Sulphur and nitrogen regulation of the protease-encoding ACP1 gene in the fungus Botrytis cinerea: correlation with a phospholipase D activity

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INTRODUCTION

Fungi sense the nutritional status of their environment via multiple mechanisms that first relay the information about particular nutrients and then trigger the appropriate cellular response (Gagiano et al., 2002; Eckert-Boulet et al., 2004; Smith et al., 2003). Three essential nutrients, namely carbon, nitrogen and sulphur, are present in the environment as readily usable sources or as complex molecules from which they can be enzymically extracted by fungi. In the case of carbon, glucose represents the favourite source but the way in which they are perceived by the fungal cell is essentially unknown.

Fungi can assimilate most of the organic and inorganic sources of sulphur that are present in their environment, and this implies different metabolic pathways, each composed of specific transporters and enzymes. However, when a fungal cell is exposed to multiple sulphur sources, it favours the one most readily metabolizable, and the expression of genes encoding proteins involved in the assimilation of the others is usually decreased (Jacobson & Metzenberg, 1977); this phenomenon has been termed sulphur catabolic repression. In the filamentous fungus Neurospora crassa, one transcriptional regulator has been found whose control over the expression of genes coding for sulphate transporters and some of the enzymes involved in sulphate assimilation has been demonstrated (Marzluf & Metzenberg, 1968; McDermott et al., 2004). This regulator, CYS3 (Fu et al., 1989; Fu & Marzluf, 1990a; Paietta, 1992), has also been shown to control production of proteases (Hanson & Marzluf, 1975) and its homologue in Aspergillus nidulans has been identified (Natorff et al., 2003). Its consensus target DNA sequence has been determined and it is found in various gene promoters (Kanaan et al., 1992; Ellenberger et al., 1992; Li & Marzluf, 1996). In the absence of sulphate or cysteine, CYS3 binds to considered the favourite sources but the way in which they are perceived by the fungal cell is essentially unknown.

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Abbreviations: AEBSF, 4-(2-aminoethyl)benzenesulphonyl fluoride; EST, expressed sequence tag; GUS, β-glucuronidase; PA, phosphatidic acid; PC, phosphatidylcholine; PE, phosphatidylethanolamine; PLD, phospholipase D.
these promoters and activates transcription. In the presence of sulphate or cysteine, sulphur catabolic repression seems to operate via ubiquitination of CYS3 and its degradation by the proteasome; this is supported by results obtained in the yeast Saccharomyces cerevisiae (Rouillon et al., 2000) and by protein similarities as well as complementation studies performed with homologous genes in filamentous fungi (Natorff et al., 1998). The signalling pathway that connects the presence of favoured sulphur sources to the changes affecting CYS3 is unknown but intracellular cysteine, or one of its derived metabolites, seems to be a necessary intermediate (Jacobson & Metzenberg, 1977; Natorff et al., 1993).

Filamentous fungi also exhibit nitrogen catabolic repression. The expression of selected genes involved in nitrogen metabolism is reduced in cells exposed to readily metabolizable nitrogen sources such as ammonia (Facklom & Marzluf, 1978; Sikora & Marzluf, 1982a, b). One transcriptional regulator, named NIT2 in N. crassa and AREA in A. nidulans, has been identified in several fungi (Fu & Marzluf, 1990b, c; Haas et al., 1995; Froeliger & Carpenter, 1996, Screen et al., 1998), and its binding to DNA is necessary for the expression of the genes involved in utilization of nitrogen sources (Scacciochcio, 2000). In the absence of ammonia, AREA binds to two closely spaced 5'-GATA sequences in various gene promoters and activates their transcription (Chiang & Marzluf, 1994; Chiang et al., 1994). Conversely, in the presence of ammonia, AREA activity is strongly reduced due to low expression of the araA gene, lower RNA stability (Morozov et al., 2001), interaction with a negative-acting regulator [NMRA in A. nidulans (Andrianopoulos et al., 1998) and NMR1 in N. crassa (Fu et al., 1988)], and/or decreased accumulation in the nucleus (Todd et al., 2005). Catabolic repression hence occurs. Again, the signalling pathway that responds to the presence of ammonia is unknown, but intracellular glutamine or glutamate have been proposed as possible intermediates (Marzluf, 1997a; Scacciochcio, 2000; Margelis et al., 2001).

Phospholipases D (PLD) are enzymes that respond to environmental signals to produce the secondary messenger phosphatidic acid (PA). In eukaryotes, several PLD-encoding genes have been isolated in recent years and they all seem to belong to the HKD gene family (McDermott et al., 2005). Members of this family preferentially catalyse the conversion of phosphatidylcholine (PC) into PA and incapacity to catalyse transphosphatidylation make the associated enzyme differ- entiated whose calcium dependence, preference for phosphatidylethanolamine (PE) over PC, and incapacity to catalyse transphosphatidylation make the associated enzyme different from the known HKD family members. The gene that codes for this non-HKD PLD is still unknown despite the availability of the whole yeast genome sequence, and it is proposed to be the first member of a novel PLD gene family and to present no homology to HKD genes (Tang et al., 2002).

The secretion of lytic enzymes is a hallmark in the biology of fungi, for these organisms rely on the degradation of polymers outside the cell for nutrition. The production of these enzymes is under the control of a sophisticated regulation system that integrates signals triggered in the cell by various chemical environmental cues. In saprophic species, large panels of enzymes enable them to thrive in very different ecological niches, and many of these enzymes are of economic interest. In pathogenic species, these enzymes participate in the penetration, progression and survival of the fungus inside its host, and understanding the regulation of their production is part of the global quest to understand fungal pathogenicity. In this study, we explored the regulation of the protease-encoding ACP1 gene of the plant-pathogenic fungus Botrytis cinerea by sulphur and nitrogen. We report on the discovery of a non-HKD PLD activity that correlates with the activity of the ACP1 promoter.

METHODS

General techniques. Cultures, Western-blot analysis and β-glucuronidase (GUS) assays were performed as previously described (Rolland et al., 2003) except that all sulphate salts in the culture medium were replaced by chloride salts. Briefly, the fungus was grown on rich medium on cellophane plates and transferred for 15 h onto the surface of 2 ml minimal medium containing 2% glucose, buffered at pH 4.0 and supplemented as indicated in the text. The medium was recovered to collect the secreted proteins by precipitation. The mycelium was frozen in liquid nitrogen and ground to prepare whole-cell crude extracts. Antibodies raised against endopolygalacturonases were used at 1/5000 dilution (Martel et al., 1996).

PLD activity assays. PLD activity was measured according to Hong et al. (2003) with the following modifications. Briefly, 50 μg crude cell extract was incubated for 2 h at 28°C in 100 μl 50 mM HEPES pH 7.5, 150 mM NaCl, 1 mM CaCl2, 0.25 mM Triton X-100 and 25 μM 1-palmitoyl-2-[1-14C]linoleoylphosphatidylethanolamine (Amersham). Following separation by TLC using chloroform/methanol/acetic acid (50:25:8) as solvent system, radioactive products were detected using a cyclone phosphorimager (Packard).

Promoter mutagenesis. Versions of the ACP1 promoter 0.4 and 0.6 kb long were generated by using PCR and appropriate primers; the corresponding DNA fragments were cloned upstream of the GUS reporter gene. Site-directed mutagenesis was also used to modify the DNA sequences corresponding to the proximal AREA and CYS3 putative binding sites in the ACP1 promoter. The consensus ‘agataa’ sequence for AREA and ‘agttgcat’ sequence for CYS3 were changed to ‘agggaa’ and ‘atgtggcat’, respectively. These mutations had been shown to disrupt the nitrogen and sulphur regulation of other genes (Li & Marzluf, 1996; Ravagnani et al., 1997).

RNA interference (RNAi) cloning. The oliC promoter was amplified from pLOB-MPD1 (kindly provided by P. Tuzdinsky, Westfälische Wilhelms-Universität, Germany) and cloned upstream of the nos terminator into pBSK (Stratagene); the primers used were OliC5’ (5'-ATATCTAGATGTTGGACCGCATTC-3’) and OliC3’ (5’-ATACTGACGGATCGATTGATGTG-3’). A 500 bp DNA fragment was amplified from B. cinerea genomic DNA using the primers PLD5’ (5’-AAGCTTGGAGATGCCTTGCGACG-3’) and PLD3’ (5’-CGCGGATCTCCCAATTCCTCAGTATGCCG-3’) designed on the basis of the EST W10A0342A12C1 (Soanes et al., 2002).
(predicted to be part of a PLD-encoding gene in *B. cinerea*). This fragment was cloned into pTOPO4 (Invitrogen), extracted by restriction with *PstI* and cloned between the olic promoter and the nos terminator to yield p66. A 300 bp DNA fragment was amplified from the *gfp* gene in p18 (Rolland et al., 2003) using GFPS’ (5'-CTCGGATCCCTTCAAGGACGACGGCAACTACAAG-3’) and GFPS’ (5’-CTCGGATCCCTGTAATGTTGTCGGCAGCTGACG-3’), digested with BamHI and cloned into p66 upstream of the *pld* sequence to yield p67. The same *pld* sequence was finally cloned into p67, upstream of the *gfp* sequence and in the opposite orientation from that of the other *pld* sequence, to yield p72. The RNAi cassette was digested with XbaI and EcoRI and cloned into pBHt2 (Mullins et al., 2001) to yield p73. *Agrobacterium tumefaciens* strain LBA1126 containing p73 was used to transform *B. cinerea* strain B05-10 as described previously (Rolland et al., 2003).

**RT-PCR.** The mycelium was frozen in liquid nitrogen, ground and suspended in 1 ml Trizol reagent (Gibco-BRL). After 5 min vigorous agitation, 200 μl chloroform was added to the samples and the mixing was repeated for 5 min. The samples were centrifuged (10 min, 13,000 g, 4 °C) and the supernate was recovered. Total RNAs were collected by further centrifugation after addition of 500 μl 2-propanol. The pellets were washed in 70% ethanol and suspended in RNase-free water. cDNA synthesis (1 h at 42 °C) was performed using AMV-RT reverse transcriptase and random primers (Promega); RNAs (5 μg) were treated with RQ1 DNase (Promega) for 2 h at 37 °C, mixed with 2-propanol and collected by centrifugation prior to the reaction. Then 1 μl cDNA was used in a quantitative amplification reaction using the ‘qPCR and Go’ kit (Qiagen), the protocol described by the manufacturer and the following primers: for the *pld* gene, 5’-TCTCAGGAACACGACACAGTAGCAG-3’ and 5’-CGGGG-GAAACACATCTTGACGACG-3’; for the *ACP1* gene, 5’-TGTAGTGTAGCAGCTAACA-3’ and 5’-GTGGTGTGGACGAGACCT-3’; for the tubulin gene, 5’-AACCTGCGGTCCTCACAATACCTGTTGTTGACGACGGCACTACAAG-3’ and 5’-CGGGATCCCTTAATACTCAGCCTCACCC-TC-3’.

**RESULTS**

The protease-encoding *ACP1* gene is regulated by sulphur

Previous studies have shown that the acid-protease-encoding *ACP1* gene is induced by plant extracts and repressed by nitrogen and neutral or alkaline pH in the two closely related necrotrophic fungi, *B. cinerea* and *Sclerotinia sclerotiorum* (Girard et al., 2004; Pousseraeau et al., 2001; Rolland et al., 2003). To investigate whether this gene also responds to the availability of sulphur to the fungal cell, we used a modified B05.10 *B. cinerea* strain (strain T3) carrying a transcriptional fusion (pACP1-GUS) able to drive an intracellular production of β-glucuronidase (GUS) that parallels that of the endogenous *ACP1* (Rolland et al., 2003). The fungus was grown in rich medium and then exposed to the absence or presence of sulphate. Acidic medium was used to avoid repression of the promoter activity by alkaline pH, and 50 mM ammonia was added to the medium to prevent transcriptional activation as a result of nitrogen starvation (Pousseraeau et al., 2001). The viability of the mycelium and the amount of proteins extracted from it were similar under all conditions. The expression of pACP1-GUS was strong in the absence of sulphate and decreased in a concentration-dependent manner when sulphate was present (Fig. 1a). Cysteine also inhibited GUS production whereas alanine had no effect. Immunodetection of ACP1 secreted by cells exposed to various amino-acid-supplemented culture media confirmed these results, and also revealed an inhibitory effect for methionine, albeit less than that of cysteine (Fig. 1b). These results demonstrate that availability of sulphur to *B. cinerea* influences the activity of the *ACP1* promoter, and that sulphur-containing amino acids act similarly to sulphate.

**Nitrogen regulation of *ACP1* appears unconventional**

GUS production was also evident in the fungal cells (Fig. 2a) when the growth medium contained sulphur but lacked nitrogen, but this production was lower than that observed in the absence of sulphur. Glutamine and glutamate, the first and second established preferred nitrogen sources for fungi (Marzluf, 1997a), repressed GUS production as ammonia did. Surprisingly, however, two non-preferred nitrogen sources (proline and alanine) also caused total repression, and only small differences between these various amino acids could be seen when the
prepared and GUS activity was measured (means ± SD of three independent experiments). The mycelium was collected, cell extracts were prepared, and GUS activity was recorded (means ± SD of three independent experiments). (b) Molecular exploration of the ACP1 promoter regulation. Four genetic constructs made of various versions of the ACP1 promoter driving the GUS gene were introduced into the B. cinerea parental strain B05.10 via A. tumefaciens-mediated transformation. Two consisted of truncated versions containing the single CYS3 proximal consensus binding site (dot), or both this site and its AREA counterpart (arrows representing the two closely spaced 5′-GATA sequences necessary for AREA binding). The two other constructs consisted of the 0.6 kb promoter into which point mutations (asterisks) were introduced to modify either the CYS3 or AREA consensus binding sites according to reports demonstrating the negative impact of the chosen mutations on gene regulation by these factors (see Methods). The first construct (mutant cys3*) drove a GUS production that responded to nitrogen limitation, but no longer to sulphur limitation (Fig. 2b), indicating that sulphur regulation occurs through the targeted CYS3 binding site. In contrast, the second construct (mutant areA*) still drove a GUS production that responded to both nitrogen and sulphur limitation. This suggests that nitrogen regulation of ACP1 relies on a 200 bp region of its promoter that contains the single proximal AREA binding site, but that the regulation would not proceed through this site. GUS production measured in the transformants carrying the areA* mutation was higher than in the other transformants, probably indicating different insertions of the four genetic constructs in the genome of each transformant, and, as a consequence, different levels of expression.

Fig. 2. Nitrogen regulation of ACP1. (a) Following growth in rich medium, B. cinerea strain T3 was transferred for 15 h or 9 h to nitrogen-free minimal medium buffered to pH 4, containing 1 mM MgSO4 and further supplemented as indicated. The mycelium was collected, cell extracts were prepared, and GUS activity was measured (means ± SD of three independent experiments). (b) Molecular exploration of the ACP1 promoter regulation. Four genetic constructs made of various versions of the ACP1 promoter driving the GUS gene were introduced into the B. cinerea parental strain B05.10 via A. tumefaciens-mediated transformation. Two consisted of truncated versions containing the single CYS3 proximal consensus binding site (dot), or both this site and its AREA counterpart (arrows representing the two closely spaced 5′-GATA sequences necessary for AREA binding). The two other constructs consisted of the 0.6 kb promoter into which point mutations (asterisks) were introduced to modify either the CYS3 or AREA binding sites. Transformants carrying each construct – ‘truncation 1’ (two isolates), ‘truncation 2’ (two isolates), ‘cys3*’ (three isolates) and ‘areA*’ (three isolates) – were grown on rich medium and then transferred for 15 h to minimal medium buffered to pH 4 and supplemented as indicated. Cell extracts were prepared and GUS activity was measured (means ± SD: truncations 1 and 2, two independent experiments; cys3* and areA*, three independent experiments).

Two PLD inhibitors affect the expression of the ACP1 gene

While exploring the mechanism of ACP1 regulation by nitrogen, we discovered that AEBSF [4-(2-aminoethyl)benzenesulphonyl fluoride] interfered with the expression of that gene. AEBSF had no effect on the activity of the GUS enzyme in vitro (data not shown), but it abolished GUS production when added to cell cultures (Fig. 3a). Identical results were obtained when the cells were continuously exposed to AEBSF (15 h), or treated for only 1 h and then transferred to control medium (14 h) (Fig. 3a). AEBSF also caused a drop in the amount of ACP1 protein secreted by the fungus, while not affecting other secreted lytic enzymes such as polygalacturonases (Fig. 3b). Known targets of AEBSF are serine proteases and PLDs (Andrews et al., 2000), and serine proteases are secreted by both S. sclerotiorum (Billon-Grand et al., 2002) and B. cinerea (G. Billon-Grand, personal communication). However from other amino acids. As this did not strongly support glutamine being the real effector in nitrogen repression (Scazzocchio, 2000), whether ACP1 was subject or not to conventional nitrogen regulation via AREA became an issue. We generated two genetic constructs in which truncated versions of the ACP1 promoter drove the GUS gene. The first truncation resulted in a 0.6 kb promoter that kept the single proximal consensus binding sites for both the nitrogen (AREA) and sulphur (CYS3) regulators. The second truncation removed an extra 0.2 kb, eliminating the AREA binding site. As shown in Fig. 2(b), and despite the weaker strength of the shorter promoters, both versions of the promoter led to minimal GUS production in the presence of both nitrogen and sulphur, and increased production in the absence of sulphur. By contrast, this production was not increased in the absence of nitrogen in cells carrying the 0.4 kb version of the promoter. To strengthen this result, we next targeted the 0.6 kb promoter through site-directed mutagenesis, and specifically modified the CYS3 or AREA consensus binding sites according to reports demonstrating the negative impact of the chosen mutations on gene regulation by these factors (see Methods). The first construct (mutant cys3*) drove a GUS production that responded to nitrogen limitation, but no longer to sulphur limitation (Fig. 2b), indicating that sulphur regulation occurs through the targeted CYS3 binding site. In contrast, the second construct (mutant areA*) still drove a GUS production that responded to both nitrogen and sulphur limitation. This suggests that nitrogen regulation of ACP1 relies on a 200 bp region of its promoter that contains the single proximal AREA binding site, but that the regulation would not proceed through this site. GUS production measured in the transformants carrying the areA* mutation was higher than in the other transformants, probably indicating different insertions of the four genetic constructs in the genome of each transformant, and, as a consequence, different levels of expression.
unlikely was the involvement of these secreted enzymes made by the irreversible effect of 1 h treatment with AEBSF, this was tested. As AEBSF retains its inhibitory action on proteases when coupled to beads (Ohkubo et al., 1994), we used AEBSF coupled to Sepharose beads as a complex whose size would be incompatible with crossing the fungal cell wall. We observed no inhibition of GUS production (Fig. 3c). We also used polyclonal anti-serine protease antibodies with inhibitory effect on the secreted protease of B. cinerea in vitro (G. Billon-Grand, personal communication), and again no inhibition of GUS production was observed (data not shown). AEBSF therefore seemed not to target a secreted serine protease. To discriminate between AEBSF targeting a serine protease or a PLD inside the fungus cell, we used curcumin, another known PLD inhibitor that does not act on serine proteases (Yamamoto et al., 1997). The low solubility of curcumin prevented its use at high concentration, but 1 mM caused partial inhibition of GUS production (Fig. 3c). Altogether, these results suggested that AEBSF could mimic the presence of ammonium in the growth medium and that it would affect the production of ACP1 by acting upon an intracellular PLD.

A PE-specific PLD activity is detected under conditions of ACP1 expression

If a PLD were involved in the regulation of ACP1 expression, we reasoned that its activity would be modulated by the same environmental conditions as those which affect that expression. B. cinerea was grown in rich medium and then exposed to both nitrogen and sulphur starvation conditions. Fungal cell extracts were prepared and fed radioactive PC, and the production of PA was measured. No such production was observed (data not shown). PE was then used instead of PC and a PA-producing activity was measured when calcium was present in the reaction (Fig. 4a). In comparison, cells supplied with nitrogen and sulphur exhibited half of this activity, and cells exposed to starvation conditions in the presence of AEBSF exhibited the same half-reduced level of activity (Fig. 4a). In parallel, AEBSF caused 44% inhibition of PA production when added in vitro to a PLD reaction performed with extracts originating from control cells (exposed to starvation conditions; Fig. 4b). We tested the capacity of the enzyme to catalyse a transphosphatidylation

![Diagram](https://via.placeholder.com/150)

**Fig. 3.** Inhibition of ACP1 expression by PLD inhibitors. (a) Following growth in rich medium, B. cinerea strain T3 was transferred to nitrogen-free minimal medium buffered to pH 4 and supplemented with AEBSF; the drug was either kept in the medium for 15 h or removed after 1 h (AEBSF-1 h) by replacing the medium with minimal medium buffered to pH 4. The mycelium was collected, cell extracts were prepared, and GUS activity was recorded (means ± SD of three independent experiments). (b) Western blot analysis of secreted proteins using anti-ACP1 (top) or anti-polygalacturonase (bottom) polyclonal antibodies. (c) Effect of curcumin and immobilized AEBSF. The fungus was treated as in (a) and GUS activity was monitored. The chemical bond between AEBSF and the Sepharose beads is unstable below pH 5; therefore the experiment had to be performed at this pH rather than pH 4. The difference in GUS production observed in the experiments arises from the lower activity of the ACP1 promoter at pH 5 than at pH 4.

![Table](https://via.placeholder.com/150)

**Fig. 4.** AEBSF-sensitive PA production correlates with the nutrient availability to the cell. Following growth on rich medium, the B. cinerea parental strain B05.10 was transferred for 15 h to minimal medium buffered to pH 4 and lacking or containing sulphur (1 mM) and nitrogen (50 mM). AEBSF was added as indicated. The mycelium was collected and cell extracts were prepared to perform phospholipase activity assays. (a) Radioanalysis of PA production from radioactive PE after TLC; the quantitative results of two independent reactions are shown in the table below. (b) Inhibition of PA production by AEBSF in extracts prepared from cells grown in the absence of sulphur and nitrogen; AEBSF was directly added to the in vitro assay; means ± SD of three independent reactions are presented.
reaction by adding butan-1-ol to the assay, and we found that this reaction did not occur (data not shown). These results indicate that cells deprived of nitrogen and sulphur respond by doubling their production of PA via an AEBSF-sensitive PE-specific PLD.

**The PE-specific PLD activity is modulated by both sulphur and nitrogen**

Having shown that sulphur and nitrogen together act upon the ACP1 promoter and modulate a PLD activity, we next investigated the effect of these nutrients individually. The fungus was grown in rich medium and then deprived of sulphate. Almost twofold higher PE-specific PLD activity was measured in these cells when compared to cells supplied with sulphate, cysteine or AEBSF (Fig. 5a). Furthermore, cysteine caused a 40 % inhibition of PA production in vitro whereas alanine had no effect (Fig. 5b). Since this inhibition could have been attributable to the reducing power of cysteine, DTT was tested at the same concentration; it showed no effect. Fungal cells grown in rich medium and then deprived of nitrogen exhibited a slightly (20 %) higher PLD activity when compared to cells supplied with ammonia or AEBSF (Fig. 5c). Neither glutamine nor alanine caused a significant reduction in PLD activity (data not shown), and these amino acids had no effect when added to the enzyme assay in vitro (Fig. 5d). Altogether, these results demonstrate that a correlation exists between the availability of sulphur or nitrogen to the cell and a decrease in the conversion of PE into PA. However, the two nutrients trigger a response of different intensity.

**The single putative PLD-encoding gene in B. cinerea seems not to be involved in ACP1 or PA production**

In order to validate our results genetically, we searched the B. cinerea EST database COGEM for a DNA fragment showing homology to a PLD gene and we found one annotated EST (number W0AA034ZA12C1). In conjunction, we also searched the now available genome sequence of this fungus (Broad Institute) and found only one gene showing homology to any known PLD genes in other organisms; this gene corresponds to the EST cited above. Oligonucleotides were designed to target the EST DNA sequence and to amplify the related DNA fragment from B. cinerea. This fragment was then used to create a PLD-RNAi construct (Fig. 6a) that was introduced into B. cinerea strain BO5.10 by A. tumefaciens-mediated transformation (Rolland et al., 2003). Several transformants carrying the RNAi construct were obtained. They all grew identically to the parental strain and they all achieved normal asexual reproduction (data not shown). As expected, the ectopic integration of the construct into the fungus genome (Rolland et al., 2003) resulted in different levels of interference (90 % in transformant R1) that were detected by quantitative real-time PCR in different transformants (Fig. 6b). However, these interferences had little or no effect on ACP1 transcription (Fig. 6c) and did not cause a reduction in PE-specific PLD activity (Fig. 6d). Unless the very small level of expression of the PLD gene in transformant R1 was sufficient to ensure wild-type level of PA production, this result rather suggests that the targeted gene does not encode the PLD responsible for the nutrient-modulated PA production observed in this study. Interestingly, this result also suggests that the AEBSF-insensitive production of PA observed in this study probably does not originate from the activity of a standard PLD, as this activity too would have been reduced in the transformants.

**DISCUSSION**

ACP1 is a protease secreted by the necrotrophic fungi S. sclerotiorum and B. cinerea during plant infection (Billon-Grand et al., 2002) or upon exposure to plant tissues (Poussereau et al., 2001; Rolland et al., 2003). Following the demonstration that the transcription of the ACP1 gene is under the control of pH and nitrogen (Poussereau et al., 2001; Rolland et al., 2003), we establish here that it also responds to changes in sulphur availability to the cell, and that it does so through a single CYS3 binding site present in the proximal 400 bp of its promoter. As already reported for other fungal proteases (Cohen et al., 1975; Farley & Ikasari, 1992; Katz et al., 1994), ACP1 is produced in response to sulphur deprivation and this probably
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represents a way for the fungus to harvest cysteine or methionine, both organic sulphur sources, from proteins present in the environment. Our results moreover indicate that ACP1 expression is higher when the fungus lacks sulphur than when it lacks nitrogen and, conversely, a 50-fold higher nitrogen concentration than that of sulphur is required to repress transcription. The regulation of ACP1 by sulphur seems therefore to be stronger than that exerted by nitrogen. The transcriptional activators CY3 and AREA respectively mediate gene regulation by sulphur and nitrogen in fungi. The presence of each nutrient activates an unknown signalling pathway whose outcome is the removal from the nucleus, and/or the degradation, of the corresponding activator, and this leads to sulphur or nitrogen catalytic repression. This study shows that cells exposed to sulphur and/or nitrogen limitation exhibit a specific conversion of PE to PA (PC is not reactive) that is sensitive to the PLD inhibitor AEBSF both in vivo and in vitro. A direct correlation is shown in this study between this activity and that of the ACP1 promoter. Moreover, two PLD inhibitors, AEBSF and curcumin, can mimic the presence of sulphur or nitrogen in the environment of B. cinerea, as deduced from the repression of the ACP1 promoter in cells exposed to these molecules. Finally, the smaller response of the ACP1 promoter to the lack of nitrogen than to that of sulphur is mirrored by a smaller modulation of the PLD activity by nitrogen than by sulphur in vivo. Altogether, these data reveal the parallel modulation of a PE-specific PLD activity and the ACP1 promoter. The concomitant inhibition of the PLD and the ACP1 promoter by AEBSF in vivo suggests that the PLD modulation could be involved in sulphur and nitrogen repression of the ACP1 gene by turning the information relating to the presence of these nutrients into a drop in intracellular PA production. The total PLD activity measured in this study is modulated up to 50% in response to the presence or absence of sulphur and nitrogen. Such changes in PLD activity in response to stimuli have been observed in yeast (Waksman et al., 1996). In fungi, PA is produced either by PLD or by phospholipase C plus diacylglycerol kinase, and PLD alone is inhibited by AEBSF (Andrews et al., 2000). Under our experimental conditions, the maximal AEBSF-sensitive production of PA accounted for 50% of the total cellular production of PA, and the AEBSF-insensitive counterpart was unaffected in a mutant whose unique PLD-encoding gene has been repressed by RNA interference. Half the production of PA therefore probably originates from a phospholipase C plus diacylglycerol kinase activity, and this activity is not modulated by nutrients.

**Fig. 6.** Analysis of PLD RNAi transformants of B. cinerea BO5.10

(a) Plasmid used in the A. tumefaciens-mediated transformation of the fungus. The PLD gene fragments are separated by a linker made of a fragment of the gfp gene and the construct is driven by the OliC promoter. The hygromycin (hph) resistance gene is driven by the TrpC promoter and both genes lie between the left and right borders of the bacterial ‘transfer DNA’. (b, c) Quantitative measurements of the targeted PLD gene expression (b) and of the ACP1 gene (c) in the parental strain (P) and three different transformants (R1, R2 and R6). The data are from two independent experiments in which the tubulin-encoding gene (tub) was used as an internal control for gene expression. (d) PA production in the parental and transformed strains (means ± SD). The strains were grown on rich medium and then transferred for 15 h to nitrogen-free, sulphur-free minimal medium buffered to pH 4.

Sulphur catabolic repression can be triggered by mineral sources, such as sulphate, or by organic sources, such as cysteine or methionine (Marzluf, 1997b). According to the current model describing this phenomenon, the intracellular pool of cysteine could act as an intermediate in the process. The PLD activity uncovered in this study is modulated in vivo by exposure of cells to sulphate, cysteine and methionine, and it is inhibited in vitro by cysteine, whereas alanine or DTT have no effect. These data strengthen the idea that the PLD activity discovered in this study could respond to changes in sulphur availability to the cell, and they would be consistent with the current model of sulphur catabolic repression (Marzluf, 1997b). More work is however required to establish whether cysteine acts directly or indirectly on the PLD activity. Amino acids as well as ammonia lead to nitrogen catabolic repression (Marzluf, 1997a), and an intracellular pool of glutamine is proposed as an intermediate in the process (Scagazzoccio, 2000; Margelis et al., 2001). Here we have shown that amino acids and ammonia modulate the activity of the ACP1 promoter. Curiously, however, glutamine seems not to play a special role in nitrogen regulation of ACP1. When compared to other amino acids tested, it is slightly more potent, but the difference is small and the PLD activity discovered in this study is not inhibited by glutamine in vitro. We used directed
mutagenesis to further investigate nitrogen repression of ACP1, and we found that disrupting one of the tandem GATA sequences that constitute the only canonical AREA binding site present in its promoter did not affect nitrogen regulation. Although the possibility remains that AREA could function through the binding of a single GATA site (Merika & Orkin, 1993), our data indicate that the nitrogen regulatory system for ACP1 is unconventional.

Our search of the recently released B. cinerea genome revealed a single gene with significant homology to the currently known PLD genes. We used this gene to successfully develop RNA interference in B. cinerea but the diminution of this gene’s expression (up to 90%) had no effect on ACP1 expression and did not affect the PLD activity discovered in this study. Together with our demonstration that this PLD does not catalyse transphosphatidylation with short-chain alcohols as acceptors, this result suggests that this PLD could be a new member of the non-HKD family of PLDs. This family of PLDs is exemplified by the yeast PLD2 protein and, possibly, a prokaryotic PLD (Ogino et al., 2001). PLD2 (Tang et al., 2002; Waksman et al., 1997) exhibits the same biochemical characteristics as the PLD discovered in this study and, despite the accessibility of the yeast genome, its encoding gene remains elusive because its sequence is unrelated to the defined HKD family of PLD genes, whose representative in yeast is PLD1 (Hairfield et al., 2001).

In conclusion, the regulation of the B. cinerea ACP1 gene by sulphur has been demonstrated. By studying the regulation of this gene by nutrients, we have also shown a correlation between the response of this gene’s promoter to sulphur or nitrogen and the activity of a PE-specific PLD. This PLD could be a new component in the signalling pathways that underlie sensing of these nutrients and catabolic repression in fungi, and PA could hence represent a secondary messenger in this system. One possible target of PA is the TOR kinase (Fang et al., 2001), and the acknowledged role of this enzyme in nutrient signalling (Rohde & Cardenas, 2004) would now merit being explored in connection with PA, sulphur and nitrogen. Finally, the PLD uncovered in this study could belong to the recently discovered non-HKD family of PLDs, and the identification of its encoding gene is of particular interest.

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