severe as previously seen for the wt strain (Fig. 8a).

Discussion

SLs were identified as communication molecules in the rhizosphere between plants and both parasitic plants and

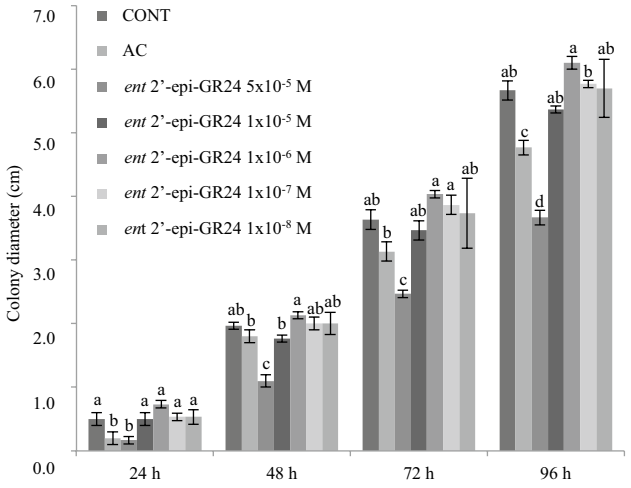

Fig. 4 Effect of (−)-*ent* 2′-epi-GR24 on *B. cinerea* wt strain growing (colony diameter, cm) on B5 (supplemented with 2 % glucose) solid medium at 20 °C in the dark after 24, 48, 72 and 96 h of inoculation. Data for each condition are presented as mean ± standard deviation. *Different letters* indicate statistically significant difference ($p < 0.05$, ANOVA) within each time point. *CONT* no GR24, *AC* acetone

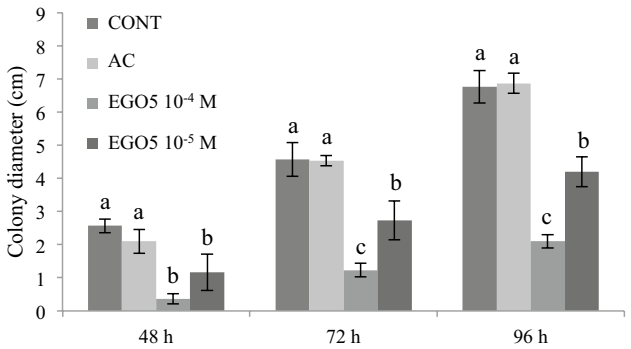

Fig. 5 Effect of EGO10 on *B. cinerea* wt strain growing on B5 solid medium at 20 °C in the dark after 48, 72 and 96 h. Data for each condition are presented as mean ± standard deviation. *Different letters* indicate statistically significant difference ($p < 0.05$, ANOVA) within each time point. *CONT* no GR24, *AC* acetone

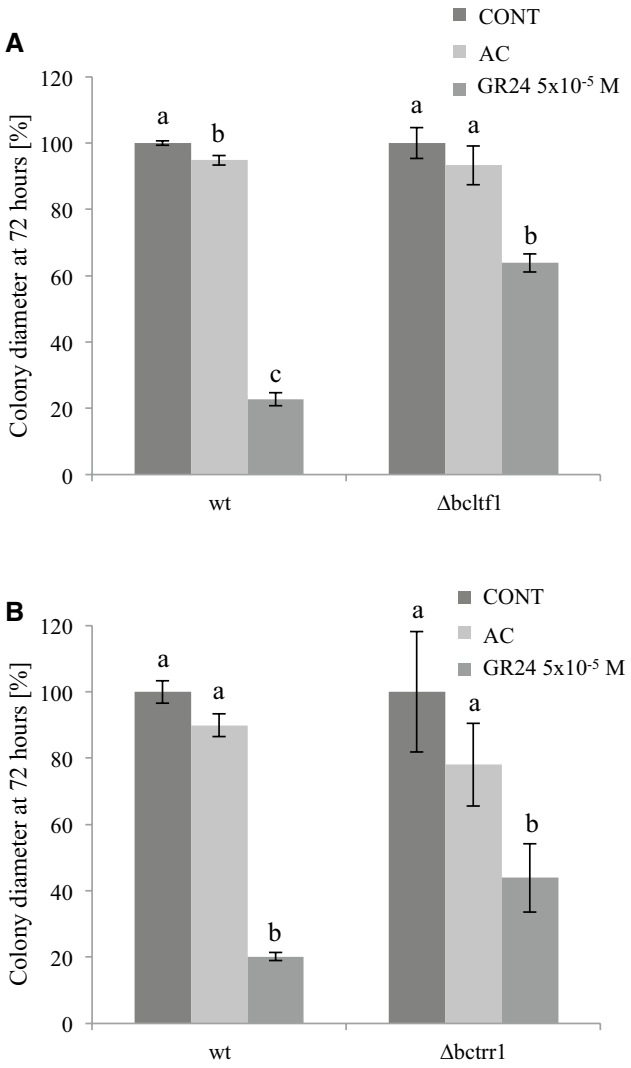

Fig. 6 Growth assay on *B. cinerea* wild type (wt) and Δ*bltf1* mutant strains growing on CM solid medium (**a**) or wt and Δ*btrr1* mutant strains growing on B5 solid medium (**b**). The colony diameter was measured after 72 h of inoculation and, to simplify the comparison, the growth on CM or B5 medium was considered to be 100 %. Data for each condition are presented as mean ± standard deviation. *Different letters* indicate statistically significant difference within genotypes (One-way ANOVA, $p < 0.05$). *CONT* no GR24, *AC* acetone

AM fungi. SLs are exuded in the soil not only by mycotrophic plants but also by non-mycotrophic plants, such as *Arabidopsis thaliana* and *Lupinus* sp. (Yoneyama et al. 2008). Such a wide distribution of SLs in the plant kingdom supports the hypothesis that they also have other important roles in the rhizosphere, possibly affecting both beneficial and pathogenic soil microorganisms (Garcìa-Garrido et al. 2009), but data on such roles are still scarce.

Dor et al. (2011) demonstrated that several phytopathogenic fungi are sensitive to GR24, a commonly used SLs synthetic analog. This supports the idea that SLs also have a more general effect on fungi. The growth of the well-studied plant-interacting fungus *B. cinerea* was shown to be affected by the exposure to GR24. This result provided us a framework to develop an alternative approach to look for fungal genes involved in SLs response. Here, we exploited the sensitivity of *B. cinerea* and *C. parasitica* to GR24, used as a racemic solution of the two enantiomers (±)-GR24, and the availability of a collection of well-characterized deletion mutants to set up a screening for the identification of strains with altered response to (±)-GR24 and, therefore, genes involved in the response to SLs.

We initially better characterized the responses to the racemic (±)-GR24 of the *B. cinerea* B05.10 wild type strain which was used to generate the gene deletion strains.

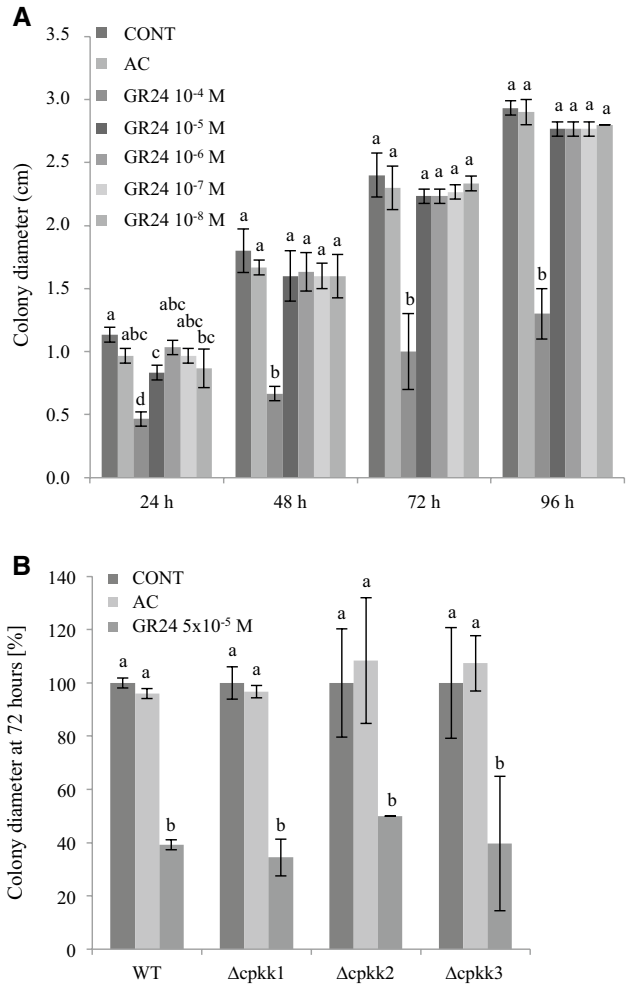

Fig. 7 Effect of (±)-GR24 on *C. parasitica* strains. **a** Growth assay on *C. parasitic*a wt strain growing on solid medium containing different concentration of (±)-GR24, acetone (AC) or only B5 medium (CONT). The colony diameter was measured at 24, 48, 72 and 96 h. *Different letters* indicate statistically significant difference within each time point ($p < 0.05$, ANOVA). **b** Growth assay on *C. parasitic*a wt and Δ*cpkk1,* Δ*cpkk2,* Δ*cpkk3* mutant strains growing on B5 solid medium. The colony diameter was measured after 72 h of inoculation and, to simplify the comparison, the growth on B5 medium (CONT) was considered to be 100 %. Data for each condition are presented as mean ± standard deviation. *Different letters* indicate statistically significant difference ($p < 0.05$, ANOVA)

In our experiments, in analogy to what has been performed by Dor et al. (2011), (±)-GR24 was embedded in the solid medium to have a uniform concentration. The radial growth of the *B. cinerea* B05.10 wild type strain was reduced, starting from 24 h of incubation, at 10^{-5} M (±)-GR24 concentrations, in agreement with Dor et al. (2011). A higher hyphal branching was also observed. This seems a rather high concentration but Besserer et al. (2006) reported that GR7, another SLs synthetic analog, stimulates branching of the AM fungus *Gigaspora rosea* at concentrations above 10^{-7} M, and that sorgolactone's effect on AM fungi is at its highest at 10^{-5} M. It is also worth to mention that the (±)-GR24 activity, when embedded in the growth medium, may decrease with time since SLs are known to be highly instable due to easy cleavage of the enol ether bond by nucleophilic agents, including water (Mangnus and Zwanenburg 1992). GR24, as a lipophilic molecule, also shows low diffusion rate in hydrophilic solutions: this may be the reason why no effect on *B. cinerea* growth was observed by Torres-Vera et al. (2014) who used a different biological assay where GR24 was poured onto a disc in front of the *B. cinerea* fungal growth.

Since in recent times stereochemistry was shown to be an important issue for SLs activity (Scaffidi et al. 2014) we also tested in a similar assay the pure enantiomer (−)-*ent*-2′-epi-GR24. This compound was also active, although it turned out to be slightly less active than (±)-GR24. On the other hand, EGO10, who was previously shown to induce branching in AM fungi (Prandi et al. 2011), led to an inhibition of the *B. cinerea* radial growth. All together these data support that SLs, in addition to AM fungi, are active on *B. cinerea*. Interestingly, also *C. parasitica* showed a growth inhibition upon GR24 exposure, confirming again a general effect of GR24 on fungi, supporting the rationale of exploiting *B. cinerea* and other fungi as an additional tool to identify genetic determinants involved in SLs responses in AM fungi.

To set up the screening of the mutants, based on the search for altered response to GR24 (impact on radial growth in solid medium), we decided to use 5×10^{-5} M (±)-GR24 concentration. We considered 32 *B. cinerea* mutants already characterized in terms of the identification of the inactivated gene since this would have simplified further investigations. Most genes are components of different signaling pathways (Table 1).

Two mutants turned out to be less sensitive to (±)-GR24: they showed a reduced growth inhibition upon (±)-GR24 exposure compared to the wt strain. The first strain is defective of the light responsive transcription factor, BcLTF1, belonging to the GATA family, which was recently described in *B. cinerea* (Schumacher et al. 2014). *Bcltf1* deletion and over-expression experiments confirmed the role of the gene in virulence, and discovered its functions in regulation of light-dependent differentiation, the equilibrium between production and scavenging of reactive oxygen species (ROS) and secondary metabolism. In addition, the deletion of *Bcltf1* was shown to affect the expression of 1539 genes: the increased expression of genes encoding alternative respiration enzymes, such as the alternative oxidase (AOX), has suggested a mitochondrial dysfunction in the absence of *Bcltf1*. The deletion mutant has an unbalanced ROS homeostasis where generation outweighs detoxification rates (Schumacher et al. 2014; Schumacher 2015).

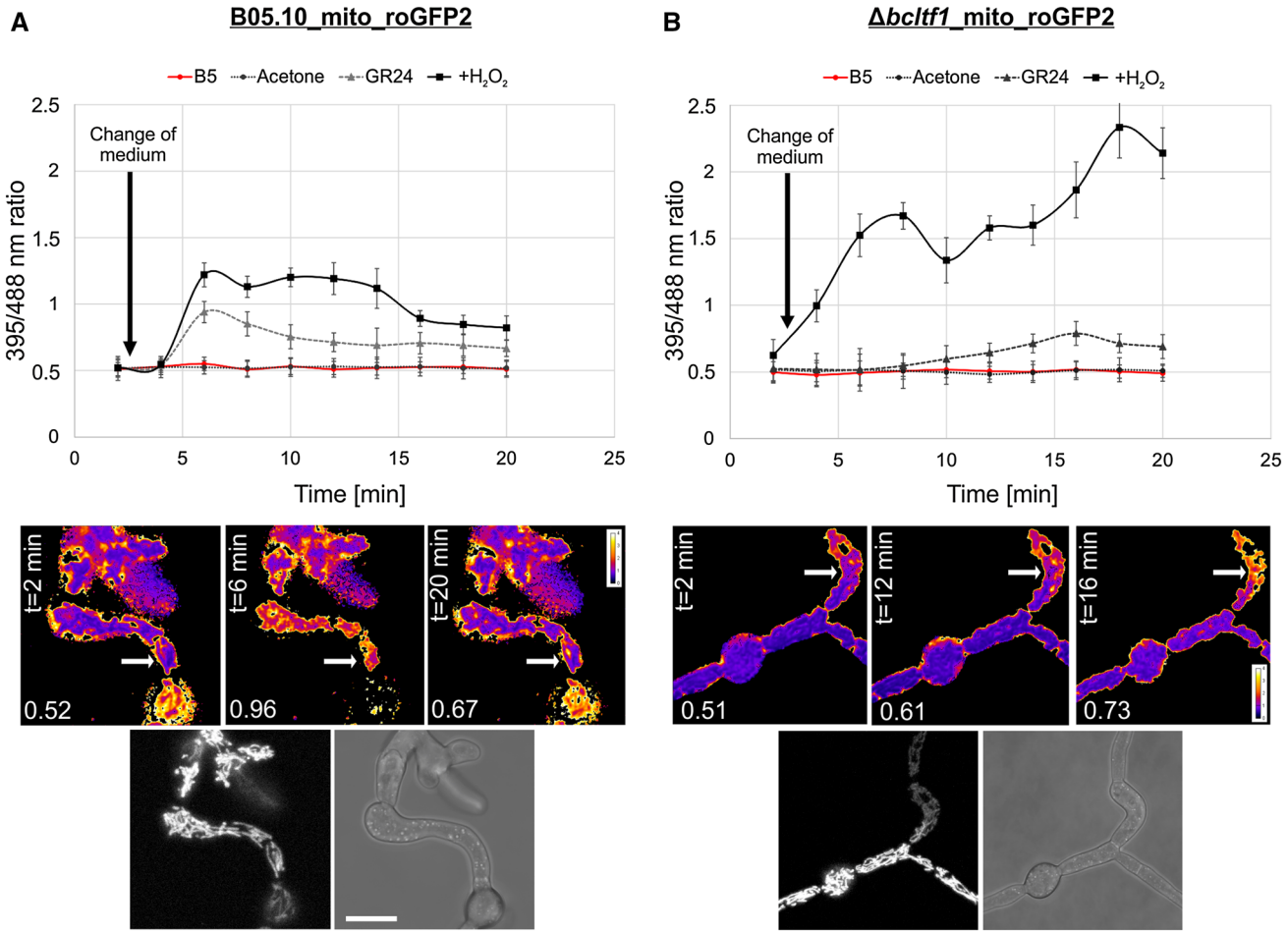

Fig. 8 The redox state in the mitochondrial intermembrane space (IMS) of *B. cinerea* is influenced by (±)-GR24. The IMS redox state of the wild type (**a**) and Δ*bcltf1* (**b**) was monitored in fluorometric measurements by CSLM in response to H_2O_2 and (±)-GR24 (controls: B5 medium supplemented with 2 % glucose or acetone). Conidia (10^5) were grown overnight on a confocal slide. For measurements, the slide is flooded with B5 medium supplemented with glucose. Subsequently, after the first measurements the control medium was removed and B5 medium supplemented with 20 mM of H_2O_2 or 5×10^{-5} M (±)-GR24 was added to the slide [indicated by the *black arrow* (3 min)]. Standard deviations were calculated from three technical replicates. From all Z-stacks images, average projections were calculated and used for ratio images. Using the software ImageJ (look up tables: LUTFire), images and numerical ratio values were prepared. The *color scale* indicates a reduced (*dark/blue*) or oxidized (*yellow*) redox state

The second strain is defective of a thioredoxin reductase, a component of the thioredoxin system which is of great importance for maintenance of cellular redox homeostasis by forming reduced disulfide bonds (Arnér and Holmgren 2000). The knock-out mutant of the *B. cinerea* gene, *Bctrr1*, was severely impaired in virulence and more sensitive to oxidative stress; moreover, Δ*bctrr1* showed enhanced H_2O_2 production and retarded growth (Viefhues et al. 2014). Also in our experiments, the Δ*bctrr1* showed a reduced growth compared to the wt strain and it was almost not responsive to (±)-GR24.

To better investigate the possible involvement of MAP kinase cascades, key components of signaling pathways, in the response to GR24 we also took advantage of *C. parasitica.* Mutant strains for three mitogen-activated protein kinase kinases (MAP2Ks), Cpkk1, Cpkk2, and Cpkk3 involved in the three main MAP cascades described in fungi, were in fact available (Moretti et al. 2014). *C. parasitica* showed a growth inhibition upon (±)-GR24 exposure; when the mutant strains were considered, they showed a behavior similar to that of the wt strain indicating that these MAP cascades are likely not involved in the response to GR24. MAP kinases are normally downstream effectors of G-protein-coupled receptors (GPCRs) that have an important sensor function in fungi (Li et al. 2007). Our results do not exclude that G signaling is involved since GTPases can transduce the signal also through downstream effectors different from MAP kinases (e.g., adenylyl cyclase, phospholipase C, and ion channels; Hamm and Gilchrist 1996). GPCRs, which act upstream heterotrimeric

GTPases, would also deserve investigations; the only *B. cinerea* mutant strain defective of a putative GPCR gene (Δ*bop1*), which was tested in our screening, showed a normal phenotype in the presence of (±)-GR24. However, the genome of fungi may contain up to 30 different GPCR genes (Li et al. 2007). So far no GPCR has been described in AM fungi.

Since both the BcLTF1 and the thioredoxin reductase deletion mutants show an altered ROS homeostasis (Schumacher et al. 2014; Viefhues et al. 2014), it is tempting to speculate that the fungal response to SLs relies on a correct ROS homeostasis. The analyses of the *B. cinerea* strain expressing a redox-sensitive GFP seem to support this hypothesis since GR24 exposure induces changes of the redox status and, in particular, at the level of mitochondria. The same construct expressed in the Δ*bcltf1* mutant confirms the possible involvement of mitochondria.

Remarkably, activation of mitochondrial metabolism upon GR24 exposure was observed in the AM fungus *Gigaspora rosea* (Besserer et al. 2008) and recently confirmed by gene expression data in *G. margarita* (Salvioli et al. 2016). Interestingly, investigations on another fungal pathogen, *Sporisorium reilianum,* also showed that 1 h GR24 application induced a burst of cell respiration and activation of genes involved in cell respiration, cell wall development, cellular growth or encoding heat shock proteins (Sabbagh 2011, 2012). Mitochondria are, therefore, emerging as targets of SLs actions. It is still to be clarified whether this is a direct or an indirect effect.

It has been suggested that the response of various pathogenic fungi to the presence of SLs (GR24) could be a stress response (Dor et al. 2011; Sabbagh et al. 2012) and that the secretion of SLs, which significantly affect fungal development, has first evolved as an external defense mechanism against potential pathogens (Dor et al. 2011). Indeed, branching of fungal hyphae is often associated with stress responses (Asante et al. 2008; Rodriguez-Urra et al. 2009; Kozlova et al. 2010).

In the case of AM fungi, SLs-induced branching may also have originated as a stress response. According to this, the activation of a fast and transient $[Ca^{2+}]_i$ increase and an elevated respiration, eventually leading to ROS production and detoxification, has also been recently interpreted in AM fungi as a response to foreign, xenobiotic molecules (Salvioli et al. 2016). Then, during the long co-evolution of the AM symbiotic association, this response might have evolved into a strategy to increase chances of the fungus to encounter a host plant.

Moving into this direction, it would be important to further characterize in AM fungi the role of the homologs of the two genes identified in *B. cinerea*. Putative homolog sequences have been identified within genomics and/or transcriptomics data of the AM fungi *Rhizophagus irregularis* and *Gigaspora margarita* and (data not shown). Further investigations are needed to decipher the role of these genes in the response to SLs and whether there are differences between beneficial and pathogenic fungi.

In conclusion, we have demonstrated that fungal pathogens, for which genetic tools are available, are sensitive to SLs and can be exploited to identify genes involved in the fungal response to SLs. In particular, the results obtained with *B. cinerea* suggest that a correct ROS homeostasis and possibly mitochondria could be mediators of this response.

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