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.
Response to Reviewers:

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<td><strong>Editor’s comments:</strong></td>
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<td>Reply: Although these are very interesting papers, we could not find an appropriate place in the manuscript to easily cite them.</td>
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Reviewer #2: This manuscript by Belmondo et al. describes the attempted identification of the molecular determinants involved in strigolactones (SLs) response in AM fungi by exploiting the SL-responsive fungal pathogens Botrytis cinerea and Cryphonectria parasitica. Two B. cinerea mutants defective of BcTrr1, a thioredoxin reductase and BcLTF1, a GATA transcription factor were turned out to be less responsive to GR24. They observed an unbalanced ROS homeostasis in these mutants, thus concluded that a correct ROS homeostasis could be mediators of response to SLs. Putative homolog sequences have been identified within genomics and/or transcriptomics data of the AM fungi Rhizophagus irregularis and Gigaspora margarita.

Although the two genes act indirectly, this is the first report of the identification of the molecular determinants involved in SLs response in fungi, and thus this is an interesting finding and the subject is well suited for Current Genetics.

Only one point that should be addressed in the paper are mentioned below. The photographs of the two B. cinerea mutants untreated (control) and treated by GR24 (5x10-5 M) should be presented to compare the morphological alterations upon GR24 treatment in wild type and the mutants.

Reply: Photographs of the two mutants treated or not with GR24 are now shown in Suppl. Fig. 2.

The text in the Results session has been modified by adding these sentences: “Hyphal branching was also monitored in germinating hyphae of the two mutants grown in liquid medium with or without 5x10-5 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 Δbcltf1, compared to the wt in the presence of GR24 (Suppl. Fig. 2).”

Reviewer #3: Dear authors,

At present many studies are running in order to discover new strigolactone (SL) functions. The possible role of SLs in plant interaction with pathogenic fungi is a new research area and only a few papers were published concerning this topic. Despite the small number of manuscripts, there are two different polar opinions about SL influence on the growth of phytopathogenic fungi: - one opinion maintains that SLs inhibit fungal growth; the other - maintains that SLs do not influence fungal growth. Therefore any data accumulated on the interaction of SLs with phytopathogenic fungi are highly important.

The results of your study are novel and interesting. The MS is written very clearly, and therefore the MS may be recommended for publication.

I have only a few very minor corrections:

1. Row 71: "Parker et al 2009". - "et al" should be removed.

2. Row 83: "Schmitz and Harrison et al 2009". - "et al" should be removed.

3. Row 359: "(colony diameter, cm)" should be moved after "strain growing".

4. Row 371: the same.

5. Rows 380, 381: "(a)" and "(b)" should be written in the capital letters.

6. Rows 386, 389: the same.

Reply: We did not make the change since we followed the rules of the Journal where bold capital letters are in the Figures while bold lowercase letters are in the figures.
7. Row 401: "Image J" in the rows 171 and 175 is written as "ImageJ". One form should be used in all places.
Reply: We have modified according to the exact word ImageJ.
8. Graph lines in Fig. 8 may be more distinguishable from each other. Probably, black, or dark grey will be preferable for the lines.
Reply: The colors in the graph have been modified to be more distinguishable.
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
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 (Ruiter-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. 2016 a). 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. 2016 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, 2103). 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, Bessérer 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\textsuperscript{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\textsuperscript{2+}]\textsubscript{i} elevation in Gigaspora margarita hyphae suggesting the occurrence of Ca\textsuperscript{2+}-based sensing mechanisms for detecting and responding to SLs (Moscatiello et al. 2014). The use of SLs analogues, 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 Germain 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 et al. 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 GFP 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 H2O and stored at 4°C, while storage of conidia was done at -20 °C in 30% (v/v) glycerol.

Cryphonectria parasitica Δcpku80 (in this work considered a wild type strain) and Δcpkk1, Δcpkk2, Δcpkk3 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 MAP2K-specific sequences that was introduced into the Δcpku80 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 1x10⁻⁴ M to 1x10⁻⁸ M] and acetone control in parallel. Petri dishes were kept in a dark room at 20 °C and at 24 h, 48 h, 72 h and 96 h the diameter of the hyphal radial growth was monitored.

Stock solution 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 10⁵ conidia / ml in GB5 medium supplemented with 2% glucose as well as 1 mM (NH₄)₂HPO₄. Droplets of 20 µl were placed on a slide and incubated in a humid chamber for 16 h. Analysis took place by using an inverted microscope (Leica DMIRE2) equipped with a Leica TCS SP2 scan head (Leica Microsystems, Wetzlar, Germany) and a 63x 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 by 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 conducted. Mean values were measured (405/488 nm ratio) with a clipping value of 2.0. The grayscale was converted to color by using the look-up table “Fire” of the ImageJ program. The threshold was set from 0-4 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 base 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', Supp 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 5x10⁻⁵ M (±)-GR24 concentration (Fig. 1) at all the time points, while a weaker inhibition was also observed for 1x10⁻⁵ 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 details at the edge of the colony by means of a stereomicroscope. The presence of (±)-GR24 led to a disordered hyphal growth (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 5x10⁻⁵ 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 5x10⁻⁵ M (Fig. 4). This molecule turned out to be slightly less active compared to (±)-GR24.

In addition, an indolyl based SL analogue 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% glucose) containing (±)-GR24 (5x10⁻⁵M 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 hours. 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 5x10⁻⁵ 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 ΔbcLtf1, 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 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 colour) to a more oxidized redox state (red/yellow colour). 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 Δbcltf1 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 \( \frac{S_{GFP2}}{S_{GFP1}} \) ratio was enhanced again (Fig. 8b). Interestingly, the effect of (±)-GR24 was not as severe as previously seen for the wild strain (Fig. 8a).

**DISCUSSION**

SLs were identified as communication molecules in the rhizosphere between plants and both parasitic plants and 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 (Garcia-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. 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 10⁻³ 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 x 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 1,539
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).

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, Δbctrr1 showed enhanced H$_2$O$_2$ production and retarded growth (Viefhues et al. 2014).

Also in our experiments, the Δbctrr1 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 functions 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 (Δbop1), 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 Δbcltf1 mutant confirms the possible involvement of mitochondria.

Remarkably, activation of mitochondrial metabolism upon GR24 exposure was observed in the AM fungus Gigaspora rosea (Bessérer 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 et al. 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 mechanisms against potential pathogens (Dor et al. 2011).

Indeed, branching of fungal hyphae is often associated to 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 with 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.
**Figure legends**

**Figure 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.

**Figure 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 5x10^-5 M (±)-GR24 (C). Pictures taken at the edge of the colony, 48 h post inoculation. Bars = 100 μm.

**Figure 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 5x 10^-5 M (±)-GR24 (C). Bars = 50 μm. Order of branching is indicated by white arrows and numbers.

**Figure 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.

**Figure 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.

**Figure 6** Growth assay on *B. cinerea* wild type (wt) and Δbltfl mutant strains growing on CM solid medium (a) or wt and Δbtrr1 mutant strains growing on B5 solid medium (b). The colony diameter was measured after 72 hours 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.
**Figure 7** Effect of (±)-GR24 on *C. parasitica* strains. (a) Growth assay on *C. parasitica* 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 hours. Different letters indicate statistically significant difference within each time point (p < 0.05, ANOVA). (b) Growth assay on *C. parasitica* wt and Δcpkk1, Δcpkk2, Δcpkk3 mutant strains growing on B5 solid medium. The colony diameter was measured after 72 hours 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).

**Figure 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 Δbcltf1 (b) were monitored in fluorometric measurements by CSLM in response to H2O2 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 H2O2 or 5 x 10^{-5} M (±)-GR24 were 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.

**Supplementary Figure 1** (a) General structures and configurations of natural (b) SLs Chemical structures of GR24 stereoisomers and EGO10 analogue.

**Supplementary Figure 2** Hyphal branching of *B. cinerea* Δbcltf1 (a, b) or Δbctrrl (c, d) mutant strains developing in the B5 liquid medium in the presence of acetone (a, c) or 5x 10^{-5} M (±)-GR24 (b, d). Bars = 50 μm. Order of branching is indicated by white arrows and numbers.
REFERENCES


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

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**Supplementary Material**

Suppl Fig 2.pdf