Identification of *Botrytis cinerea* Genes Up-Regulated During Infection and Controlled by the G α Subunit BCG1 Using Suppression Subtractive Hybridization (SSH)

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The G α subunit BCG1 plays an important role during the infection of host plants by *Botrytis cinerea*. $\Delta bcg1$ mutants are able to conidiate, penetrate host leaves, and produce small primary lesions. However, in contrast to the wild type, the mutants completely stop invasion of plant tissue at this stage; secondary lesions have never been observed. Suppression subtractive hybridization (SSH) was used to identify fungal genes whose expression on the host plant is specifically affected in *bcg1* mutants. Among the 22 differentially expressed genes, we found those which were predicted to encode proteases, enzymes involved in secondary metabolism, and others encoding cell wall-degrading enzymes. All these genes are highly expressed during infection in the wild type but not in the mutant. However, the genes are expressed in both the wild type and the mutant under certain conditions in vitro. Most of the BCG1-controlled genes are still expressed in adenylate cyclase (*bac*) mutants in planta, suggesting that BCG1 is involved in at least one additional signaling cascade in addition to the cAMP-depending pathway. In a second SSH approach, 1,500 clones were screened for those that are specifically induced by the wild type during the infection of bean leaves. Of the 22 BCG1-controlled genes, 11 also were found in the in planta SSH library. Therefore, SSH technology can be successfully applied to identify target genes of signaling pathways and differentially expressed genes in planta.

Additional keyword: pathogenicity.

Gray mold rot or Botrytis blight, caused by the ascomycete *Botrytis cinerea* Pers.:Fr. (teleomorph *Botryotinia fuckeliana* (de Bary) Whetzel), affects nearly all species of dicotyledons, including most vegetable and fruit crops, flowers, woody ornamentals, and greenhouse-grown crops. The fungus uses a wide range of infection strategies that allow it to directly penetrate mature-to-senescent leaves and other tender tissues, such as seedlings, floral organs, and mature fruits. The fungus generally infects host tissues in cool damp weather (10 to 25°C) in water droplets (Prins et al. 2000), but it also can germinate at high humidity in the absence of water droplets (Williamson et al. 1995).

So far, the underlying mechanism for pathogenicity and the genes involved in its development are poorly understood. Loss of a single cell wall-degrading enzyme, such as cutinase (van

der Vlugt-Bergmans et al. 1997; van Kan et al. 1997), has no effect on pathogenicity, but the deletion of other genes (e.g., one of the six polygalacturonase genes, *bcpgl*) (ten Have et al. 1998) or the pectin methylesterase gene (Valette-Collet et al. 2003) reduced the virulence of the pathogen. In contrast to the necrotrophic fungus *Botrytis cinerea*, the disruption of the only two, closely linked, polygalacturonase genes found in the biotrophic fungus *Claviceps purpurea* resulted in nearly total loss of pathogenicity (Oeser et al. 2002).

The disruption of genes encoding catalases (Schouten et al. 2002) or superoxide dismutases (Rolke et al. 2004), all enzymes responsible for protecting the fungus from oxidative stress, had only small effects on virulence, suggesting that *B. cinerea* is a robust pathogen adapted to high levels of H₂O₂ and other active oxygen species. Several ABC- and MFS-transporter-encoding genes were identified and disrupted in *B. cinerea*. However, none of these enzymes seem to play a crucial role in detoxification of specific plant defense compounds, such as saponins and phytoalexins, and, therefore, have no dramatic influence on pathogenicity (Hayashi et al. 2002a and b; Schoonbeek et al. 2001). The role of the *B. cinerea*-specific phytotoxin botrydial (Colmenares et al. 2002) in the infection process is not well understood because the genes of the biosynthetic pathway are not yet cloned. One reason for relatively low effects of single-gene disruptions is the redundancy of cell wall-degrading and detoxifying enzymes as well as membrane transporters in *B. cinerea* and other plant-pathogenic fungi (Ahn et al. 2001). Deletion of transcriptional regulators, such as SNF1 of *Cochliobolus carbonum* (Tonukari et al. 2000) and *Fusarium oxysporum* (Ospina-Giraldo et al. 2003), or of conserved signal transduction components, such as G α - and G β -subunits of heterotrimeric G-proteins (Gao and Nuss 1996; Horwitz et al. 1999; Jain et al. 2003; Liu and Dean 1997; Parsley et al. 2003; Regenfelder et al. 1997), MAP kinases (Di Pietro et al. 2001; Mey et al. 2002; Zheng et al. 2000), adenylate cyclases (Choi and Dean 1997; Gold et al. 1994; Klimpel et al. 2002) or PKA-encoding genes (Gold et al. 1997; Kang et al. 1999; Mitchell and Dean 1995; Yang and Dickman 1999) results in much stronger reduction of pathogenicity because these proteins normally affect whole sets of target genes. In *B. cinerea*, four genes encoding signaling components have been disrupted so far: two G α subunit-encoding genes, *bcg1* and *bcg2* (Schulze Gronover et al. 2001), the adenylate cyclase-encoding gene, *bac* (Klimpel et al. 2002), and the MAP kinase-encoding gene, *bmp1* (Zheng et al. 2000).

The strongest effect on pathogenicity was achieved by the deletion of *bmp1*: mutants, which showed a total loss of ability to penetrate plant tissue and to develop symptoms (Zheng et

al. 2000). *Bac* mutants could penetrate and develop primary and secondary lesions, but the infection process was much slower than in the wild type, and conidia never were observed on rotted plant tissue (Klimpel et al. 2002).

Deletion of the *bcg1* gene, encoding one of the two Gα subunits in *B. cinerea*, resulted in reduced pathogenicity on bean and tomato, loss of protease secretion, and changed colony morphology (Schulze Gronover et al. 2001), but germination of conidia and penetration of plant tissue were not disturbed. However, after apparently overcoming certain physical and chemical barriers set up by the host plant, the infection process stopped after formation of primary lesions and no spreading soft rots developed. Addition of cAMP restored the wild-type colony morphology but not the ability to produce extracellular proteases, demonstrating that BCG1 is involved in at least one additional signaling cascade in addition to the cAMP pathway.

Because *bcg1* mutants are, in contrast to *bmp1* (MAPK) mutants (Zheng et al. 2000), still able to penetrate plant tissue, it is possible to compare in planta gene expression patterns between the wild type and *bcg1* mutants. Using suppression subtractive hybridization (SSH), we have identified 20 in planta-induced genes which are no longer expressed in the $\Delta bcg1$ -infected bean leaves. In this article, we describe genes encoding six putative proteases, two xylanases, and one maltose permease, as well as genes involved in secondary metabolism obtained by this approach. In addition, a second SSH library containing in planta-induced genes was constructed. Among the 1,500 clones sequenced so far, 11 of the 22 BCG1-controlled clones also were found in this SSH library.

RESULTS

Generation and analysis of subtracted cDNAs.

Up to the end of primary lesion formation, the infection process of the wild type and *bcg1* mutants was identical. Following germination of conidia, the hyphae grew on the surface of bean leaves and penetrated. After 24 h, the first small necrotic spots were visible, which developed to primary lesions with a diameter of approximately 3 mm after 40 to 48 h. In the wild type, these primary lesions spread and developed soft rots under conditions of high humidity and temperatures between 10 and 15°C. In the *bcg1* mutants, however, the infection process ceased after penetration of the cuticle and appearance of first symptoms, allowing us to compare the gene expression of the mutant and wild-type strains exactly at this time point. Leaf material from the primary lesions of both strains was collected and used for mRNA isolation and SSH library construction. The *B. cinerea bcg1* and the *Phaseolus vulgaris* glutamine synthetase (accession number X12738) genes were used as probes to quantify the ratio of fungal and plant RNA in total RNA preparations.

The resulting SSH cDNA fragments were cloned into the cloning vector pCR 2.1, whereby insert sizes varied from 300 to 1,000 bp. These clones were candidates for transcripts specifically up-regulated in the wild type just before soft rot lesions started to develop.

A comparison of the sequences revealed 39 contigs of different genes: 6 of them are plant genes and 4 encode ribosomal genes. The differential expression of the remaining genes was confirmed by Northern blot analysis using RNA from the same stage of infection of wild type and $\Delta bcg1$ mutant as for the con-

Fig. 1. Confirmation of putative differentially expressed suppression subtractive hybridization (SSH) clones by Northern blot analysis. Each lane contains 15 µg of total RNA from infected plant material (bean leaves, 48 h post inoculation) inoculated with conidia of the wild-type B05.10, the *bcg1* mutant, and the *bac* mutant. Each blot was hybridized to the indicated radioactively labeled SSH clone or rDNA (loading control).

struction of the library. In addition to the wild type and the $\Delta bcl1$ mutant, we included RNA prepared from primary lesions caused by the adenylate cyclase (*bac*) mutant (Klimpel et al. 2002). We wanted to know whether genes down-regulated in the *bcl1* mutant in planta also were down-regulated or missing in the *bac* mutant. The results indicated that 20 of the 29 genes were differentially expressed in planta. They were highly enriched only in the wild type, but not in the *bcl1* mutant (Fig. 1). From these 20 genes, only four SSH clones (SSH A06, SSH H08, SSH B03, and SSH C09) were significantly down-regulated, and four clones (SSH3, SSH B06, SSH E10, and SSH G02) were slightly down-regulated in the *bac* mutant, demonstrating that one of the signaling pathways controlled by BCG1 is the cAMP pathway. The expression level of all the other genes was not significantly decreased in the *bac* mutant, indicating that BCG1 directly or indirectly regulates at least one additional signaling pathway (Fig. 1).

The differentially expressed genes were grouped into different categories by their putative function (Table 1). Only four genes (SSH12, SSH C01, SSH H08, and SSH H12) could not be classified because they did not have significant hits in the databases. Among the classified genes, we found six different undescribed proteases not expressed in the $\Delta bcl1$ mutant. This finding supports the results of the milk agar-plate tests (Schulze Gronover et al. 2001) that showed the loss of protease secretion in *bcl1* mutants.

Another interesting group of clones are those with putative functions in secondary metabolism. Among them are genes encoding a polyketide synthetase and three P450 monooxygenases. Six of the genes encode enzymes of carbohydrate metabolism, such as cell wall-degrading enzymes, carbohydrate permeases, and a putative tartrate dehydrogenase (Table 1). Three of these six genes belong to the group of genes controlled by BCG1 via the cAMP signaling pathway.

In order to identify genes which are up-regulated in planta in the $\Delta bcl1$ mutant, a reverse subtraction ($\Delta bcl1$ – wild type [WT]) was performed. Two clones, SSH(–) B01 and SSH(–)

C12, encoding a putative glucose sensor and a pectate lyase, appeared several times in this approach. Northern blot analysis confirmed that both genes are up-regulated in the $\Delta bcl1$ mutant during the infection process (Fig. 1).

To get more information about the time course of the expression of different SSH genes in planta, RNA was isolated at specific stages of infection (primary lesion, outgrowth into spreading lesion, and soft rot) and hybridized with the specific clones. Two types of expression patterns were obtained. SSH clones encoding the putative P450 monooxygenases (SSH B06, SSH E10, and SSH G02) and the putative tartrate dehydrogenase (SSH E06) had an expression maximum at 44 hours postinoculation (hpi) when the fungus started to form spreading lesions. The expression of all the other genes increased with the development of the soft rot. Some of them are not expressed at the stage of primary lesions (e.g., SSH A06 and SSH H08), whereas others were already weakly expressed at this time point (e.g., SSH 22 and SSH 8) (Fig. 2).

In vitro transcriptional characterization of differentially expressed genes.

To obtain more information about regulation of expression of the selected genes under different cultivation conditions in vitro, the wild type and the $\Delta bcl1$ mutant were incubated in synthetic media with or without nitrogen, with low (0.5%) or high (3%) concentration of sucrose, with or without cAMP, and also in media with different plant extracts, such as V8 juice, soy bean, and bean leaf extracts. Northern blots were prepared from mycelium grown for 40 h in malt medium followed by incubation for 5 h in minimal Czapek Dox medium before transfer into the eight different media. As a control, RNA from infected bean leaves at the end of the formation of primary lesions was loaded. To compare the expression of the differentially expressed protease genes with a protease-encoding gene not found in this SSH approach, we used the *B. cinerea* homologue of *pepE*, an aspartic protease gene of *Aspergillus oryzae* (Kunihiro et al. 2002) as a control gene (Fig. 3).

Table 1. Differentially expressed suppression subtractive hybridization (SSH) clones, not expressed (SSH), or up-regulated (SSH(-)) in *bcl1* mutants in planta

Clone ^a	Blast result	E value
Secondary metabolism		
SSH 3**	Polyketide synthase (BFU68716)	0
SSH B06	Cytochrome P450 monooxygenase (BAB59027)	2E-22
SSH G02	Cytochrome P450 monooxygenase (AAK11528)	2E-18
SSH E10	Averantin oxidoreductase (P450 enzyme) (Q12732)	6E-41
Proteases		
SSH 8*	24-kDa metalloproteinase precursor (P46073)	3E-10
SSH 17*	Aorsin (BAB97387)	4E-57
SSH 22*	Polyporopepsin (P17576)	1E-11
SSH 26	Acid protease (AAF34754)	2E-05
SSH F11*	Penicillolysin precursor (P47189)	5E-13
SSH G04*	Tripeptidyl-peptidase I precursor (O14773)	3E-62
Carbohydrate transport, degradation and metabolism		
SSH A06**	Maltose permease (CAB46745)	2E-08
SSH B03**	Endo-1,4- β -xylanase (P56588)	7E-92
SSH C09**	Endo-1,4- β -xylanase A (P35809)	9E-65
SSH F05*	Endo- β -1,4-glucanase (AAL88714)	5E-91
SSH D07*	High-affinity gluconate transporter (Q92339)	4E-21
SSH E06*	Probable tartrate dehydrogenase <i>ttuC</i> (P70792)	1E-108
Other genes		
SSH 12	Putative multicopper oxidases (NP_759933)	8.1
SSH C01*	Hypothetical protein (Q09762)	6
SSH H08*/**	Hypothetical protein (AAN35381)	0.071
SSH H12*	Hypothetical 38.1-kDa protein (O94635)	1E-06
Up-regulated genes		
SSH(–) B01	Putative glucose sensor (O13477)	2E-05
SSH(–) C12	Pectate lyase C (Q00843)	9E-24

^a Clones which were found also in the in planta SSH library = * and clones which are under control of BCG1 via BAC = **.

None of the differentially expressed SSH clones was up-regulated under extreme nitrogen and carbon starvation conditions. Most of them were expressed best in media with 0.5 and 3% sucrose and in planta in the wild type. In the mutant, most of the genes were strongly up-regulated with 3% sucrose and were much less expressed in the presence of 0.5% sucrose. Additional studies on pH regulation in combination with varying sucrose concentrations showed that the general modus of pH regulation of the SSH clones is the same in both strains (high expression up to pH 4, very low expression at pH 5 and higher). However, in the $\Delta bcg1$ mutant only, the expression of these genes was additionally affected by the sucrose concentration. The genes showed a better expression level in the presence of 3 than 0.5% sucrose, even at pH 5 (Fig. 4). These results confirmed the specific effect of sucrose on the expression of the SSH genes and correspond with our finding that the $\Delta bcg1$ mutant has an aberrant colony morphology (small compact colonies) on agar media with 0.5% sucrose, but grows like the wild type on media with 3% sucrose (Schulze Gronover et al. 2001). cAMP applied to the sucrose medium had no or only a small effect on gene expression. As expected, the main effect was the loss of gene expression of 20 genes in the *bcg1* mutant in planta and on the plant extract media, in marked contrast to the control gene encoding the aspartic protease, *bcepeE*.

Construction of an SSH library of in planta-induced genes.

In addition to genes which are differentially expressed at the stage of primary lesions between the wild type and the $\Delta bcg1$ mutant, we also were interested in genes which are up-regulated in the wild-type strain in planta from the beginning of the infection process up to the soft rot formation. Therefore, we constructed a second SSH library using wild-type infected plant material 24, 48, and 60 hpi for mRNA preparation ("tester"). mRNA from uninfected bean leaves ("driver") was subtracted from mRNA of the three time points (Fig. 5).

Approximately 10% of the 1,500 analyzed clones contained plant-derived sequences, mostly encoding pathogenicity-related (PR) proteins (Table 2). Approximately 10% of the SSH clones represented novel *B. cinerea* sequences not contained in the *B. cinerea* public EST library. Some of the sequences, categorized into groups according to their putative function, are shown in Table 2. Most of the genes belong to the group of cell wall-degrading or carbohydrate transport proteins. Several genes with homology to cytochrome P450 monooxygenases, reductases, or oxidoreductases suggest involvement in secondary metabolism. Another group of genes showed high homology to enzymes responsible for detoxification of plant saponins and phytoalexins, but also of fungicides and reactive oxygen species (ROS), such as ABC and MFS transporters, glutathione S-transferase, and a copper-zinc superoxide dismutase (*bcsod1*) (Rolke et al. 2004). Several components of signaling pathways, such as *cdc42*, a ras-like GTPase, and several protein kinases or phosphatases also were identified. Of high interest is a gene with homology to the integral membrane protein, Pth11, from *Magnaporthe grisea*, which is essential for pathogenicity on rice (De Zwaan et al. 1999).

Interestingly, 11 of the 22 genes which are differentially expressed in the $\Delta bcg1$ mutant in planta also were found among the 1,500 sequenced clones of the in planta SSH library, demonstrating that both SSH approaches lead to identification of PR genes.

DISCUSSION

B. cinerea is a pathogen with an extremely broad host spectrum. Despite the enormous yield losses in fruit, vegetable, and flower production, the identification of PR genes is only beginning. Several putative pathogenicity genes, encoding cell wall-degrading enzymes (ten Have et al. 1998, 2001; Wubben et al. 1999), ABC and MFS transporters (Hayashi et al. 2002a

Fig. 2. Time course expression pattern of differentially expressed suppression subtractive hybridization (SSH) clones. Bean leaves were inoculated with conidia of the wild type B05.10 and harvested at distinct hours post inoculation (hpi) (30 hpi, formation of primary lesion; 44 hpi, first spreading from primary into secondary lesion; 68 hpi, total soft rot of the plant tissue). Each lane contains 15 μ g of total RNA from infected plant material inoculated with conidia of the wild type B05.10.

and b; Schoonbeek et al. 2001), saponinases (Quidde et al. 1999), and enzymes degrading or generating active oxygen species such as catalases (Schouten et al. 2002; van der Vlugt-Bergman 1998), superoxide dismutases, and glucose oxidases (Rolke et al. 2004) have been cloned. However, a significant reduction in virulence has been obtained only for mutants of the endopolygalacturonase, *bcpgl*, and the pectin methyl-esterase, *bcpme1* (ten Have et al. 1998; Valette-Collet et al. 2003). So far, only the disruption of signaling components, such as the MAP kinase-encoding gene *bmp1* (Zheng et al. 2000), the G α subunit encoding gene *bcg1* (Schulze Gronover et al. 2001), and the adenylate cyclase gene, *bac* (Klimpel et al. 2002) resulted in total loss (*bmp1*) or a drastic reduction of pathogenicity. However, the transcription factors or target genes activated by these signaling proteins are not yet known.

As described above, *bcg1* mutants can successfully penetrate the plant cuticle and produce primary lesions as the first visible symptom in an identical manner to the wild type. However, the mutants stop the infection process at this time point, although the hyphae are still alive inside the plant tissue. In addition to this significant reduction of pathogenicity, the mutants were no longer able to secrete proteases on milk agar and showed a more compact colony morphology on potato sucrose agar with 0.5 to 1% sucrose but not with 3% sucrose (Schulze Gronover et al. 2001). In addition, *bcg1* mutants lost their ability to produce the sesquiterpenoid phytotoxin botrydial (C. Schulze Gronover and B. Tudzynski, unpublished data) demonstrating that BCG1 also regulates the production of secondary metabolites. The influence of G protein-mediated signal-

ing on production of toxic secondary metabolites was shown in *Aspergillus* spp. (Calvo et al. 2002). FadA, a homologue of BCG1, was found to act positively on penicillin production, whereas it negatively influenced aflatoxin and sterigmatocystin production. The constitutively activated FadA (*fadA*^{G42R}) also was able to increase trichothecene mycotoxin production in *Fusarium sporotrichoides* (Tag et al. 2000).

It is not yet known which types of genes are needed to overcome the stage of primary lesions and for production of rapidly spreading soft rot lesions. It was shown clearly that the MAP kinase Bmp1 is responsible for the first stages of plant infection, including differentiation of germlings and penetration (Zheng et al. 2000). In contrast to $\Delta bcg1$ mutants, $\Delta bmp1$ mutants developed no symptoms on bean or tomato leaves, and this makes it difficult to analyze the corresponding target genes.

Using the polymerase chain reaction (PCR)-based SSH method (Diatchenko et al. 1996), we wanted to find genes that were induced specifically in planta at the stage of primary lesions in the wild type just before development of spreading lesions.

Among the analyzed genes, 20 were expressed in planta only in the wild type but not in the mutant, suggesting that their expression is dependent on the active BCG1. Six of them were protease-like genes, whereby this result corroborates the finding that *bcg1* mutants do not produce or secrete proteases on milk agar. We suggest that these proteases have a function in degrading the cell wall and PR proteins of the host plant, and also in nutrition of the mycelium during the following invasive growth. It is well known that one of the plant responses to fungal infection is the production of ROS, resulting in cross-linking of cell wall proteins (Otte and Barz 1996). *B. cinerea* is able to detoxify ROS by several enzymes, such as catalases (Schouten et al. 2002; van der Vlugt-Bergman 1998) and superoxide dismutase (Rolke et al. 2004), but cannot avoid the stabilization of plant cell walls. This group of different protease-like enzymes may be a powerful tool to overcome the network of cell wall proteins and to use intracellular proteins for invasive growth, but biochemical studies are needed to characterize them in detail.

In addition to the proteases, we found genes with a putative role in secondary metabolism (Table 1). Interestingly, all of these genes were slightly down-regulated in the *bac* mutant (Fig. 1), suggesting a partial involvement of the cAMP signal-

Fig. 3. Northern blot analysis of some of the differentially expressed suppression subtractive hybridization (SSH) clones under in vitro cultivation conditions. The wild-type strain, B05.10, and the *bcg1* mutant were grown on a rotary shaker for 40 h in liquid malt medium. The mycelia then were transferred to minimum Czapek-Dox (CD) medium without nitrogen (N-) and carbon (C-) sources for 5 h, and then incubated for 2 h in the following media: 1, CD without N- and C- sources; 2, CD + (NH₄)₂SO₄; 3, CD + (NH₄)₂SO₄ + 3 mM cAMP; 4, CD + 0.5% sucrose; 5, CD + 3% sucrose; 6, CD + V8 juice; 7, CD + 0.5% soya meal; 8, CD + bean leaf extract; 9, RNA from infected bean leaves 48 h post inoculation (in planta control). Each blot was hybridized to the indicated radioactively labeled SSH clone or rDNA (loading control).

Fig. 4. pH-dependent expression of two differentially expressed suppression subtractive hybridization (SSH) clones (putative protease encoding genes). The wild-type strain, B05.10, and the *bcg1* mutant were grown on a rotary shaker for 40 h in liquid malt medium. The mycelia then were transferred to minimum Czapek-Dox (CD) medium without nitrogen and carbon sources for 5 h, and then incubated for 2 h in the following media at indicated pH: a, 0.5% sucrose; b, 3% sucrose. Each blot was hybridized to the indicated radioactively labeled SSH clone or rDNA (loading control).

ing pathway in secondary metabolite production as it was shown for *Aspergillus* spp. (Calvo et al. 2002). The polyketide synthetase (PKS) (SSH 3) already was partially sequenced (J. van Kan, *unpublished data*) (accession number BFU68716), but it is not yet known within which polyketide biosynthetic pathway the PKS is involved. The P450 monooxygenases could be involved in biosynthesis of terpenoids, such as the phytotoxin botrydial (Colmenares et al. 2002), or abscisic acid (Okamoto et al. 1988), or of unknown *Botrytis* products. Regulatory studies showed that the genes encoding the three P450 monooxygenases (SSH B06, SSH G02, and SSH E10) are expressed under botrydial producing conditions in the wild type but not in the $\Delta bcl1$ mutant (data not shown). This result fits very well with our finding that the $\Delta bcl1$ mutants totally lost the ability to produce botrydial.

The fact that these genes have their maximal expression level at the change from primary to secondary lesion let us suggest that the produced metabolites (e.g., botrydial) could play a crucial role for the spreading of the fungus from the primary lesion.

Several other SSH genes seem to have a function in plant cell wall degradation, sugar transport, or carbohydrate metabolism. Interestingly, in addition to genes that were down-regulated in the $\Delta bcl1$ mutant in planta, we identified two genes which had a significantly increased expression level in the mutant.

$G\alpha$ subunits have been suggested to activate or inactivate the adenylate cyclase activity and thus to affect the cAMP signaling pathway (Gao and Nuss 1996; Ivey et al. 1999; Klimpel et al. 2002), but no data are available on the regulation of other signaling cascades, such as MAP kinase pathways, by a $G\alpha$ subunit in filamentous fungi. The Northern blot analysis presented here for the 22 BCG1-dependent SSH clones demonstrates clearly that the expression of only some genes was down-regulated in both the *bcl1* and the *bac* mutant, whereas the others were expressed in the *bac* mutant in a similar manner as in the wild type. Therefore, BCG1 probably controls even more signaling cascades that have not yet been identified.

The genes for $G\beta$ subunits were cloned in some fungi (e.g., *Cryphonectria parasitica* [*cpgb-1*]) (Kasahara and Nuss 1997)

and recently in *F. oxysporum* (*fgb1*) (Jain et al. 2003). From the phenotype of deletion mutants, one can suggest that $G\alpha$ and $G\beta$ subunits have some common but also some distinct functions, and that the loss of one subunit can affect the level of the other. In *C. parasitica*, disruption of either *cpg-1* or *cpgb-1*, as well as introduction of dominant-activated *cpg-1* gene copies (Segers and Nuss 2003), consistently reduced the level of its presumptive partner protein (Kasahara et al. 2000; Parsley et al. 2003). In *Neurospora crassa*, $G\alpha$ protein levels are reduced in *gnb-1* ($G\beta$) null mutants, and a *gna-1/gna-2* double knock-out resulted in reduction of $G\beta$ (Ivey et al. 1999; Yang et al. 2002). These results suggest that the absence of a correctly functioning partner affects the accumulation of the other subunit due to a yet unknown mechanism of post-transcriptional regulation of G protein signaling (Parsley et al. 2003). Therefore, it is possible that some of the changes in expression levels of SSH clones in the *bcl1* mutant are the result of the decreased accumulation of $G\beta$. On the other hand, a dual role in signaling of BCG1 similar to the small G protein Ras2 in *Saccharomyces cerevisiae* (Pan and Heitman 1999) that functions in both the MAP kinase and cAMP signaling pathways also is possible. Recently, we cloned the *B. cinerea* $G\beta$ -encoding gene, *bgb1* (C. Schulze Gronover and B. Tudzynski, *unpublished data*). It will be of great interest to identify the downstream effectors of the $G\beta\gamma$ signaling pathway and to compare them with the $G\alpha$ targets.

In addition to the screening for BCG1-controlled genes, we were interested in finding genes which are involved in early (penetration), medium (formation of primary lesions), and late (spreading lesions, invasive growth, sporulation on macerated leaves) stages of infection. Therefore, a second SSH approach was used for identification of in planta-expressed genes. Among the 1,500 clones, we found 11 of the 22 already identified BCG1-dependent genes (Table 1), such as the cytochrome P450 monooxygenase- (clone 9), the endoglucanase- (clone 22), and the tartrate dehydrogenase- (clone 35) encoding genes (Table 2). Approximately 10% of the clones were not yet identified in any differential cDNA approaches and are novel *B. cinerea* sequences. Some of them, like the new glutathione S-transferase (clone 5), the cellobiohydrolase I (clone 16), and

Fig. 5. Scheme for the isolation of total RNA from infected ("tester") and uninfected ("driver") bean leaves. The RNA was used for the construction of an in planta cDNA library by the suppression subtractive hybridization (SSH) approach.

Table 2. In planta induced putative pathogenicity-related (PR) genes

Clone	Blast result	E value	Frequency
Detoxification of plant compounds			
1	ABC transporter (<i>atrB</i>)	8E-20	1
2	MFS transporter of unknown specificity	6E-15	2
3	Glutathione S-transferase I	4E-46	13
4	Copper-zinc superoxide dismutase	4E-61	5
Secondary metabolism			
5	Isopentenyl pyrophosphate:dimethylallyl pyrophosphatase	1E-24	1
6	CPRD8 protein	8E-76	1
7	Cytochrome P450	2E-23	1
8	Bifunctional P-450:NADPH-P450 reductase	3E-49	1
9	Related to cytochrome P450 3A7	7E-10	1
10	Putative peptide synthase protein	1E-29	1
11	Oxidoreductase	3E-05	4
12	TRP-1	5E-08	3
Cell wall degradation, carbohydrate and lipid metabolism and transport			
13	1,4-β-D-glucan cellobiohydrolase B precursor	2E-10	1
14	Cellobiohydrolase I	1E-32	10
15	Cellobiohydrolase II	3E-03	1
16	Cellulase precursor	3E-38	6
17	Exocellulase precursor	7E-26	4
18	β-Glucosidase	5E-34	3
19	Exoglucanase I precursor (EC 3.2.1.91)	4E-44	1
20	Endoglucanase I	5E-68	1
21	β-Glucosidase homolog	2E-21	1
22	Endoglucanase EG-II precursor (EC 3.2.1.4)	1E-79	2
23	Endopolygalacturonase 1	1E-152	20
24	Endopolygalacturonase 6	7E-56	2
25	Putative α-xylosidase	4E-65	3
26	Possible xylan degradation enzyme	1E-03	2
27	Glycosyl hydrolase, invertase	6E-78	5
28	General α-glucoside permease	6E-10	1
29	Lactose permease	7E-36	1
30	Maltose permease MAL3T	8E-18	1
31	Probable sugar transporter	9E-57	1
32	Cutinase	6E-29	17
33	Lipase 2 precursor	2E-23	1
34	Mannitol-1-phosphate dehydrogenase (EC 1.1.1.17)	5E-34	4
35	Probable tartrate dehydrogenase ttuC'	1E-108	6
36	Metabolite transport protein	2E-37	2
37	Polyporopepsin (EC 3.4.23.29)	1E-10	1
38	Vacuolar aspartic protease precursor	2E-33	1
39	Penicillolysin precursor (EC 3.4.24.39)	5E-13	4
40	24-kDa metalloproteinase precursor	3E-10	6
41	Polyporopepsin (EC 3.4.23.29)	1E-11	6
42	Aspartyl proteinase	1E-164	1
43	Ubiquitin conjugating enzyme Spm2	1E-40	1
44	Ubiquitin	3E-67	1
45	Small GTP-binding protein YPTI	3E-98	1
46	RING-H2 finger protein RHA2A	4E-13	1
47	Secretory pathway GDP dissociation inhibitor	1E-23	1
Signal transduction			
48	Integral membrane protein, Pth11-like protein	1E-08	1
49	Serine-threonine kinase, receptor-associated	5E-22	1
50	Serine/threonine protein phosphatase 2A	1E-53	1
51	GTPase CDC42	1E-106	3
52	Ras-like protein	5E-27	6
53	Calmodulin	3E-78	1
PR enzymes of plants			
54	Serine proteinase inhibitor	8E-29	1
55	Thaumatococcus-like protein PR-5b precursor	1E-17	1
56	Protease inhibitor 2	5E-07	1
57	Calmodulin	2E-48	1
58	Intracellular pathogenesis related protein	1E-74	1
59	Lignin forming anionic peroxidase precursor	4E-53	1
60	Glutathione S-transferase GST 14 (EC 2.5.1.18)	1E-62	1
61	Superoxide dismutase [Cu-Zn] (EC 1.15.1.1)	2E-37	1
62	Intracellular pathogenesis related protein	2E-82	14
63	NADPH:isoflavone reductase	1E-122	10
64	Pathogenesis-related protein STH-2	8E-20	9
65	Pathogenesis-related protein 1 (PvPR1) (PR2)	8E-82	2
66	Chalcone synthase 17 (EC 2.3.1.74)	7E-47	3
67	Pathogenesis-related protein PR1	2E-52	2
68	Transcription factor	3E-23	2
69	PDR5-like ABC transporter	4E-89	1
70	PDR-like ABC transporter	3E-27	1

the cutinase (clone 32) were found at least 10 times in the library. Only 10% of the genes were of plant origin and mostly were PR genes (Table 2). This demonstrates the usefulness of the SSH approach for searching for PR fungal genes involved in different stages of the infection process.

Many genes highly expressed in the plant-pathogen interaction have been cloned in the recent years from fungi. The authors successfully used methods such as expressed sequence tags (Trail et al. 2003), mRNA expressed differential display (Kim et al. 2000; Yang et al. 2000), or SSH (Beyer et al. 2002; Thara et al. 2003; Wulf et al. 2003; Yang et al. 1999) to identify genes specifically up-regulated in the infection process. The comparison by SSH of in planta gene expression profiles between the wild type and a mutant blocked in a particular signal transduction pathway recently was shown to be a powerful tool to identify targets of a MAP kinase pathway (Lev and Horwitz 2003). In this article, we report a similar SSH approach for identification of genes, which are directly or indirectly (e.g., through the changed level of the corresponding G β moiety) controlled by the G α subunit BCG1. These BCG1-controlled genes obviously play an important role at the stage of development of spreading lesions and invasive growth. Therefore, subtractive hybridization is a powerful method for enriching differentially expressed genes in planta and genes whose expression is under specific control of signaling pathway components.

We were not only able to identify 22 BCG1-regulated genes, but also could show, for the first time, that BCG1, which is essential for full pathogenicity in *B. cinerea*, controls more signaling pathways than the cAMP pathway. One of them could be the G β -controlled signaling cascade, suggesting that the G β accumulation is decreased in the *bcg1* mutant in a similar way as it was described for *C. parasitica* *cpg-1* disruptants and mutants expressing a dominant active CPG-1 protein (Parsley et al. 2003; Segers and Nuss 2002). Searching for specific transcription factors from the cAMP and the unknown signaling pathways is the next step in our research program.

MATERIALS AND METHODS

Fungal strains and growth conditions.

Botrytis cinerea Pers.:Fr., anamorph of *Botryotinia fuckeliana* (de Bary) Whetzel, strain B05.10, was derived from wild-type strain SAS56 by treatment with benomyl for haploidization (Quidde et al. 1999). The strain $\Delta bcg1$ is a knockout transformant of B05.10 concerning the α -subunit (*bcg1*) of a heterotrimeric G-Protein (Schulze Gronover et al. 2001). The fungus was grown in liquid media or on agar plates containing 1.5% malt extract, 0.5% glucose, 0.01% yeast extract, caseamino acids, peptone, and 0.001% ribonucleic acid (malt medium). For shift experiments, mycelium was grown for 40 h in malt medium, then transferred to a salt solution (Czapek-Dox without carbon and nitrogen source, pH 4.5) for 5 h, then transferred to salt solution plus the inductive test components for at least 2 h.

Infection assays on bean plants.

Infection assays were performed on bean plants as described previously (Schulze Gronover et al. 2001). First necrotic symptoms became visible after 24 to 30 h, the so-called primary lesion. After 40 to 48 h, the fungal hyphae grew out of the primary lesion to form the secondary lesion, which ended in the typical soft rot. Time points varied in different experiments depending on factors such as temperature or humidity and on the fitness of the plants and the fungus. All infection assays were prepared in parallel for in planta expression analysis between different strains.

RNA isolation and Northern blots.

Total RNA was isolated from mycelium or soft rot lesions of *Botrytis*-infected bean leaves using the RNA easy extraction kit (Promega, Mannheim, Germany). mRNA was purified with the Oligotex mRNA Kit (Qiagen, Hilden, Germany). The methods used for RNA electrophoresis, gel blotting, probe labeling, and hybridization also have been described elsewhere (Church and Gilbert 1984; Sambrook et al. 1989).

SSH.

SSH (Diatchenko et al. 1996) was carried out using the Clontech (Palo Alto, CA) PCR Select-Subtraction cDNA kit. For construction of the *Botrytis* spp. in planta cDNA library, the same amounts of cDNA from *Botrytis* (B05.10)-inoculated bean leaves harvested 24, 48, and 60 hpi were used as a "tester" and cDNA from noninfected bean leaves was used as a "driver".

In a second approach (WT- $\Delta bcg1$ and $\Delta bcg1$ -WT), cDNA from wild type (B05.10)-infected bean leaves was used as a "driver" and cDNA from $\Delta bcg1$ -infected bean leaves was used as a "tester" or vice versa ($\Delta bcg1$ -WT). Both cDNA populations derived from infected bean leaves were harvested before forming soft rot lesions. PCR products were analyzed on a 2% agarose gel and cloned into the pCR2.1 cloning vector (Invitrogen, Groningen, The Netherlands) for sequencing.

Computational analysis.

Sequence analysis was performed using the NCBI database server. Protein homology was based on BlastX searches (Altschul et al. 1997). For further investigations, the programs of DNA STAR Inc. (Madison, WI, U.S.A.) were used.

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AUTHOR-RECOMMENDED INTERNET RESOURCE

Genoscope public *Botrytis cinerea* EST library: www.genoscope.Cns.fr/