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Identification of up-regulated genes of the hyphomycete, *Beauveria* bassiana, during infection of Leptinotarsa decemlineata

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I. Introduction

The last century has seen both the introduction of chemical insecticides into agriculture as well as its backlashes. Indeed, the intensive use of these chemicals has been shown to cause numerous deleterious effects on the environment (e.g. adverse effects on nontargeted insects, birds, fish and aquatic invertebrates) and on human safety. Agricultural chemicals may be significant contributors to various ailments including cancer, hypothalamic disorders and neurological disorders such as Parkinson disease.

Moreover, numerous pests developed resistance to chemical insecticides, including the Colorado potato beetle, *Leptinotarsa decemlineata* (*L. decemlineata*), which is an economically important pest of potato and other solanaceous (i.e. tomato, aubergine) throughout the world (Figure 1A). The Colorado potato beetle developed high resistance against chemical pesticides in Canada, Germany, but also the cotton and tobacco white flies in Pakistan, Israel, South-western U.S.A., the mosquitoes in California and the diamondback cabbage in Tasmania.

These observations have provided a strong impetus for the development of microbial control agents for use in integrated control of insect pests. Several microorganisms are currently under consideration as control agents of insects, including viruses, bacteria, protozoa and fungi.





(A) Three stages of development of *L. decemlineata*; the larval and adult stages being responsible for the devastation of the potato cultures. (B) Two adults after the complete infection process of *B. bassiana*.

1. The hyphomycete Beauveria bassiana

1.1. The organism

Fungi are a phylogenetically diverse group of microorganisms that are all heteroptrophic eukaryotes, unicellular (i.e. yeasts) or hyphal (i.e. filamentous), and reproduce by sexual and/or asexual spores.

As most entomogenous of terrestrial insect species, *Beauveria bassiana* Basalmo (Vuillemin) belongs to the class of the Hyphomycetes (division: Deuteromycetes). The members of this class are characterized by mycelial forms, that bear asexual resting spores, termed "conidia". These spores are resistant to environmental extremes and are the infective stage of the fungal life cycle. The most common entomopathogenic genera of hyphomycetous fungi are *Aspergillus, Beauveria, Culicinomyces, Hirsutella, Metarhizium, Nomuraea, Paecilomyces, Tolypocladium* and *Verticillium*. Each of these taxa is defined by its characteristic conidiogenesis.

B. bassiana is found naturally on some plants and in the soil throughout the world. It is known as the white muscardine fungus because infected insect larvae eventually turn white or grey. In the soil, it occurs as a saprophyte.

1.2. The hosts

Some entomopathogenic fungi have restricted host ranges, i.e. *Aschersonia aleyrodis* infects only scale insect and whiteflies, while other fungi have a broad host range with individual isolates being more specific, i.e. *Metarhizium anisopliae* (*M. anisopliae*) and *B. bassiana*.

The *B. bassiana* strain 252 (ARSEF), used in this study, is highly virulent against the Colorado potato beetle, *L. decemlineata* (100 % mortality in about 5 days in laboratory conditions) (Figure 1B) but is not pathogenic against the wax moth and the cockhafer

(between 0 and 3 % mortality), the *B. bassiana* strain 447 is highly virulent aginst ants, and *B. bassiana* CS-1 is used as a biological control agent of the diamond back moth.

B. bassiana has already been used for control of the Colorado potato beetle in the former UdSSR (Feng et al., 1994), and more recently in the USA (Wraight & Ramos, 2001). *B. bassiana* strain 447 is actually approved for indoor use as an insecticide in bait stations to control individual fire ants and other ants (but not colonies) in USA (U.S. Environmental Protection Agency).

Besides these two examples, the exhaustive list of *B. bassiana* hosts includes important pests as whiteflies, thrips, aphids, grasshoppers, termites, Mexican bean beetle, Japenese beetle, boll weevil, cereal leaf beetle, bark beetles, European corn borer, codling moth, lygus bug, and chinch bug (Figure 2).

The tse-tse fly, vector of the sleeping sickness, and the kissing bug, vector of the Chagas disease in South of America, are also susceptible to some *B*. *bassiana* isolates.



Tse-tse fly



Imported fire ant

С

A

B



Grasshopper



Kissing bug



Termite



European corn borer



Western flower thrip

Figure 2. Insect hosts of *B.bassiana*, chosen examples.

(A) Insect hosts of medical importance with the Tse-tse fly, vector of the sleeping sickness, and the kissing bug, vector of the Chagas disease. (B) The two main urban insect hosts. (3) Insects host of agricultural importance.

2. Pathogenesis of Hyphomycetes

Among entomopathogens, only fungi have acquired the ability to invade insects through the external cuticle. Indeed, the way of infection of other insect-pathogenic microorganisms, such as virus, bacteria, protozoan, nematodes, is limited to their ingestion by the host.

2.1. Prepenetration events

Conidia contact insects passively with the aid of agents as wind and water. Conidia of most insect-pathogenic fungi adhere strongly to the insect cuticle. The mechanism of attachment of the spores is thought to be due to non-specific hydrophobic forces. But more specific recognition systems (e.g. glycoprotein, enzyme) were detected on conidia surface and may be involved in the attachment of conidia (Boucias and Pendland, 1991). For example, Grula et al. (1984) reported hemagglutinins on the conidial surface of *B. bassiana*, which were inhibited by various haptene sugars (i.e. glucose, glucosamine, and N-acetyl-glucosamine); incubating *B. bassiana* conidia with these hapten sugars reduced their affinity to insect larvae. In *M. anisopliae*, adhesion of the spores is enhanced via the secretion of a mucilaginous coat during conidial hydratation (St Leger, 1993). In some strains, adhesion of the spores is host-specific (Fargues, 1984).

Once in contact with the appropriate cuticle, and under favourable conditions (i.e. nutrients, moisture, temperature), the conidium germinates and may form some infection structures (e.g. germ tube swelling, appressorium or extracellular sheath) from which the penetration hypha is formed (Figure 3).

In vitro germination of deuteromycete insect-pathogenic fungi with large host range as *B. bassiana* occurs in response to non-specific sources of carbon and/or nitrogen (Smith and Grula, 1981; St Leger et al. 1986a) (St Leger 1986b). However, the ability to utilize

the lipids that make up the outer layer of the cuticle may be fundamental for pathogenesis. Nutrient-mediated germination of *B. bassiana* conidia on the integument of the bark beetle, has been shown by Hunt et al. (1984). They also demonstrated that fatty acids and lipids influences germination (Hunt, 1986). Entomopathogenic fungi with restricted host ranges have more specific requirement for germination. For example, *N. rileyi*, which primarily infects lepidopteran responds to diacylglycerols and polar lipids (Boucias and Pendland, 1984). Successful germination presupposes also a tolerance of potentially toxic compounds in the outer layers of the cuticle (e.g. short-chain fatty acids, phenols) (St Leger et al., 1990) (Figure 3), and of antagonism from the saprophytic flora on insect cuticle (Charnley, 1989).

Many insect pathogenic fungi (e.g. *M. anisopliae, Paecilomyces farinosus, Erynia radicans, Nomuraea rileyi*) have been reported to secrete mucilage during germ tube and/or appressorial formation. In many cases, the secretion of this mucilage is though to bind the appressorial cells to the cuticle surface. This mucilage is hygroscopic and may also create a favourable environment for the exocellular enzymes released by these structures (Boucias and Pendland, 1991).



Figure 3. Structure and composition of insect cuticle, and mode of penetration of entomopathogenic fungi. Adapted from Clarkson, Charnley (1996), and Charnley, St. Leger (1991).

2.2. Penetration into the host

In *M. anisopliae* the invasion process requires the formation on an infection structure, called appressorium. Contrary to the germination, the differentiation of the appressorium has specific nutrients requirement. Differentiation is stimulated by low levels of nitrogenous compounds and, in many isolates, is catabolite repressed by carbon compounds. In presence of high level of nitrogen and carbon sources, the germ-tube continues to elongate and forms a hyphal mat (St Leger et al., 1989, 1992). In addition to nutrition, surface topography influences the formation of an appressorium. Upon contact with flexible cuticle, germ tubes produces an appressorial cell which gave raise to a penetration tube (Wraight et al., 1990). *B. bassiana* does not produce appressorial structure but penetrate the insect via an infection germ tube. Pekrul and Grula (1979) reported that conidia on heavily sclerotized regions (head capsule) produce germ tubes that grow over the surface until they contact flexible interscleral regions. The physical and chemical stimuli that responsible for this directional tropism are unknown.

Germlings of *M. anisopliae* possess cyclase, tyrosine protein kinase, phosphoprotein phosphatase, and an ATPase that act to change second messenger levels (cAMP or Ca²⁺) or are themselves activated by second messengers to trigger differentiation (St Leger et al., 1989c,d, 1990a,b,c).

Insect-pathogenic fungi enter the cuticle by the infection peg formed underneath the appressorial cells (e.g. *M. anisopliae*) or directly by germ tubes (e.g. *B. bassiana*). In *M. anisopliae*, the wax layer of the wireworm cuticle beneath the appressoria disappears, and in *B. bassiana* some circular holes are present at the point of entry of the germ tubes into *Heliothis zea* cuticle (Zacharuk, 1970a; Pekrul and Grula, 1979). While penetration of the epicuticle occurs mainly by enzymatic degradation, penetration of the procuticle involved both enzymatic degradation and the mechanical separation of the lamellae (Goettel et al., 1989a). Indeed, ultrastructural studies have shown that hyphae tend to grow between the cuticular lamellae of the exocuticle mechanically cleaving their way along lines of least resistance (Zacharuk, 1970b) (Figure 3).

2.3. Extracellular enzymes

Many pathogens can produce a range of cuticle-degrading enzymes corresponding to the different polymers of the insect cuticle, protein, chitin and lipids (St Leger et al., 1986; Charnley and St Leger, 1991). When M. anisopliae, B. bassiana and V. lecanii are grown on ground cuticle in liquid medium, they secrete sequentially esterase and proteolytic enzymes (endoprotease, aminopeptidase, and carboxypeptidase) (< 24h), followed by N-acetylglucosaminidase and finally chitinase and lipase (3-5 days later). The order of appearance of the enzymes is supported by the sequence of cuticle constituents solubilized into the culture medium. Chitinase is an inducible enzyme, and in cuticle, the chitin is masked by protein; so the late appearance of chitinase is presumed to be the result of the induction by the chitin after the degradation of the cuticle proteins. The late detection of lipase seems to be due to the fact that the enzyme is generally cell bound in young culture (St Leger et al., 1986a,b, c). Among the first enzymes produced by *M. anisopliae* on exised blowfly wings are endoproteases and aminopeptidases that are produced at the same time than the formation of the appressoria. N-acetylglucosaminidase is produced slowly compared with proteolytic enzymes.

The major protein produced during appressorium development in *M. anisopliae in vitro* is Pr1. Pr1 is a serine endoprotease which possess a broad primary specificity for amino acids with a hydrophobic side group at the second carbon atom (e.g. phenylalanine, methionine and alanine) but also possess a secondary specificity for extended hydrophobic peptide chains with the active site recognizing at least five subsite residues. Pr1 is also a good general protease with activity against a range of proteins (casein, elastin, bovin serum albumin, collagen) and insect cuticle (St Leger et al., 1987b). Binding of Pr1 to negatively charged groups of the cuticle is linked to its basic nature. After the adsorption, the active site comes into contact with susceptible peptide bonds and then the solubilized peptides are further degraded until a chain length of about five (St Leger et al., 1986c).

Pr1 is produced rapidly (< 2h) in culture by carbon and nitrogen derepression alone. Extracellular levels of Pr1 are enhanced in cultures supplemented with insect cuticle or other insolubles polymers (e.g. cellulose) when in amount not sufficient to produce catabolite repression. Addition of more readily utilized metabolites (glucose, alanine) repressed extracellular protease production. Thus, the production of Pr1 is constitutive but repressible (St Leger et al., 1988a). Similarly, the addition of readily utilizable metabolites during growth of *M. anisopliae* on insect cuticle, prevent the synthesis of Pr1 and penetration of the fungus. Nutrient starvation is therefore likely to be a key environmental signal for the switch from a saprophytic to a pathogenic mode of growth, possibly after depletion of nutrients on the insect surface. Synthesis of Pr1 during maturation of appressoria and production of penetration pegs exceeds largely the synthesis of others proteins. Simultaneous application of turkey egg white inhibitor and conidia significantly delayed mortality of *Manduca sexta* larvae compared to larvae inoculated with conidia, supporting the importance of Pr1 in penetration (St Leger et al., 1989b). Studies with inhibitors of some protein, RNA, DNA synthesis in M. anisopliae indicates that the control of enzyme production is at the level of transcription as it is sensitive to actinomycin D and 8-azaguanine. In vitro translation of poly(A) RNA isolated from appressoria using a rabbit reticulocyte system confirmed *de novo* synthesis of Pr1-specific mRNA during differentiation (St Leger et al., 1991a).

The Pr1 cDNA has been cloned and revealed that PR1 is synthetized as a large precursor (52 kDa) containing a signal peptide, a propeptide and a mature 30 kDa protein. Pulselabelling experiment demonstrated that about seven minutes were required for [35 S]methionine to be processed into extracellular Pr1. Isolates of four other entomopathogenic fungi including *B. bassiana* (*V. lecanii, P. farinosus and Tolypocladium niveum*) produced Pr1-type enzymes during nutrient deprivation. A pr1-like gene in *B. bassiana* has been cloned and sequenced (Joshi et al., 1995). The cDNA sequence revealed that *B. bassiana* Pr1 is also synthetized as a large precursor (Mr 37 460) containing a signal peptide, a propeptide and the mature protein predicted to have a Mr of 27 kDa.

The promoter of the gene encoding PR1 of *M. anisopliae* contains putative binding sites for regulatory proteins similar to carbon-catabolite repressor (CREA) and nitrogenmetabolite regulator (AREA) of *Aspergillus nidulans* and to the eukaryotic CREB (Clarkson and Charnley, 1996).

2.4. Production of toxins

When the fungus reaches the haemolymph of the insects, it may grow as yeast-like blastospores or hyphal bodies, rather than in a mycelial form. This may facilitate the dispersion and colonization of the haemocoel, optimise nutrient assimilation and dispate the efforts of defense immune system. This immune response of the insect may be humoral (e.g. phenoloxidase, lectins), and/or cellular (e.g. phagocytosis, encapsulation).

Before the fungus can proliferate in the haemocoele, it must generally overcome the insect defense response via the production of toxins. *B. bassiana* produces various toxic compounds including beauvericin, bassianolide and oosporein, and *M. anisopliae* produces numerous cyclic depsipeptides, termed destruxins, which lead to an immunosuppression or a tetanic paralysis (Bradfish et al., 1990). The death of the insect host may result from a combination of actions such as depletion of nutrients, physical obstruction or the organ invasion and toxicosis.

Once the fungus has killed its host, the fungus grows saprophytically within the host, and metabolites that they produced may be involved in the competitive exclusion of competing microorganisms from the cadaver. Soon after the host death and under favourable conditions, hyphae grow back out through the softer part of the cuticle and produce millions of infective spores that are released.

Epizootics of hyphomycetous fungi can occur in field populations of insects and can be important in natural regulation of pest populations. An epizootic is the result of a complex interactions between the host, the pathogen and the environment over time; but relatively few is known about fungal epizootics for most fungal species.

3. Mycoinsecticides

3.1. Main advantages and constraints

Main advantages of entomopathogenic fungi over chemical pesticides are their significantly higher host specificity, the reduction of hazards (e.g. contamination of the water system), and the theoretically impossibility for the insects to develop resistance, as fungi use simultaneously several mode of actions, and as a "living pesticide" is subjected to adaptation too.

However, there are also some constraints to the use of insect-pathogenic fungi. The time delay to reach the onset of mortality is only 3 to 4 days for chemicals. The use of fungi under optimal conditions leads also to the death of an insect between 3 to 5 days (from the time of application), however, in field environment, death can take substantially longer (Figure 4). This is generally due to conditions of suboptimal environment. For example, disease progression by *B. bassiana* is arrested in Mormon crickets incubated at 37° C, but resumed when the crickets are transferred to 25° C, suggesting that the mycosis can be delayed in field by high temperatures and thermoregulation. The relative humidity is also an important factor for germination of the spores, also different formulations of spore suspension (e.g. oil, emulsion) are under investigations.

Thus, the use of insect-pathogenic fungi has not always provided a consistent suppression of insect pests. However, in Greenhouse, where the environmental conditions can be controlled, several taxa, i.e. *B. bassiana*, *M. anisopliae*, *V. lecanii*, have shown good results in suppression of insect pests, and *B. bassiana* (Botanigard®) has been recently registered against an array of greenhouse pests.



Figure 4. Mortality of the Mormon crickets inoculated with *B. bassiana* conidia.(A)Cumulative mortality among fifth-instar Mormon crickets in a bioassey of *Beauveria bassiana*.(B) Cumulative mortality among adult Mormon crickets treated with *Beauveria bassiana* in the laboratory and reared in the laboratory or in field cages (Streett and Woods).

3.2. Field application of B. bassiana for managing L. decemlineata

As mentioned previously, the Colorado potato beetle, *L. decemlineata*, developed resistance against several insecticides; this promoted sudies about an alternative method to control this pest, including the use of *B. bassiana*.

Poprawski et al. (1997) observed that foliar application of *B. bassiana* early in the season reduced the densities of older larvae, which was then 10 larvae per plant, against 21 larvae per plant when an insecticide was used and 41 larvae per plant for the control treatment. Furthermore, the predatory Heteroptera and other non-target insects showed a good survival compared to the insecticides treatment. In North of America, the use of *B. bassiana* in conjunction with the insect-pathogenic bacterium *Bacillus thurigensis* (*B. thurigensis*) led to a yearly decreased of beetles per plot compared to others treatments (Drummond and Groden, 1996). Several insectides have also been tested in combination with *B. bassiana* and some showed an additive effect (Anderson et al., 1997).

Applications of *B. bassiana* on the foliage or directly in the soil led to the killing of the over-wintering adults in the soil and, thus reduced populations of emerging adults (Watt and Lebrun, 1984; Cantwell et al., 1986; Anderson et al., 1988).

However, if these cited studies have shown significant suppression of the Colorado potato beetle, others revealed a low or absence of efficacy using *B. bassiana* in field trials. Several reasons have been suggested to explain this low efficacity in some field trials, but only limited research have focused on the constraints on disease development. The presence of a microclimate could be an important factor for efficacy (Lacey et al., 1999a).

3. 3. Prospects for strain improvement of entomopathogenic fungi

As entomopathogenic fungi have a very slow speed of kill compared to the chemical and quite variable efficacy in natural environment, any consideration of the suitability of a fungus for commercial purposes leads to the possibility of improving its performance.

The characteristics that might benefit from genetic improvement are:

(1) an improvement of the virulence (speed of kill), via modification of receptors that detect the presence of the host, of enzymes that facilitate penetration of the host, of gene products that inactivate host defenses, of toxins that are required for disease symptoms;

(2) a modification of their host specificities, i.e. with manipulation of genes responsible of host adhesion, of the exploitation of nutrients, humidity, specific recognition factors on the cuticle surface, of resistance to inhibitory compounds;

(3) a reduction of the inoculum via increasing the production of cuticle-degrading enzymes (St Leger et al., 1994, 1996b);

(4) the alteration of persistence, e.g. via the introduction of spores-killing factors, double-strand RNA, virus (Koltin et al., 1987);

(5) a tolerance to environmental constraints, especially a resistance to dessication and temperature extremes;

(6) a resistance to certain fungicides for the use of fungi as part as an integrated pest management programme.

However, to date, little is known concerning the biochemical or molecular basis of most of the mentioned characteristics that could be subjected to genetic engineering.

4. PhD research project

The project consisted in the identification of up-regulated genes of *B. bassiana* (strain ARSEF 252) during the infection process of the Colorado potato beetle, *L. decemlineata*. This study tended to focus especially on the early steps of the pathogenic process when the fungi germinate on the insect cuticle and begin its degradation by the production of numerous extracellular enzymes. Two different approaches have been used, with: (1) *B. bassiana* in an artificial context of infection with its culture in presence of ground cuticle of the Colorado potato beetle, and, (2) *B. bassiana* in a natural context of infection with the inoculation of *L. decemlineata* axenic larvae.

The chosen method was the cDNA representational difference analysis (cDNA RDA) corresponding to a process of subtraction coupled to an amplification, leaving only the differences between the two compared mRNA population representations (Figure 5).



Figure 5. Schematic diagram of the cDNA representational difference analysis.

Hatched boxes show the oligonucleotides used to generate difference products. The process is shown up to the first difference product. To generate the second and third difference products, the products of the previous round are reintroduced, into the scheme at the tester stage, in the proportions indicated in Materials and Methods (adapted from Hubank and Schatz (1994)).

The main advantages of the cDNA RDA are the higher probability to identify part of the coding sequence of a gene, and the lower rate of false positive compared to an differential-display approach.

The attempt to study gene expression of *B. bassiana* in a natural context of infection failed, probably due to the method, which might be not adapted to this particular study. However, several genes specifically expressed or over-expressed by *B. bassiana*, in the established artificial context of infection, have been identified. These genes encode putative hydrolases, transporters, a probable metabolic enzyme, a presumed V-ATPase and two putative mucin-like proteins that could play an important role in the pathogenic process of *B. bassiana*.

II. Results

The project of this PhD consisted in the identification of the virulence factors of the *B*. *bassiana* strain ARSEF 282, highly virulent toward the Colorado potato beetle, *L*. *decemlineata*. The first part of the research was performed in an artificial context of infection, with *B*. *bassiana* grown in a medium containing ground cuticle of the Colorado potato beetle to induce the pathogenic process. The second part of the research was performed in a natural context of infection, with *B*. *bassiana* grown in a medium containing ground cuticle of the research was performed in a natural context of infection, with *B*. *bassiana* grown in a medium containing grown in axenic larvae of the Colorado potato beetle. Both studies were performed at the gene expression level using the method termed cDNA representational difference analysis (cDNA RDA).

1. Identification of up-regulated genes of B. bassiana in cuticle medium by cDNA RDA

1. 1. Establishment of the procedure to create an artificial context of infection

The artificial context of infection by *B. bassiana* was achieved via transfer of the fungus in different media, each medium containing less nutrients than the previous one. The final medium contained additionally the ground cuticle of the Colorado beetle to induce the infectious process of *B. bassiana*. This transfer experiment coupled a large production of germinated spores and a progressive decrease of the concentration of the carbon and nitrogen sources in the successive media (Figure 23).

The production of germlings was chosen because penetration of the host cuticle occurs via the germ tube of the conidia that germinate on the insect surface.

The transfer experiments began with cultures in two rich media (containing 2 % sucrose, and then 1% sucrose, 0.2 % sodium nitrate) to amplify rapidly the amount of spores, and thus, isolate enough RNA for the cDNA RDA.

The last cultures were performed in the basal salts media 2 very poor in carbon and nitrogen sources (0.1 % sucrose, 0.02 % sodium nitrate) supplemented, or not, with 0.5 % or 1 % (w/v) cuticle from adult Colorado potato beetle.

In the first case, the presence of the cuticle in the culture medium of the germinated spores, via its intrinsic chemical and physical signals, should induced the pathogenic process of *B. bassiana*. The mRNA population isolated from this culture was then used to form tester representation in the cDNA RDA experiment.

In the second case, the last culture was performed in the same basal salts medium containing 0.1 % sucrose and 0.02 % sodium nitrate, but not supplemented with the cuticle of the Colorado potato beetle. The mRNA population isolated from this culture was then used to form the driver representation in the cDNA RDA, population of representated mRNA that is substracted from the tester population (Figure 5).

The last step of the transfer experiment was the culture of the germinated spores in a basal salts medium, very poor in carbon and nitrogen sources, supplemented or not with the cuticle of the insect. Both cultures were performed in the same medium as a basis, thus, the differences between the two mRNA populations used for the RDA could only be linked to the presence, or not, of the cuticle. This medium contained a low amount of nutrients to avoid a too big stress of the fungal cells transferred in this medium without cuticle.

However this carbon and nitrogen source concentration should be maintained at a low level to avoid that the amount of nutrients in the medium supplemented in cuticle represses the induction of the pathogenic process; indeed the entomopathogenic fungus *M. anisopliae* was shown to infect its host only when it need to establish a nutritional relationship with it.

Between the cultures of *B*. *bassiana* in the relatively rich medium (1 % sucrose, 0.2 % sodium nitrate) and the cultures in the poor medium (basal salts medium 2: 0.1 % sucrose, 0.02 % sodium nitrate), *B*. *bassiana* was grown in a medium containing 0.2 % sucrose and 0.05 % sodium nitrate (basal salts medium 1). This intermediate culture was realized to avoid a too important stress of the cells by their direct transfer from a

relatively rich medium into a very poor medium, and in parallel to favour the germination of the spores, spores remaining dormant under unfavourable environmental conditions.

1.1.1. Impact of the culture conditions on *B. bassiana* at the cellular level

The two first steps of the transfer experiment, (culture on YPS plates and culture in complete medium, (Figure 6 B), permit a rapid amplification of the number of spores, and their relative homogenization in term of viability. The spores were then transferred into the basal salts medium 1 (0.2 % sucrose and 0.05 % sodium nitrate). This medium allowed 18-24 % of the spores to germinate within 23 h (Figure 6 A). At this time point, most of the germlings were harbouring two short germ tubes, one of each side of the spores. The germ tubes of few germinated spores were longer and already produced few lateral branches.

After the germination of the spores in the basal salts medium 1 (0.2 % sucrose, 0.05 % sodium nitrate), the germlings (all coming from one same culture) were transferred either into the basal salts medium 2 (0.1 % sucrose, 0.02 % sodium nitrate), or into this same basal salts medium but supplemented with the ground cuticle of the Colorado potato beetle. To estimate the difference of growth, some of these germlings were also transferred into a rich undefined medium containing 2 % sucrose.

After 3.5 h culture in the basal salts medium not supplemented with the cuticle of the Colorado potato beetle, several observations could be performed (Figure 6 C). Most of the spores had two germ tubes; generally one of these germ tubes sporulated after a short growth only. When the germ tubes developed lateral branches, these last ones sporulated very fast. The germination rate is either very low or null during this culture time.



After the 3.5 h culture in the basal salts medium 2 supplemented with cuticle (Figure 6 D), most of the germlings had two germ tubes, with one longer than the other and forming generally a unique long lateral branch. The sporulation of the germ tubes or lateral branches was very seldom. Germination of further spores seemed to occur during this culture period.

After 3.5 h culture in the rich medium (Figure 6 E), most of the germlings had two germ tubes, and most of the germ tubes had one or several lateral branches. In some cases, the lateral branches already formed secondary branches. The sporulation of the germ tubes was very seldom. The presence of swollen spores and spores with short gem tubes, proved that germination occurred during this time.

To summarize, the hyphal growth of the germlings, which had germinated in a common culture, was different in the three media. The germ tubes were growing in the three different media and producing some lateral branches. However, in the basal salts medium 2 without cuticle, the lateral branches were short and sporulated very fast; in the basal salts medium 2 with cuticle, the lateral branches were limited to one or two per germling, but were growing exponentially; finally, in the rich medium, the germ tubes had several lateral branches, about the same number than in the medium without cuticle for a similar germ tube length, but growing exponentially and making sometimes already some secondary branches.

Considering that the basal salts medium 2 supplemented with the cuticle of the insect had a nutrient concentration intermediate to the basal salts medium 2 and the rich medium, it was unexpected that the germlings generally developed just one long lateral branch (when they developed often five lateral branches, for a similar hyphal length, in the poor or rich medium). This growth characteristic seemed to be directly linked to the physical and/or chemical signals intrinsic to the cuticle and not to the nutrient source that it also formed. 1.1.2. Impact of the culture conditions on B. bassiana at the molecular level

Studies of the entomopathogenic fungus *M. anisopliae* have previously shown that its pathogenic process was linked to the production of the chymoelastase protease PR1. This subtilisin-like protease, was shown to be produced by the differentiated germlings and the infection hyphae of the fungus during the pre-penetration events, and at the beginning of the penetration of the insect (Figure 4) (St Leger et al., 1989a; St Leger et al. 1989b, Goettel et al., 1989).

As the Pr1 homologous cDNA and genomic sequence were cloned from *B. bassiana* (Joshi, et al., 1995), Western blot analysis and quantitative RT-PCR could be performed to follow Pr1 expression at the protein and RNA levels at different steps of the transfer experiment.

1.1.2.1. Western blot analysis of PR1 production

Total protein extracts were isolated from the germlings cultivated in the four different liquid media of the transfer experiment. Protein isolation from the culture in complete medium was performed 25 h after the inoculation, time allowing the conidia to form two germ tubes but no lateral branches neither spores. The cultures in the basal salts medium 1 and in the basal salts medium 2 with or without 0.5 % cuticle were strictly performed according to the procedure of the transfer experiment, and the proteins isolated after the normal incubation time, 23 h and 3.5 h, respectively. The two cultures in basal salts medium 2 with and without cuticle were performed in parallel and with the same source of germinated spores.

The Western blot analysis showed that the PR1 production was repressed in the complete medium (Figure 7).



Figure 7. Production of PR1 during the transfer experiment of *B. bassiana*.

Total protein extracts were isolated from germlings, cultivated in complete medium (CM), basalt salt medium 1 (BSM 1) and 2 (BSM 2) with or without cuticle. They were then separated on a 12.5 % SDS-PAGE gel and stained with blue Coomassie. (B) Immunoblot analysis of the same protein samples using an antisera against Pr1. The arrow indicates the mature PR1.

Twenty-three hours after the transfer of the spores from the complete medium into the first basal salts medium, the production of PR1 was detectable by the Western analysis but was still very low. A very faint band was indeed detected on the Western blot at an estimated molecular mass of about 30 kDa, which is the size of the mature protease in *M. anisopliae*.

After the 3.5 h culture of the germlings in the basal salts medium 2 with and without cuticle, the quantity of PR1 was 4-fold higher than in the basal salts medium 1.

The quantity of PR1 in the germlings cultivated in the basal salts medium 2 with the insect cuticle appeared not significantly different than in the germlings grown in the same medium without cuticle. This difference could be here estimated to be about 1.2-fold higher (density measurements).

The Western blot analysis permit to detect several other proteins, whose expression was derepressed in the basal salts medium 2. Some of these proteins appeared to be more produced in the medium supplemented with the insect cuticle than in the same medium without cuticle. A co-immunoprecipitation experiment could permit to know if one of these proteins is immunologically related to PR1 and might be the PR1 precursor. In *M*.

anisopliae, the precursor of PR1 was detected by pulse-chase analysis and found to be 11 kDa larger than the mature protease. The Western blot revealed here one faint band at about 42 kDa detectable only in the basal salts medium 2 (with or without cuticle). This protein might be the precursor of PR1 in

B. bassiana.

1.1.2.2. Analysis of Pr1 expression by quantitative RT-PCR

Total RNA isolation was performed after the cultures of the germlings in complete medium (25 h after the inoculation with spores), in basal salts medium 1 (23 h after the inoculation with the spores), and in basal salts medium 2 with or without 0.5 % cuticle (3.5 h after the inoculation with the germinated spores). The two last cited cultures without and with cuticle were performed in parallel and using the same source of germinated spores. Total RNA were also isolated from (1) spores formed in complete medium and (2) germlings grown in the rich YPS medium after their transfer from the basal salts medium 1 (as in the transfer experiment procedure).

The quantitative RT-PCR was repeated at least one time for each sample; the quantification of Pr1 expression in the basal salts medium 2 with and without cuticle was also performed with 2 independent RNA samples.

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Culture medium	СМ	BSM 0.2 %	BSM 0.1 %	CUT 0.5 %	YPS
Culture time	25 h	23 h	3.5 h	3.5 h	3.5 h
Nb of Pr1 ss cDNA mol.	565	3560	8080	4460	511

Figure 8. Quantification of Pr1 transcription at the different steps of the transfer experiment by real-time PCR.

(A) Different concentrations of the PCR product, using the primers RT-Pr1.rev and RT-Pr1.for, were used as template to establish the standard curves. (B) The first-strand cDNA synthetized from the total RNA of the different cultures was used as template for PCR amplification using the same primers as in (A).

(C) Number of molecules of the Pr1 transcripts in the different PCR reactions.

(CM) complete medium; basal salts medium 1 (BSM 1); basal salts medium 2 (BSM 2); basal salts medium with cuticle (CUT).

The quantitative RT-PCR demonstrated that Pr1 mRNA synthesis was very low in the ungerminated spores and in the rich media containing 1 % carbon source or more (Figure 8). In the basal salts medium 1, containing 0.2% sucrose and in the basal salts medium 2, containing 0.1 % sucrose, the number Pr1 transcripts was at least 6-fold and 12-fold higher than in the rich media, respectively. On the other hand, when the germlings were transferred into the basal salts medium 2 supplemented with 0.5 % ground cuticle and cultivated for 3.5 h, the Pr1 expression was only increased by 1.3-fold compared to the previous culture in the basal salts medium 1, that is to say, by 8-fold compared to the expression in the rich media.

Thus, the expression patterns of Pr1 at the RNA and at the protein level did not evolved in a parallel manner.

1.2. Identification of up-regulated genes of *B. bassiana* in cuticle medium by cDNA RDA

Expression of *B. bassiana* genes in an artificial context of infection via the transfer experiment was studied via three different cDNA RDA. Two experiments were performed using low stringency conditions of the subtractive hybridization steps, i.e. with relatively low ratios tester:driver. One low-stringency RDA (cDNA RDA LS1) used RNA isolated from cultures in basal salts medium 2, with or without 1 % ground cuticle of the Colorado potato beetle. The second low stringency RDA (cDNA RDA LS2) used RNA isolated from cultures in basal salts medium 2 with or without only 0.5 % cuticle. The third cDNA RDA was achieved with RNA isolated from culture in basal salts medium 2 supplemented, or not, with 0.5 % ground cuticle of the Colorado beetle (Figure 23), but using high stringency conditions of the subtractive hybridizations (cDNA RDA HS). These high stringency conditions should favour the identification of genes whose expression is more strongly

up-regulated in the cuticle medium than in the poor medium (in comparison with the experiment using low stringency subtractive hybridisation).

1. 2. 1. Identification of up-regulated genes of *B. bassiana* in 1 % cuticle medium by low stringency cDNA RDA (LS1)

Total RNAs were isolated from the cultures in basal salts medium 2 without cuticle and in basal salts medium 2 containing 1 % cuticle (Figure 9 A). After purification of the $poly(A)^+$ RNA, cDNA synthesis was performed.



Figure 9. Total RNA and cDNA from *B. bassiana* grown in medium with or without cuticle of the Colorado potato beetle.

(A) Total RNA isolated from *B. bassiana* grown in the basal salts medium with (2) or without (1) cuticle. "M" indicates the 0.24-9.5 Kb RNA ladder (Invitrogen). (B) double-strand cDNA (1d) and (2d) synthetized from the corresponding RNA sources (1) and (2). "M" indicates the DNA molecular size marker IV (Roche). (C) (Ms) Single-strand and (Md) double-strand cDNA synthetized from the 0.24-9.5 Kb ladder. (1s) Single-strand and (1d) double-strand cDNA synthetized from the previously mentioned RNA (1). (2s) Single strand and (2d) double-strand cDNA synthetized from the previously mentioned RNA (2).

The first-strand cDNA were synthetized using oligo(dT) as primer. The second-strand cDNA were synthetized using the RNase H method. The patterns of double-strand cDNAs (ds cDNA), synthetized from the cultures in basal salts medium 2 with or without cuticle, were very similar (Figure 9 B, C). Most of the ds cDNA had a size

between 1 and 3.5 kbp. A gene was highly transcribed in both culture conditions and even slightly more in the medium with cuticle. The size of the corresponding ds cDNA was about 2.1 kbp. Thus this cDNA could not correspond to Pr1, as *B. bassiana* Pr1 mRNA, has a size of about 1300 b (Joshi et al., 1995), and *M. anisopliae* Pr1 mRNA, a size of 1500 bp.

The ds cDNAs were then restricted with the 4-cutter enzyme DpnII, ligated with a 12/24-mer adaptor and amplified by PCR using the 24-mer as primer (Figure 5). The population of amplified DpnII fragments, that represented the mRNA from the culture in medium without cuticle, formed the driver representation. The population of amplified DpnII fragments, that represented the mRNA from the culture in medium with 1 % cuticle, formed the tester representation. The size of the majority of the DNA fragments from both representations was between 300 and 1.000 bp, as expected (Figure 11).

In parallel to the cDNA RDA experiment, that permit the subtraction of the driver representation from the tester representation, a control cDNA RDA was achieved. In this control experiment, the tester representation was formed from the mRNA from the culture in the medium without cuticle, as described above. The driver representation was constituted of the same tester representation, which had been later artificially spiked with DpnII fragments of the pyruvate decarboxylase gene from our *B. bassiana* strain. Four DpnII fragments had been spiked to form the driver population of the control RDA: a 910, 480, 260 and a 200 bp fragments. These fragments had been chosen knowing that they should have a size between 200bp and 1000 bp to be amplified with the RDA PCR condition. The 910 bp DpnII fragment was spiked at a ratio "spiked fragment: tester representation" of 1: 10.000, the 480 bp fragment at a ratio 1: 100.000, the 260 and the 200 bp fragments at a ratio of 1:200.000. This ratio was calculated considering that the mean size of the DpnII fragments in the tester representation was 500 bp.

The control and the cDNA RDA experiments were performed in parallel. Three subtractive hybridizations have been achieved with the tester:driver ratios 1:10, 1:100 and 1:5.000, respectively.

After the completion of the three subtraction/amplification steps, the third difference product of the control experiment showed an enrichment of two fragments

(Figure 10). Their molecular sizes were about 900 and 500 bp, as visible on the agarose gel. However, if the two big spiked fragments have been enriched during the cDNA RDA, the two smaller spiked fragments, of 260 and 200 bp, could not be found at the end of the experiment.



Figure 10. cDNA RDA control experiment.

In this test expriment, the driver representation was formed from the RNA of *B. bassiana* grown in the basal saltss medium without cuticle. The same representation was spiked with 4 *Dpn*II fragments of 910, 480, 260 and 200 bp, and used as the tester representation. The first, second and third difference products (DP1, DP2 and DP3) were obtained using the tester: driver ratios of 1:10, 1:100 and 1:5000, respectively. Only the two biggest spiked *Dpn*II fragments were recovered after the 3 cycles of subtraction/amplification.

The cDNA RDA experiment was performed using the cDNAs from the culture without cuticle for the driver representation and the cDNAs from the culture with cuticle for the tester representation. The two driver and tester representations were similar in size distribution and repartition, what is essential for a successful RDA (Figure 11). After the three subtraction/amplification steps, the third difference product, separated on a 2 % agarose gel, appeared to be composed of four main bands of about 600, 500, 450 and 400 bp, respectively.



Figure 11. Difference products obtained from the low-stringency cDNA RDA 1 (cDNA RDA LS1) using RNA from *B. bassiana* grown in medium with and without 1 % cuticle of the Colorado potato beetle.

(A) The driver representation made from the fungus grown in medium without cuticle [DR (- cuticle)] and the tester representation made from the fungus grown in medium with 1 % (w/v) cuticle [TR (+ cuticle)] are shown. (B) The first, second and third difference products (DP1, DP2 and DP3) were obtained using the tester: driver ratios of 1:10, 1:100 and 1.5000, respectively.

The cloning of these four main bands showed that they were composed of numerous different sized fragments. In order to verify that these cDNA RDA fragments corresponded to genes, whose expression is higher in cuticle medium than in the medium without cuticle, some of them were used as probe for Northern blot analysis. The expression of the genes was also estimated in the rich medium YPS. Only two of the tested cDNA RDA fragments appeared to correspond to genes over-expressed in the cuticle medium compared to the medium without cuticle (Figure 12). All the other tested RDA fragments were shown to be false positives; one corresponded to a gene over-expressed in the basal salts medium without cuticle rather than in the medium with cuticle; the others corresponded to genes expressed at the same level in the basal salts media with and without cuticle (data not shown). These false positives could be due to a residual DNA contamination in the RNA samples, or more likely, to cross-

contamination of the samples during the cDNA RDA procedure and/or the necessity of a fourth subtraction/amplification cycle before the cloning of the difference products.

The LS1-1 RDA fragment used as probe in Northern blot hybridized on a transcript of about 4.8 kb. The LS1-1-gene was over-expressed 4-fold more in the cuticle medium than in the basal salts medium (Table 1); however, the expression patterns in the cuticle medium and in the rich medium were similar (Figure 12). The size of the cloned LS1-1 fragment was about 470 bp. Sequencing analysis did not allow to determine more exactly the size of LS1-1 as the GATC *Dpn*II restriction site was found only at one side. Alignment of the deduced amino acid sequence revealed a significant homology with 86 % identities and 91 % similarity with the vacuolar (H⁺)-ATPase 98 kDa subunit of the ascomycete fungus *Neurospora crassa* (*N. crassa*). The observation that the expression of this gene was up-regulated in the same proportions in the cuticle medium and in the rich medium suggested that the gene regulation respond to the nutrient quantity provided by the cuticle rather than the nutrient quality.



Figure 12. Northern blots analysis of two cuticle up-regulated genes detected by low stringency cDNA RDA (cDNA RDA LS1).

(A) Northern blot analysis: to confirm the differential gene expression, total RNA from the basal saltss medium 2 without cuticle (1), with cuticle (2), and from a rich medium (3) were hybridized with the DP3 fragments. (B) The 25S RNAs were used as control for equal loading. (C) The putative protein encoded by the gene from which was amplified the RDA fragment is indicated.

The LS1-2 fragment, used as probe in Northern blot, hybridised on a transcript of about 5.2 kb. The LS1-2-gene was expressed specifically in the cuticle medium. As for LS1-1, the sequencing of LS1-2 allowed to find the GATC restriction site only on one side of the cloned fragment. The deduced amino acid sequence showed the highest homology with the mucin-like protein MUC2 precursor of the rat, *Rattus Norvegicus*. The amino acid identity and similarity were respectively 25 % and 38 %, with an e-value closed to "0". This relatively low score might be due to the distance between these 2 species, or to

the presence of some hypervariable regions (seen even among mucins from the same organism) (Di Noia et al, 1998). LS1-2 was used as probe in Southern blot analysis of B. bassiana genomic DNA restricted with different restriction enzyme. This experiment showed that this gene was present in only one copy in the genome (data not shown). In order to get the all LS1-2 gene sequence, and perhaps a further better alignment with a mucin protein, LS1-2 was used as probe to screen a lambda library of genomic DNA from *B. bassiana*. However, among numerous restriction enzyme double digests of the selected positive clone, only one restriction enzyme mix produced a easily clonable fragment. This 2.6 kbp fragment was cloned and sequenced. Unfortunately, only few bp of these fragment aligned more inside the coding region of MUC2 from Rattus norvegicus, the sequence on the other side of the known LS1-2 corresponded to the 3'non-coding region of the gene and aligned with any other sequence of the database. However, the alignment of the subcloned genomic fragment allowed to find the second DpnII restriction site. The deduced amino acid sequence of this newly defined LS2-2 GATC fragment showed the same homology with the mucin-like MUC 2 protein precursor of *Rattus norvegicus* than the previous one.

1. 2. 2. Identification of up-regulated genes of *B*. bassiana in 0.5 % cuticle medium by low stringency cDNA RDA (LS2)

The first cDNA RDA experiment gave rise to a lot of false positives, that were probably due to cross-contamination and/or the need of a fourth subtraction/amplification cycle before the cloning of the difference products. Thus, a second cDNA RDA was performed with the same procedure than previously with the exception of the addition of a fourth subtraction/amplification cycle. The tester:driver ratios of the subtractions were then 1:10, 1:100, 1:5000 and 1:25.000, respectively.

The LS1-1 fragment was revealed to be part of a gene, over-expressed in the cuticle medium compared to the basal salts medium, and in the rich medium. This modification of gene expression could be induced by the increased level of nutrient in the cuticle medium compared to the medium without cuticle, and not by a specific signal from the cuticle. To limit the risk of inducing expression of genes regulated by nutrient

availability, the percentage (w/v) of cuticle in the medium was decreased to 0.5 % instead of 1 %. All the other media were prepared as mentioned previously.



Figure 13. Difference products obtained from the low-stringency cDNA RDA 1 (cDNA RDA LS2) using RNAs from *B. bassiana* grown in medium with and without 0.5 % cuticle of the Colorado potato beetle.

(A) The driver representation from the fungus grown in medium without the insect cuticle [DR (- cuticle)] and the tester representation from the fungus grown in medium containing 0, 5 % (w/v) insect cuticle [TR(+ cuticle)] are shown. (B) The first, second, third and fourth difference products (DP1, DP2, DP3 and DP4) were obtained using the tester:driver ratios 1:10, 1:100, 1: 5.000 and 1: 25.000, respectively.

Separation of the difference product 4 on a 2 % agarose gel showed three main bands of 450, 350 and 300 bp (Figure 13). Five different clones from the transformation of each of the three main bands were used as probe against the initial tester and driver representations in Southern blot experiments (Figure 14). All the tested probes hybridized more on the tester representation (medium plus cuticle) than on the driver representation (medium minus cuticle). The fragments were then used as probe in Northern blot experiments and sent for sequencing. The hybridization pattern of the cloned difference products on the representations were in all cases confirmed by the Northern blot analysis on the original RNA populations. The sequencing of the cuticle-specific difference products permit to identify four different fragments harbouring the *Dpn*II recognition site at each extremity. These RDA fragments had homologies with an
genes encoding ABC multidrug transporter, the oligopeptide transporter Opt2 from *S*. *cerevisiae*, a serine peptidase and a choline sulfatase.

Sequencing of the LS2-1 RDA fragment described a fragment of 385 bp with the two GATC restriction site of *Dpn*II. The deduced amino acid sequence of LS2-1 showed 69 % identities and 83 % positives (99/119 amino acids) with an ATP-binding cassette transporter (ABC transporter) of the fungus *Aspergillus nidulans* (*A. nidulans*). This ABC transporter has been reported to be involved in multidrug resistance of *A. nidulans* (Del Sorbo et al., 1997) (Table 1). Northern analysis, using the LS2-1 difference product as probe, permited to estimate the LS2-1-gene mRNA size at about 4.8 kb. The LS2-1-gene was expressed 2.5- fold more in the culture with cuticle than in the culture the without cuticle (Figure 14). The expression levels of this gene in the poor medium without cuticle and in the rich medium were similar. Thus over-expression of this putative ABC transporter in *B. bassiana* grown in the cuticle medium was probably not a response to the increase amount of nutrients in the medium, but a response to cuticle physical and/or chemical intrinsic signals.



Figure 14. Southern and Northern blots analysis of 4 cuticle up-regulated genes detected by low stringency cDNA RDA. (A) Northern blot analysis: to confirm the differential gene expression, total RNA from the basal saltss medium 2 without cuticle (1), with cuticle (2), and a rich medium (3) were hybridized with the DP3 fragments. (B) The 25 S RNAs were used as control for equal loading. (C) The putative protein encoded by the gene from which was amplified the RDA fragment is indicated.

The LS2-2 was found to be a *Dpn*II fragment of 326 bp. Its deduced amino acid sequence showed the highest homology (55 % identities, 78 % positives - 68/87 amino acids) with a hypothetical protein from the fungus *N. crassa*. The first protein with an identified function and showing homology with LS2-2 was the oligopeptide transporter Opt2p from *Saccharomyces cerevisiae* (*S. cerevisiae*). Alignment of the amino acid sequence of Opt2p and LS2-2 highlighted 47 % identities and 69 % positives (63/91 amino acid). Both the hypothetical protein of

N. crassa and Opt2 of *S. cerevisiae* aligned with the amino acid sequence deduced from the same frame of the LS2-2 nucleic acid sequence. Northern blot analysis, using the LS2-2 fragment as probe, revealed that the transcript of this probable oligopeptide in *B. bassiana* had a molecular size of 4.5 kb and was specifically expressed in the cuticle medium culture.

The LS2-3 cDNA RDA difference product was identified to be a 384 bp DpnIIfragment. The deduced amino acid sequence of LS2-3 aligned with 52 % identities, 61 % positives (62/101 amino acids) on a hypothetical protein of *N. crassa*, with 31 % identities, 41 % positives (33/79 amino acids) on an alpha beta hydrolase fold family member of *Caenorhabditis elegans* (*C. elegans*), and 22 % identities, 43 % positives on a putative serine peptidase of *Oryza sativa* (*O. sativa*). In the three cases, the translation from the same frame aligned on the respective protein fragment. The alignment of the whole sequence of the hypothetical protein of *N. crassa* on the serine peptidase sequence of *O. sativa* gave a similar homology result with 28 % identities and 43 % positives (204/ 471 amino acid).

The transcript, corresponding to the LS2-3 difference product, was estimated to have a molecular size of 3.5 kb. It was detected in the culture in cuticle medium, but not in the basal salts and rich medium cultures.

The last difference product characterized, LS2-4, was a *Dpn*II fragment of 510 bp. Homology analysis with the protein database revealed 77 % identities, 91 % positives (88/96 amino acids) with a hypothetical protein of *N. crassa* and 63 % identities, 78 % positives (75/95 amino acids) with a choline sulfatase of *Pseudomonas aeruginosa* (*P. aeruginosa*). The homology of the protein sequence deduced from LS2-4 and the bacterial sulfatase was high for such different organisms. Northern analysis allowed the detection of a transcript of about 4.4 kb in the cuticle medium only, suggesting this over-expression of the HS-3-gene was induced specifically by the cuticle. This low stringency cDNA RDA allowed the identification of four genes over-expressed in *B*. *bassiana* grown in medium with 0.5 % insect cuticle compared to *B*. *bassiana* grown in the same medium without cuticle. These four genes encode putative ABC multidrug transporter, oligopeptide transporter, serine peptidase and choline sulfatase, respectively. Northern blot analysis showed that three of these genes were specifically expressed in the cuticle medium culture, and one was slightly over-expressed in the cuticle medium.

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A Choline sulfatase $Pseudomonas aeruginosa$ $NP_248721.1$ (4) $3 \times 10E-35$ $63 \% - 78 \%$	D	LS2-4	Hypothetical protein	Neurospora crassa	EAA36031.1	Unpubl.	3 x 10E-48	77 % - 91 %	CS
	A		Choline sulfatase	Pseudomonas aeruginosa	NP_248721.1	(4)	3 x 10E-35	63 % - 78 %	

Table 1. Sequence homologies of the difference product fragments obtained with the 2 low stringency cDNA RDAs LS1 and LS2.

The homology of the difference product fragments were analysed with the reanslated BLAST searches (NCBI). The table shows the best homology and, when necessary, the highest homology with an identified protein. Northern analysis was performed using each RDA fragment as probe. The level of over-expression of the gene in the cuticle medium compared to the basal salt medium 2, or the specific expression of the gene in the cuticle medium are indicated by "x-fold" and "cs" (cuticle specific), respectively. Note: the level of over-expression was estimated by density measurement.

<u>1. 2. 3. Identification of up-regulated genes of *B. bassiana* in 0.5 % cuticle medium by high stringency cDNA RDA (HS)</u>

The last cDNA RDA was realized using low stringency conditions. The used tester:driver ratios of the four subtractive hybridisations were 1:10, 1:100, 1:5.000 and 1:25.000. The advantage of these conditions is the obtention of a difference product in most of the cases. In order to favour the identification of genes highly over-expressed in the cuticle medium culture, the cDNA RDA was repeated using high stringency subtraction conditions. The used tester:driver ratios of the subtractive hybridisations were then 1:100, 1:800 and 1:10.000. After the third subtraction/amplification cycle, the third difference product was composed mainly of fragments of about 500, 400 and 300 bp (Figure 15).



Figure 15. Difference products obtained from the high-stringency cDNA RDA (cDNA RDA HS) using RNAs from *B. bassiana* grown in medium with and without 0.5 % cuticle of the Colorado potato beetle.

(A) The driver representation from the fungus grown in medium without the insect cuticle [DR (- cuticle)] and the tester representation from the fungus grown in medium containing 0,5 % (w/v) insect cuticle [TR(+ cuticle)] are shown. (B) The first, second and third difference products (DP1, DP2 and DP3) were obtained using the tester:driver ratios 1:100, 1:800 and 1: 10.000, respectively.

Each of these three groups of fragments was subjected to cloning. Five to ten clones from each transformation were used as probe in Southern analysis to see if they were more abundant in the tester representation than in the driver one (Figure 16). After this verification, the difference products were sent for sequencing and used as probe in Northern blot experiments. Six different fragments have been identified to represent genes over-expressed in the cuticle medium. A seventh one was not used as probe for Northern blot analysis, but had same alignment results than another tested RDA fragment, suggesting that they were 2 different *Dpn*II-fragments from the same gene.

The HS-1 difference product was a 333 bp DpnII-fragment. The deduced amino acid sequence showed the highest homology with a hypothetical protein of *N. crassa* with 59 % identities and 71 % positives (72/99 amino acids). The highest amino acid homology of HS-1 with a defined protein was with the pyruvate decarboxylase Pdc5p of *S. cerevisiae*. The HS-1 translated sequence and Pdc5p shared 51 % identities and 63 % positives (62/98 amino acids) (Table 2). Northern blot analysis showed that the transcript hybridising with HS1 was 4.5 kb long. It was revealed to be more abundant in the cuticle medium culture than in the rich culture, and more abundant in the rich medium than in the poor medium. The HS1-gene was over-expressed 6-fold more in the cuticle medium than in the basal salts medium without cuticle (Figure 16).

Sequencing of the HS-2 difference revealed a 322 pb *Dpn*II-fragment. The deduced amino acid sequence of HS2-2 aligned on the oligopeptide transporter Opt2 of *S*. *cerevisiae* with 46 % identities and 68 % positives (64/94 amino acids). The nucleic acid sequence of HS-2 aligned exactly on the previous LS2-2 RDA fragment. HS2-2 was also used as probe in Northen blot experiment. The corresponding transcript of about 4.6 kb was detected only in the cuticle medium as previously shown for the LS2-2-gene.

The HS-3 difference product was a 508 bp DpnII-fragment. The deduced amino acid sequence showed 23 % identities, 35 % positives (42/117 amino acids) with the excretory/secretory mucin MUC-3 from *Toxocara canis* (*T. canis*). Northern analysis revealed a transcript of 3.8 kb. The HS-3-gene was not expressed in the rich medium culture and only at a low level in the poor medium without cuticle. The HS- 3-gene was expressed 9-fold more in the cuticle medium then in the poor medium suggesting that this expression was induced specifically by the cuticle.



Figure 16. Southern and Northern blots analysis of 4 cuticle up-regulated genes detected by high stringency cDNA RDA (HS RDA). (A) Southern analysis: the driver (1) and the tester (2) representations were hybridized with the subcloned DP3 fragments. (B) Northern blot analysis: to confirm the differential gene expression, total RNA from the basal saltss medium 2 without cuticle (1), with cuticle (2), and a rich medium (3) were hybridized with the DP3 fragments. (C) The 25 S RNAs were used as control for equal loading. (D) The putative protein encoded by the gene from which was amplified the RDA fragment is identified.

Sequencing of HS-4 revealed a 236 bp DpnII-fragment. The deduced amino acid sequence aligned on a putative alpha beta hydrolase fold family member from C. elegans with 31 % identities and 46 % positives (29/63 amino acids). The homology between HS-4 and the putative hydrolase of C. elegans was similar than the homology between LS2-3 and another putative hydrolase of C. elegans. However, LS2-3 aligned also on a hypothetical protein of N. crassa and a putative serine peptidase of O. sativa (Table 1), and the RDA fragment HS-4 did not share such homologies. The HS-4 fragment, used as probe, detected a transcript of about 3,9 kb, the LS2-3 detected a transcript of about 3.5 kb. The Northern blot hybridisation patterns of LS2-3 and HS-4 were also different. The transcript hybridising with LS2-3 could not be detected in cultures in both media without cuticle, while transcription of the HS-4 gene was shown in all the three different cultures. The lowest transcription level of the HS-4-gene was detected in the basal salts medium culture. The expressions in the cuticle medium and in the rich medium were similar and 2.5-fold more important than in the basalts salts medium. Enhancement of the transcription of this putative α/β hydrolase might depend on the presence of carbon and/or nitrogen sources in the medium.

The HS-5 difference product was a 505 bp *Dpn*II-fragment. HS-5 aligned on a hypothetical protein of *S. cerevisiae* with 31 % identities, 46 % positives (37/80 amino acids), and on a siderophore transporter for enterobactin of *S. cerevisiae* with 26 % identities, 45 % positives (37/82 amino acids). Northern blot analysis showed that the HS-5 transcript of 4.4 kb, was not present in rich medium culture and in low-copy number in the poor medium. Expression of the HS-5 gene was estimated to be 4-fold higher in the cuticle medium culture compared to the poor medium culture.



Figure 17. Northern blot analysis of two cuticle up-regulated genes detected by high stringency cDNA RDA.

(A) Northern blot analysis: to confirm the differential gene expression, total RNA from the basal saltss medium 2 without cuticle (1), with cuticle (2), and a rich medium (3) were hybridized with the DP3 fragments. (B) The 25 S RNAs were used as control for equal loading. (C) The putative protein encoded by the gene from which was amplified the RDA fragment is indicated.

Sequencing of HS-6 revealed a 184 bp DpnII fragment. The deduced amino acid sequence aligned on a hypothetical protein from *N. crassa* with 72 % identity and 87 % similarity (Acc. N° CAC0902.2) (Table 2). The fragment HS-6, used as probe in Northern blot, hybridised on a transcript of about 3.3 kb (Figure 17). Expression of the HS-6-gene was cuticle-specific.

Sequencing of the fragment HS-7, of 181 bp, revealed homology with the same hypothetical protein (Acc. N° CAC09402.2) sharing homology with HS-6. The percentage of identity and similarity were identical too. However, sequences of HS-6 and HS-7 were different suggesting that these fragments were restricted from the same cDNA of from paralogue genes cDNA. No Northern analysis was performed using HS-7 as probe.

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		Homologies of the RDA frag	ments				Level
							of over-
ıt							expression
	Protein	Source organism	GenBank	Ref.	E-value	Identities –	
			accession n°.			Positives	
HyJ	pothetical protein	Neurospora crassa	EAA30481.1	Unpubl.	1 x 10E-27	58 % - 72 %	6-fold
Pyı	uvate decarboxylase Pdc5p	Saccharomyces cerevisiae	NP_013235.1	(5)	2 x 10E-20	51 % - 63 %	
OI	igopeptide transporter Opt2	Saccharomyces cerevisiae	NP_015520.1	(9)	8 x 10E-16	48 % - 68 %	CS
Ex	cretory/secretory mucin MUC-3	Toxocara canis	AF167708_1	(2)	0.66	23 % - 35 %	9-fold
Pu	tative alpha beta hydrolase fold	Caenorhabditis elegans	NP_501601.1	Unpubl.	2.3	31 % - 46 %	2.5-fold
Hy	pothetical protein	Saccharomyces cerevisiae	P25596	Unpubl.	0.019	31 % - 46 %	4-fold
Sic	derophore transporter for enterobactin	Saccharomyces cerevisiae	NP_014484.1	(8)	0.21	26 % - 45 %	
H	pothetical protein	Neurospora crassa	CAC09402.2	Unpubl.	7 x 10E-21	72 % - 87 %	CS
H	pothetical protein	Neurospora crassa	CAC09402.2	Unpubl.	2 x 10E-15	71 % - 87 %	NT

Table 2. Sequence homologies of the difference product fragments obtained with the high stringency cDNA RDA (HS).

The homology of the difference product fragments were analysed with the translated BLAST searches (NCBI). The table shows the best homology and, when necessary, the highest homology with an identified protein. Northern analysis was performed using each RDA fragment as probe. The level of over-expression of the gene in the cuticle medium compared to the basal salt medium 2, or the specific expression of the gene in the cuticle medium are indicated by "x-fold" and "cs" (cuticle specific), respectively. Note: the level of over-expression was estimated by density measurement. The cDNA RDA experiments performed with RNA from B. bassiana cultivated in medium, supplemented or not with cuticle of the Colorado potato beetle, permit to identify eleven genes over-expressed in the cuticle medium culture. Six of these eleven genes appeared to be specifically expressed in the cuticle medium culture. Three genes were over-expressed 2 - to 5 fold more in the cuticle medium than in the poor medium, and two genes were over-expressed 5- to 10-fold more in the cuticle medium. On the eleven genes identified to be over-expressed in the cuticle medium compared to the poor basal salts medium, only three of them appeared to have a similar level of expression in the rich medium; these three concerned genes encode a probable vacuolar (H^+) ATPase, pyruvate decarboxylase and a hypothetical α/β - hydrolase. This result indicated that enhanced transcription of most of these genes in the cuticle medium culture did not answer to the increased nutrient availability compared to the poor medium, but was due to intrinsic physical and/or chemical signals of the cuticle. Three genes seemed to be involved in the cuticle degradation, and three might encode transporters. Two genes might encode highly O-glycosylated proteins, termed mucins. The three last difference products cloned were shown to align on a pyruvate decarboxylase of S. cerevisiae, on a vacuolar $(H^{\dagger})^{-}$ ATPase subunit of N. crassa and on a hypothetical protein of *N. crassa*.

These experiments have shown that identification of genes of *B. bassiana* overexpressed in an artificial context of infection using cDNA RDA was realisable. However, this transfer experiment constituted only a rough model of infection. Another RDA experiment was also undertaken with *B. bassiana* in a natural context of infection.

2. Identification of up-regulated genes of B. bassiana during infection of L. decemlineata axenic larvae by cDNA RDA

To identify genes of *B*. *bassiana* over-expressed in the natural context of infection, infected L2 (second instar) larvae were chosen to form the further tester population of the cDNA RDA experiment. Two main technical problems had to be solved to permit this study.

First, the sensitivity of this PCR-based method obliged to work with axenic larvae. A protocol to obtain sterile hatching eggs of the Colorado potato beetle, and then a procedure for a sterile rearing of the axenic larvae, have been established in collaboration with the laboratory of Dr Zimmermann at the "Federal biological research centre for agriculture and forestry" in Darmstadt.

Second, the use of infected living insects led to isolation of RNA from the both insect and fungus at the same time. In other words, the further tester representation would be composed of cDNA *Dpn*II-fragments from both *L. decemlineata* larvae and *B. bassiana*. The driver population should also be made of cDNA synthetized from a RNA mix constituted of *B. bassiana* RNA and *B. bassiana*-uninfected axenic larvae RNA (Figure 18). After the choice of the growth conditions of *B. bassiana*, whose RNA would be part of the driver representation, determination of the fungal RNA quantity in the RNA isolated from the infected larvae was performed using real-time PCR. This quantification allowed the addition of RNA from *B. bassiana* in the infected larvae. The cDNA synthesis and the cDNA RDA experiment were then achieved.



Figure 18. cDNA RDA with a mixed driver representation.

The tester representation should be formed from the RNA isolated from the axenic larvae of the Colorado potato beetle infected by *B. bassiana*. In order to enrich the difference products of the further cDNA RDA in genes over-expressed in response to the fungal infection, the driver representation had to be formed from RNA isolated from the fungus and the insect. After the quantification of the fungal RNA in the infected larvae, the same ratio "fungal RNA:insect RNA" was mixed to form in a later step the driver representation. The mRNA purification, cDNA synthesis and synthesis of the representations were then performed according to the normal protocole.

2.1. Preliminary experiment to the cDNA RDA

2.1.1. Procedure to obtain axenic larvae of L. decemlineata

The cDNA RDA, and then the Northern blots, required a lot of RNA and so the preparation of numerous axenic larvae at a same time. No procedure was previously established to obtain surface-sterilized eggs of the Colorado potato beetle able to hatch normally. Rearing on artificial diet has been reported (Hsiao and Fraenkel, 1968, Wardojo, 1969, Domek, 1997, Gelman et al., 2001); however, these diets were mostly expensive and/or laborintensive and contained preservatives or antibiotics. Thus, it was necessary to establish a simple and rapid procedure to obtain axenic larvae. This procedure consisted first in the surface-sterilization of the eggs, and then in the rearing of the hatched larvae with sterile food.

2.1.1.1. Surface sterilization of the eggs before hatching

Superficial sterilization of the eggs was performed in two steps: sterilization with formaldehyde vapour for 24 h, and then sterilization in a solution containing streptomycine, chloramphenicol and Tween 80 (wetting agent) for 30 min (Figure 19). Eggs were then extensively rinsed with sterile distilled water, transferred onto a filter paper in a Petri dish and stored in the incubator until hatching.



2.1.1.2. Rearing of the axenic larvae

In order to verify the absence of any bacterial or fungal contamination, each five axenic larvae hatching from the surface-sterilized eggs were grown in LB agar plates. It is known that hatching larvae first feed on their own eggshell before feeding on the leave. Even beetles have been observed to feed on their own eggs. Therefore, the rearing of the axenic larvae was performed with gamma-irradiated egg masses.

2.1.2. Determination of the ratio fungal RNA/insect RNA in the *B. bassiana*-infected larvae

Quantification of the RNA from the fungus in the infected larvae was achieved by realtime PCR. The copy number of the transcript of an actin gene from the fungus and an actin gene from the insect was used as reference of the total mRNA of each of them in the infected larvae.

Degenerated primers were designed to amplify a part of one actin gene from B. *bassiana*. Other degenerated primers were designed to amplify the corresponding part of a gene encoding a cytoplasmic actin of *L. decemlineata*. The two amplified fragments were cloned into *pUC*18 and sequenced. Two specific primers were then designed to amplify a 103 bp fragment in the fungus and a 114 bp fragment in the insect. The two reverse primers were chosen in the same variable region of the sequences from the fungal and insect actin gene, respectively. The two forward primers were drawn with the same requirement.





the insect actin cDNA in the infected larvae, respectively. (-RT) indicates that no reverse transcription was performed before the PCR amplification, (+RT) indicates the corand RNA from the fungus grown on dead larvae were mixed at different ratios and used as template for PCR using the fungal actin primers (C) and the insect actin primers of the fungal actin and insect actin genes were realised using different numbers of molecules of the corresponding PCR products as template. RNA from the axenic larvae (D) respectively. The different quantities of fungal RNA spiked in 2 µg of insect RNA are indicated. The sample indicates the amplification of the fungal actin cDNA and responding samples for which a reverse trancription was performed. No indication means that the RT-PCR was performed normally. As the primers had been designed in the coding sequence of the actin gene, for each RNA samples, a control of amplification without a previous reverse transcription was performed. The number of molecules of the actin single-strand cDNA (ss cDNA) was determined for the fungus and for the insect in the reverse-transcribed RNA sample isolated from the infected larvae. These values, determined according to their respective standard curves, were 360 molecules of the fungal actin ss cDNA and 1.870.000 molecules of the insect actin ss cDNA(Figure 20; Table 3).

	test	: ng funga	sample					
Amount of spiked	0.5 1 1.5				3 3			
fungal RNA (ng)								
Reverse transcription	+	- +			+	-	+	+
Nb of fungal actin	510	1.000 2.00		0	2.490	21	358	24
ss cDNA mol.								
			-					
	test: ng fungal RN			A spiked/ reaction			sample	
Amount of spiked	1.5			1.5				
fungal RNA (ng)								
Reverse transcription	+			+			+	-
Nb of insect actin	1.919.500			0			1.866.050	0
ss cDNA mol.								

 Table 3. Quantification of the molecules of an actin transcript from *B*. bassiana and *L*.

 decemineata, in a control reaction and in the infected larvae.

In other words, about 0.02 % of the mRNA isolated from the infected larvae was fungal RNA. In parallel, two micrograms of RNA from the axenic larvae was spiked with 0.025 %, 0.05 %, 0.075 %, 0.1 %, 0.125 % and 0.15 % of RNA from the fungus grown on gamma-irradiated larvae as substrate. Real-time PCR was performed with the same master mix used for the determination of the fungal actin molecules in the infected larvae, and at the same time. About 510 molecules of the fungal actin ss cDNA were detected in the sample spiked with 0.025 % of ss cDNA from the fungus. According to these results, if the sample would have been spiked with only 0.02 % of the fungal actin ss cDNA, about 380 molecules of the fungal cDNA would have been detected, value tightly closed to the 360 molecules determined previously in the infected larvae sample.

2.1.3. Procedure for the cDNA synthesis

Total RNA was isolated from the axenic larvae infected with *B. bassiana* conidia 1.5 days after inoculation. Total RNA was also isolated from axenic larvae (instar 2) and from *B. bassiana* conidia grown 1.5 days on γ -irradiated larvae (Figure 21). *B. bassiana* was grown on gamma-irradiated larvae to mimic as closed as possible the growth conditions of *B. bassiana* infecting the axenic larvae (physical signals of the cuticle; quality, quantity and availability of the nutrients).

Total RNA from the axenic larvae was spiked with 0.025 % of the fungal RNA prior to the $poly(A)^+$ RNA purification and the cDNA synthesis. The monitor reactions of the cDNA synthesis experiment showed that most of the synthetized ds cDNA had a size between 1 to 2.5 kbp, and that some of them reaching a molecular size of about 9 kbp (Figure 21).



Figure 21. Total RNA and cDNA synthesis from the axenic larvae, *B. bassiana* and the axenic larvae infected with *B. bassiana*.

(A) Total RNA were isolated from (1) the axenic larvae, (2) the axenic larvae infected with *B. bassiana*, and, (3) from *B. bassiana* grown on dead larvae. The position of the 25S and 18S rRNA from *B. bassiana* are indicated. (B) Double-strand cDNA synthetized from (Cds) the control formed by the 0.24-9.5 kb RNA ladder (Invitrogen), ((1+3)ds) the RNA mix from the axenic larvae and *B. bassiana* and (2) the RNA from the infected larvae.

2. 2. Identification of up-regulated genes of *B. bassiana* in the infected larvae by high stringency cDNA RDA

Double-strand cDNA, were synthetised from the RNA of infected larvae and from the RNA mix from axenic larvae and *B. bassiana*. They were then restricted by DpnII, ligated with a 12/24-mer adaptor and amplified by PCR to form the tester and the driver representation, respectively (Figure 5). The tester:driver ratios of the three successive subtractive hybridisations were 1:100, 1:800 and 1:10.000, respectively. The difference products one, two and three were obtained after the three rounds of subtraction/amplification cycle using the stringency conditions mentioned above. Unlike the previous cDNA RDA experiments, electrophoresis of the difference products two and three did not revealed less but more intense bands, but more less intense bands for the second difference product and a diffuse pattern of DNA fragments for the third difference product (Figure 22).



Figure 22. Difference products obtained from the cDNA RDA using mix of RNA from the axenic larvae and the fungus and the RNA from the infected larvae.

The driver representation made from the mix of RNA "uninfected larvae + fungus" [DR(UL+F)], the tester representation made from the RNA of the axenic larvae infected by *B. bassiana* are indicated. The first, second and third difference products (DP1, DP2 and DP3) were obtained using the tester:driver ratios of 1:100, 1:800 and 1:10.000, respectively.

III. Discussion

1. Transfer experiment and PR1 expression

The identification of up-regulated genes of *B. bassiana* during its pathogenic process was first achieved in an artificial context of infection. Previous studies showed that the culture of entomopathogenic fungi, such as B. bassiana, M. anisopliae and V. lecaani, in basal salts medium containing ground insect cuticle as sole carbon and nitrogen source, induces the secretion of diverse hydrolases, such as proteases, aminopeptidases, esterases and lipases, into the media (St Leger et al., 1986a). The activity of one subtilisin-like protease, called PR1, was particularly high in the cuticle medium and was further studied. When M. anisopliae was cultivated in media containing one percent of sucrose and/ or N-acetylglucosamine, the PR1 activity could not be measured. The transfer of the mycelia in a basal salts medium containing neither carbon nor nitrogen source was derepressing the PR1 activity. Finally, the growth of *M. anisopliae* in a basal salts medium containing one percent of ground cuticle of the locust gave rise to an increased activity of PR1 compared to this in the basal salts medium without cuticle. It was also suggested that PR1 production was under a general carbon and nitrogen repression mechanism, and moreover was induced by the cuticle of the insect host (St Leger et al, 1988c; St Leger et al., 1991a; St Leger et al., 1993; Paterson et al., 1994).

Taking these observations as a basis, a transfer experiment was established in order to induce the expression of genes involved in the pathogenic process (Figure 23). Spores of *B. bassiana* were first amplified by culture in rich medium. The study being achieved at the RNA level and consisting in the subtraction of two mRNA populations, a big care had to be taken to not stress the cells in the cultures and to have the insect cuticle as sole difference between the two media at the last step of the experiment. After a first amplification of the spores, these ones were transferred into a defined complete medium with half concentration of sucrose. The spores obtained by this second amplification, were then transferred in a fresh medium (basal salt medium 1) with again half concentration of sucrose and sodium nitrate (the nitrogen source) than the previous one

and allowed to germinate in 23 hours. Finally, the germinated spores were transferred into a medium containing or not the host cuticle in order to obtain the RNA for the future tester or driver representations, respectively, of the cDNA RDA. This medium (basal salts medium 2) contained also half concentrations of carbon and nitrogen sources compared to the basal salts medium 1.

The analysis of PR1 production at the protein level, by Western blot (Figure 7), and at the transcription level, by real-time PCR (Figure 8), showed quite different expression patterns. In the rich media, no signals were detected on the Western blot and only a low basal level of Pr1 transcription was measured. These results were expected as PR1 expression is under a general carbon and nitrogen repression mechanism. After the 23 h incubation in the basal salt medium 1, PR1 production seemed to be slightly derepressed and a discrete band was visible on the Western blot. The comparison of PR1 production between the basal salts medium 1 and 2 showed an increase of 4-fold at the protein level and 2-fold at the RNA level. Finally, the comparison between the basal salts medium 2 containing or not the ground cuticle revealed a not significant increase in PR1 production at the protein level (1.2-fold), but even a decrease of the Pr1 transcripts in the cuticle medium. The number of Pr1 transcript molecules in the cuticle culture was between this obtained from the basal salt medium 1 and the basal salts medium 2 cultures.

The different PR1 expression pattern between the western blot and real-time PCR analysis might come from the different sensitivity of the two methods. In *M. anisopliae*, the increase in Pr1 mRNA was shown to be parallel to an increased of transcription during deprivation. A quantification of the ratio mRNA/total RNA or a previous $poly(A)^+$ RNA before the reverse transcription should allow to determine if the lower number of Pr1 transcript in the cuticle medium culture is linked to a higher rRNA/mRNA ratio.

Transcription of Pr1 is already detected after two hours culture of *M. anisopliae* in a basal medium supplemented with cuticle (St Leger et al., 1992). The detection of Pr1 mRNA after 3.5 h culture in the cuticle medium, and also in the poor medium was thus in agreement with this study. However, measurement of PR1 activity in *M. anisopliae* cultures showed that PR1 is over-expressed in a medium containing insect cuticle compared to a medium without cuticle. In the present study, the enhancement of PR1

protein in the cuticle medium did not appear to be significant and even a slow decrease of the transcripts molecules was observed. One hypothesis is that hydrolysis of the cuticle particles during the sterilization (5 min at 115°C) liberates some readily utilizable nutrients which partially repressed Pr1 transcription during the first hours of culture.

2. Putative genes specifically- or over-expressed in the L. decemlineatacuticle medium cultures of B. bassiana

2.1. Hydrolases

2.1.1. Choline sulfatase

The second low-stringency cDNA RDA experiment permit to identify a DpnII fragment, called LS2-4, showing significant homology (78 % similarity) with a choline sulfatase from *Pseudomonas aeruginosa* (Stover at al., 2000) (Table 1). The choline sulfatase function of this protein from *P. aeruginosa* was predicted from the sequence. In *B. bassiana*, expression of this putative choline sulfatase appeared to be cuticle-specific. The hybridization signal was however very weak, and it cannot be excluded that the LS2-4-gene was expressed at a low level in the poor and/or the rich media (Figure 14).

Choline is a major component of membranes and a structural component of some microbial cell wall polymers. Choline was also shown to stimulate hyphal extension and to inhibit initiation of branching in the fungus *Fusarium graminearum* (F. *graminearum*) (Wiebe et al., 1989).

The choline-O-sulfate is assumed to constitute both an osmoprotectant and an endogenous reserve of sulfate and choline in fungi (Markham et al., 1993). The

conversion of choline-*O*-sulfate into choline and sulfate is catalysed by the choline sulfatase. After the 3.5 h culture of *B*. bassiana in the basal salts medium containing the beetle cuticle, the germlings were forming at most two ramifications, but which could be very long (Figure 6). This observation could support the hypothesis of an increased concentration of choline in the cuticle medium following the increased synthesis of choline sulfatase.

The choline produced from the choline-O-sulfate can be further metabolised in glycine betain by the successive actions of a choline- and a betain aldehyde dehydrogenases (Osteras et al., 1998). Culture of the mold *Penicillium fellatanum* (*P. fellatanum*) in sulfat-deficient medium in presence of L-[*methyl*-¹³C]methionine permit to detect a decrease of choline-O-sulfate and a parallel accumulation of glycine betain. When the sulfate content of the medium was adjusted to 5 mM sulphate, the near depletion in glycine betain and the return of cytoplasmic choline-O-sulfate were observed (Park et al., 1999). These results suggest that choline accumulates as choline-O-sulfate when the medium contains sufficient sulfate, but that choline is stored in the cytoplasm as glycine betain in a sulfate-deficient medium.

As the fungal continued to grow in the basal salts medium supplemented in cuticle compared to the basal salts medium without cuticle, where the fungus was sporulating, the medium might became quickly sulfate-deficient. A hypothesis is that the hydrolysis of the choline-*O*-sulfate by the newly synthetized choline sulfatase could then supply *B*. *bassiana* in sulfat and choline. The choline could then have been totally or partially stored as glycine betain, as observed for *P*. *fellatanum* when grown in sulfat-deficient medium.

The LS2-4 gene encoding a putative choline sulfatase was shown to be expressed only in the cuticle medium, by Northern analysis. This enzyme catalyses the formation of choline and sulfat from the choline-*O*-sulfate. The increase of the choline concentration having an impact on the hyphal extension and on the frequency of branching of hypha, as well as the supply with sulfat of the fungus in the probably sulfat-deficient medium, could be 2 beneficial effects of this choline sulfatase in the cuticle medium and perhaps during the infection process.

2.1.2. Alpha/beta-hydrolase fold enzymes

Two different cDNA RDA fragments, termed LS2-3 and HS-4, were shown to be part of a gene encoding a putative α/β -hydrolase fold enzyme. The LS2-3 deduced amino acid sequence showed 41 % similarity with a putative α/β -hydrolase fold family member from *C. elegans* and 43 % similarity with a putative serine peptidase from *O. sativa* (Table 1). Expression of the LS2-3-gene was detected only in the cuticle medium (Figure 14). The HS-4-gene shared 46 % similarity with another putative α/β -hydrolase fold enzyme from *C. elegans* (Table 2). The HS-4-gene was expressed about 2.5-fold more in the cuticle medium than in the basal salts medium. However, its expression levels in the cuticle medium and in the rich medium were not significantly different (Figure 16).

The α/β -hydrolase fold family of enzymes is one of the largest group of structurally related enzymes with various catalytic functions. The canonical α/β -hydrolase fold is an eight-stranded mostly parallel α/β structure, and all the enzymes have a Nucleophile-His-Acid catalytic triad. However, they can operate on substrates with completely different chemical and physicochemical properties, and act in diverse biological contexts. Theoretically, the HS-4-gene could be a putative lipase, thioesterase, serine peptidase, proline oligopeptidase, proline iminopeptidase, hydroxynitrile lyase, and others. Leopold and Samsinakova (1970) detected the enzyme activities present in the culture filtrates of *B. bassiana* grown in medium containing insect cuticle. They could measure the activities of extracellular enzymes corresponding to the main constituents of insect cuticle, i.e. proteins, chitin and lipids. The HS-4-gene might be most probably an α/β -hydrolase fold enzyme with an endoproteases, amino peptidase, carboxypeptidase, lipase or esterase activity.

The LS2-3-gene showed a closed percentage of similarity both with a serine peptidase and with a α/β -hydrolase fold enzyme. Thus, this gene could encode a serine carboxypeptidase with the typical Nucleophile-His-Acid catalytic triad of the α/β hydrolase fold enzyme (Holmquist, 2000). Another significant possibility is that this gene might encode a serine protease from the trypsin or the subtilisin families. In this last case, the protein should present the invariant Ser-His-Asp catalytic triad of these 2 families (Blow et al., 1969; Wright et al., 1969). The activity of all of the serine carboxydase, trypsin protease (e.g. Pr2) and subtilisin protease (e.g. Pr1) have been detected in the culture lysates of *M. anisopliae* and *B. bassiana* grown in medium containing cuticle of the locust (St Leger et al., 1986b; St Leger et al. 1987c).

The HS-4- and the LS2-3-genes might encode 2 putative hydrolases that are presumably responsible for the degradation of the insect cuticle. The putative hydrolase encoded by the HS-4-gene seemed to belong to the α/β -hydrolase fold family, but its hydrolytic activity was not identified. The LS2-3-gene encodes a putative serine protease, which could belong to the α/β -hydrolase fold family as suggest the alignment results, but also to the trypsin and subtilisin families. Sequencing of the entire ORF and identification of the catalytic triad residues should support one or the other hypothesis.

2.2. Transporters

2.2.1. Siderophore transporter

The amino acid sequence deduced from the RDA fragment HS-5 shared 45 % similarity with a siderophore transporter for enterobactin in *S. cerevisiae* (Table 2). The HS-5 gene was expressed 4-fold more in the cuticle medium than in the basal salt medium. Its expression was not detected in the rich medium (Figure 17).

Siderophores are low molecular weight high-affinity iron chelators. They are excreted by microorganisms under iron depletion conditions to capture extra-cellular iron, which is mostly present in virtually insoluble compounds (hydroxides, oxides). Siderophores complexes are taken up by specific transport systems. Expression of the siderophore transporters, MIRA and MIRB, of *A. nidulans* were shown to be regulated by iron, regulation mediated by the transcriptional repressor SREA (Haas et al., 2003). The expression pattern of the HS-5-gene could correlate with this of a siderophore transporter, which is regulated by iron. The growth rate of the fungus was higher in the cuticle medium than in the basal salt medium without cuticle, that implied a more important requirement in iron. The cuticle did not constitute a directly available source of iron creating probably rapidly iron starvation conditions (< 4 h) for the cells. The cellular response could also have been an up-regulation of genes encoding siderophores and siderophores transporters. The HS-5 RDA fragment showed homology with a siderophore transporter for enterobactin, a siderophore produced by enterobacteria. The alignment performed a 505 bp sequence and revealed 45 % similarity, so the gene could be a transporter for another siderophore than enterobactin; however several observations would support the hypothesis of the synthesis of a siderophore transporter for enterobactin by *B. bassiana*.

Iron-siderophores complexes are taken up by specific transport systems, however some micro-organisms have also developed transport system for heterologous siderophores produced by other species. For example: *E. coli* synthetize transporters not only for the homologous enterobactin and aerobactin siderophores, but also for hydroxamate siderophores, which are produced by fungi (Howard, 1999). The filamentous ascomycete *A. nidulans* possesses MIRB, which transports exclusively the native siderophore triacetylfusarine C, and MIRA, which transports specifically the heterologous siderophore enterobactin C.

The phytopathogen *Ustilago maydis* (*U. maydis*) utilizes also both native and heterologous siderophores. Under iron stress, *U. maydis* synthetizes normally two different siderophores; however, under laboratory conditions, it also uses a bacterial siderophore (Ardon et al., 1997, Ardon et al., 1998). Utilization of this bacterial siderophore probably does not affect *in vivo* recruitment of iron by the phytopathogenic fungus, but its saprophytic existence in soil could be influenced. Competition for heterologous siderophores might have an important role in microbial system.

HS-5-gene might encode a siderophore transporter and even a siderophore transporter for enterobactin. The HS-5-gene expression pattern supported this hypothesis.

The synthesis of a siderophore transporter for enterobactin by *B*. *bassiana* might play different roles. A saprophytic flora might grow on the insect surface; bacteria that could

take advantage of the penetration of the fungal infectious peg to enter the insect. Competition between the bacteria and the fungus for iron among others salts and nutrients might be significant during germination of B. bassiana on the host surface and during penetration of the cuticle. Expression of this putative siderophore for enterobactin might also play a role in competition with the opportunistic colonizers of the insect cadavers.

2.2.2.Oligopeptide transporters

Both cDNA RDA, using low and high stringency conditions for the subtractive hybridisations, permit to identify a fragment (termed respectively LS2-2 and HS-2) of a gene sharing amino acid sequence homology with oligopeptide transporters. The deduced amino acid sequence from LS2-2/HS-2 showed 68 % and 66 % similarity with the peptide transporters Opt2p and Opt1p, respectively, from *S. cerevisiae* and 57 % similarity with Isp4p from *S. pombe* (Table 1, 2). Expression of this putative oligopeptide transporter in *B. bassiana* appeared to be cuticle-specific, as it was neither detected in the poor medium nor in the rich medium (Figures 14, 16).

The cellular uptake of small peptides is fundamental to nutrition and the economy of amino acids in many organisms. Peptide transporters translocate peptides (2-6 residues in length) across the cellular membrane in an energy dependant manner. After uptake, the internalised peptides are rapidly hydrolysed by peptidases and used as source of amino acids, nitrogen or carbon. Peptide transport systems can also have a role in the sensing of population density (Rudner et al., 1991; Koide and Hoche, 1994), and in aiding organisms to evade the host immune system (Parra-Lopez et al., 1993). Peptide transporters fall into three distinct families: the ATP-binding cassette (ABC) superfamily (Higgins, 1992), the peptide transporter (PTR) family (Steiner et al., 1995), and the oligopeptide transporter (OPT) family (Lubkowitz et al., 1998).

The OPT family members have no significant sequence homology or regions of homology with the ABC and PTR peptide transporters. To date, members of the OPT family have been identified in yeast (i.e. *Candida albicans*, CaOpt1p (Lubkowitz et al.,

1997); Schizosaccharomyces pombe, Isp4p (Lubkowitz et al., 1998); Saccharomyces cerevisiae, Opt1p and Opt2p (Hauser et al., 2000) and in one plant (i.e. Arabidopsis thaliana, AtOPT1 to AtOPT9 (Koh et al., 2002). The OPT transporters have 12 or 14 transmembrane domains. The signature motif SPYxEVRxxVxxxDDP(S/T) was identified in the yeast OPT family members. The OPT transporters identified in *A. thaliana* share a high homology with CaOPt1p (49 % to 53 % similarity), but formed a distinct subgroup compared to the yeast OPT members. The OPT proteins translocate peptides of 3 and/or 4 and/or 5 amino acids residues, e.g. CaOpt1p transports peptides. Hydrolysis of ATP provides the energy for the peptide transport by the ABC transporters, and the proton-motive force drives the peptide translocation in the case of the PTR family members. The oPT family.

The LS2-2/HS-2 deduced amino acid sequence had significant homology with the peptide transporters Opt2p and Opt1p from *S. cerevisiae*, and with Isp4p from *S. pombe*. The oligopeptide transporter function of Opt2p has only been predicted from the amino acid sequence. Investigations on the Opt1p from *S. cerevisiae* revealed that it is a 12-14 TMs integral membrane protein transporting four to five amino acids oligopeptides. Opt1p was shown to have high affinity for enkephalin, an endogenous mammalian pentapeptide, and for gluthatione, an antioxidant. Opt1p seemed to be normally expressed only during sporulation, but little is known about the genes and proteins involved in the regulation of *OPT1* expression (Hauser et al., 2001).

Isp4p from *S. pombe* is a tetrapeptide transporter whose gene expression is up-regulated during nitrogen starvation (Lubkowitz et al., 1998). As meiosis in *S. pombe* can be induced by nitrogen starvation, Isp4p might play a role in meiosis, but that remains to be demonstrated.

Expression of the LS2-2/HS-2-gene was only detected in the cuticle medium culture of *B. bassiana*. In *S. pombe*, Isp4p is produced in response to nitrogen starvation. Both cultures, in the basal salts medium with or without cuticle, were limited in nitrogen; thus, nitrogen starvation could not alone be the inductor of the expression of this gene. However, as expression of this probable oligopeptide transporter was not detected in the rich medium culture, its expression is likely under a carbon and/or nitrogen catabolite

repression. One hypothesis emerging is that nitrogen starvation might be a component of the signal inducing the expression, another part of the signal being linked to the presence of the cuticle.

The LS2-2/HS-2-gene is probably encoding an oligopeptide transporter of tri-, tetraand/or pentapeptide. Expression of this gene was only detected in the *B. bassiana* cultures in the medium supplemented with its host cuticle. Thus, the function of this putative oligopeptide transporter is presumably the up-take of the peptides produced by the degradation of the beetle cuticle by proteases and peptidases secreted by the fungus. The subtilisin-like protease PR1 was shown to be secreted by *B. bassiana* in basal salt media containing insect cuticle (St Leger et al., 1987b,c). This protease hydrolyases the cuticle into pentapeptides, then endo-peptidases degrades the solubilized peptides further until a chain length of about 5 is obtained (St Leger et al., 1986c). The fungus might produce this putative oligopeptide transporter for the up-take of the small oligopeptides, products of the degradation of the cuticle. Once imported into the cells, the oligopeptides are likely further hydrolysed by peptidases and used as amino acid, nitrogen or carbon source. This hypothesis is supported by the investigations of St Leger et al. who highlighted the use of the *Manduca sexta* cuticle as nutrient source during the infectious process of *M. anisopliae* (St Leger et al., 1989a).

2.2.3. ABC multidrug transporter

The LS2-1 RDA fragment deduced amino acid sequence showed significant homology with fungal protein belonging to the ABC transporter superfamily: an ABC transporter from *A. nidulans* (83 % similarity), Atr5p from *Mycosphaerella graminicola* (*M. graminicola*) (75 % similarity), bfr1p from *S. pombe* (67 % similarity), and to a less extent, to Snq2p from *S. cerevisiae* (65 % similarity) (Del Sorbo et al., 1997; Stergiopoulos et al., 2002; Nagao et al., 1995; Goffeau et al., 1996; Jacq et al., 1997) (Table 1). Northern analysis revealed that the LS2-1-gene was expressed about 2.5-fold more in the cuticle medium than in the basal salt and the rich media (Figure 12).

The ABC transporter superfamily is ubiquitous and present in both prokaryotic and eucaryotic kingdoms. The ABC transporters are able to drive the uptake or efflux of compounds by hydrolysis of ATP (Higgins et al., 2001). They have important functions in cellular metabolism (e.g. with the transport of amino acids, sugars, oligopeptides, ions as Cl^- , K^+) and detoxification by transporting actively compounds across the plasma membrane and organellar membranes. ABC transporters are able to extrude toxic compounds, preventing their intracellular accumulation and thus protecting the cells from the deleterious effects of the toxicants. ABC transporters can also be involved in establishment of resistance to a wide range of structurally and functionally unrelated compounds; this phenomenon is termed multi-drug resistance and is due to the overproduction of ABC transporter(s).

The structure of the ABC transporters includes at least one nucleotide binding domain (NBD), and several transmembrane segments (TMS). The TMSs and NBDs are generally arranged in a duplicated forward $(TMS_6-NBD)_2$ or reverse $(NBD-TMS_6)_2$ configuration, but numerous half-size transporters are also known. ABC transporter proteins are characterized by the presence of highly conserved amino acid sequence in their NBDs. Consensus sequences comprise the Walker A $[G-(X)_4-G-K-(T)-(X)_6-I/V]$ and Walker B $[R/K-(X)_3-L-(hydrophobic)_4-D]$ motif (Walker et al., 1982), and the ABC signature $[L-S-G-G-(X)_3-R-hydrophobic-X-hydrophobic-A]$ (Ames et al., 1990).

The MDR-type ABC transporters, like the well characterized Pdr5p or Snq2p, are fullsize transporters with usually twelve predicted TMSs. Snq2p (and Pdr5p) is a plasma membrane protein which mediates MDR by extruding hundreds of structurally and functionally unrelated compounds. Snq2p was also shown to modulate resistance to some metal ions like Na⁺, Li⁺ and Mn2⁺ (Wolfger et al., 2001). The LS2-1 fragment shared also high homology with an ATP transporter from *A. nidulans* and the ATPtransporter BF1 from *S. pombe*, both involved in multidrug resistance. Even more interestingly, LS2-1 showed significant homology to the ABC transporter MgAtr5 from the wheat pathogen *M. graminicola*. Deletion mutants of MgAtr5 show an increase in sensitivity to a putative wheat defence compound (resorcinol) and to a grape phytoalexin (resveratol), suggesting a role for this ABC transporter in protecting the fungus against plant defense compounds (Zwiers et al., 2003). A similar role during host infection is assumed for the ATP-transporter ABC1 from the phytopathogen Magnaporthe grisea (M. grisea) responsible for the rice blast disease (Urban et al., 1999).

The putative ABC transporter identified by the cDNA RDA was expressed about 2.5fold more in the cuticle medium than in the 2 others media without cuticle (Figure 14). Over-expression of the LS2-1-gene was specifically induced by the insect cuticle. The presence of some potentially toxic compounds in the outer layers of the cuticle, such as phenols and short-chain fatty acids, might up-regulate the expression of this putative ABC transporter of the MDR-type during the early stage of the pathogenic process. Another hypothesis is that this putative ABC transporter could extrude a fungal toxin in response to a chemical or physical signal intrinsic to the cuticle. This last hypothesis is more unlikely as a basal expression of this putative ABC transporter was detected in the basal salt medium and in the rich medium and the basal salt medium containing no potential toxic compounds according at least to their concentration.

The LS2-1-gene presumably encodes an ABC transporter of the MDR-type. To test the hypothesis of a role of this putative ABC transporter in pumping-out toxic compounds present in the insect cuticle, a preliminary experiment would consist in a Northern analysis of the LS2-1-gene expression after treatment of *B. bassiana* with various anti-fungal compounds (e.g. benomyl, cycloheximide, miconazole, hygromycin). Sensitivity to toxic compounds of promoters and deletions mutants of the LS2-1-gene would then confirm the function of the putative ABC transporter in the protection of the fungus against toxic compounds. A further study using the potential toxic compounds of the cuticle should then be realized.

2. 3. Metabolic enzyme: pyruvate decarboxylase

The deduced amino acid sequence from the HS-1 RDA fragment showed 63 % similarity with the pyruvate decarboxylase Pdc5p from *S. pombe*, the paralogue of Pdc5p from *S. cerevisiae* (Table 2). Northern analysis revealed that the HS-1-gene was expressed 6-fold more in the cuticle medium than in the basal salt medium. The

was not significantly different (Figure 16).

difference of the HS-1-gene expression in the cuticle medium and in the rich medium

The cuticle of the insect is mainly composed of proteins, chitin and lipids. The chitin is a β (1-4)-linked polymer of glucosamin. The entomopathogenic fungi secrete N-acetyl glucosaminidase to degrade the chitin in the insect cuticle. Activity of the N-acetyl glucosaminidase has been identified in culture of the entomopathogenic fungus *M*. *anisopliae* in presence of its host cuticle (St Leger et al., 1986c). The N-acetyl glucosamine is transformed in fructose-6-phosphate, glucose-6-phosphate, and finally, in glyceraldehyde-3-phosphate. The glyceraldehydes-3-phosphate can enter the pentose cycle and formation of pyruvate can occur.

The 2 major fates of the pyruvate are its oxidation to CO_2 via the TCA cycle, and, its transformation to ethanol catalysed by the pyruvate decarboylase and the alcohol dehydrogenase. Before entering the TCA cycle, pyruvate undergoes an oxidative decarboxylation catalysed by the pyruvate dehydrogenase, a mitochondrial enzyme complex. In most yeast, oxidation is predominant in aerobic conditions while the transormation of the pyruvate into ethanol occurs under anaerobic conditions (Flores et al., 2000). However, the pyruvate-bypass phenomena was also observed in yeast (Pronk et al., 1996). This process permit the formation of acetyl CoA in the cytosol through the concerted action of pyruvate decarboxylase, acetaldehyde dehydrogenase and acetyl CoA synthetase. This acetyl CoA is then imported by the mitochondria. The significance of the pyruvate-bypass phenomenum has been shown in *S. cerevisiae* (Flikweert et al., 1996; Van der Berg and Steensma, 1995).

In *S. cerevisiae*, several genes encode pyruvate decarboxylases. However, eighty percent of the activity depends on Pdc1p. The pyruvate decarboxylase activity increases in the presence of glucose due to an enhanced transcription of *PDC1* but also of *PDC5* (Hohmann, 1997). The HS-1-gene is presumably a paralogue of *PDC5*. Over-expression of the putative pyruvate decarboxylase of *B. bassiana* in the rich medium, containing 2 % sucrose, and in the cuticle medium, containing chitin, and thus providing glucose, compared to the basal salt medium, which contained only 0.01 % sucrose, could be due to the pyruvate-bypass.

The HS-1-gene is probably the paralogue of *PDC5* from *S. pombe* and *S. cerevisiae* encoding a pyruvate decarboxylase. The hydrolyse of the chitin present in the insect cuticle very likely led to the formation of glucose and then to pyruvate. The cultures of *B. bassiana* were performed under aerobic conditions. The pyruvate could then been transformed to acetyl CoA in the mitochondria, through the action of the pyruvate dehydrogenase complex, and enter directly the TCA cycle. But the pyruvate might also have been transformed into acetyl CoA into the cytosol, via the so-called pyruvate-bypass, implicating the pyruvate decarboxylase. The enhanced transcription of *PDC5* in *S. cerevisiae*, in response to glucose, supports this hypothesis.

2.4. Others fungal proteins

2.4.1. Vacuolar (H⁺)-ATPase

The LS1-1 RDA fragment amino acid sequence shared 91 % similarity with the vacuolar (H⁺)-ATPase (V-ATPase) subunit of 98 kDa from the ascomycete *N. crassa* (Table 1). Expression of this gene was expressed about 4-fold more in the cuticle medium than in the basal salt medium, as estimated by Northern blot. Expression of the LS1-1-gene was very similar in the cuticle medium and in the rich YPS medium (Figure 12).

Vacuolar (H⁺)-ATPases are a family of ATP-dependent proton pumps that generate the energy and the appropriate pH for the various compartments of the vacuolar system of the cell (Stevens and Forgac, 1997; Nelson and Harvey, 1999). The V-ATPase is composed of a peripheral V₁ domain responsible for the ATP hydrolysis and an integral V₀ domain responsible for the proton translocation. The 98 kDa V-ATPase subunit probably encoded by the LS1-1-gene is one of the five subunits composing the V₀ domain, termed subunit a. Interestingly, in the yeast, all subunit of the V-ATPase are encoded by a single gene except for the subunit a which is encoded by two genes, *VPH1* and *STV1*. Vph1p localizes to the central vacuoles whereas Stv1p seemed to localize mainly to the *trans*-Golgi. This suggest that the subunit a target the V-ATPase to
different compartments in the cell. Yeast mutants lacking Vph1p or Stv1p have been constructed (Perzov et al., 2002). In the *STV1* deletion mutant, an over-production of Vph1p in the vacuolar membrane was observed. This over-production could have occurred at the transcriptional level as in the *B. bassiana* cultures. However, if more Vph1p was synthetized in the *STV1* deletion mutant, less holoenzymes were present; thus, a decreased activity was observed.

Thus, depending on the simultaneous over-expression, or not, of the other subunits of the V-ATPase, an enhanced transcription of the subunit a might have an effect on B. *bassiana* grown in the cuticle- and rich-media.

Moreover, the multiplicity of the V-ATPase functions, via its effects on endocytosis, on vacuolar acidification, multiply the number of hypothesis. The observation that this presumed V-ATPase subunit was over-expressed in both cuticle- and undefined rich media, suggest that this enhanced transcription of the LS1-1-gene was a response to the higher amount of nutrients compared to the basal salt medium 2.

2.4.2. Mucin-like proteins

Two RDA fragments, called LS1-2 and HS-3, showed some homology with mucin proteins. LS1-2 shared 38 % similarity with MUC2 from the rat, *Rattus norvegicus* (Table 1), and HS-3 shared 35 % similarity with MUC3 from the ascarid nematode *Toxocara canis* (Table 2). In both cases, the genes seemed to be cuticle-specific, as they were neither detected in the basal salts medium 2 nor in the rich medium (Figure 12, 16).

Mucins are a family of structurally related proteins involved in mucus formation and in cell adhesion (Hilkens et al., 1992). They are glycoproteins characterized by repetitive sequences rich in threonine and serine residues (Lambrechts et al., 1996), or regions with repeated threonine and/or serine (Di Noia et al., 1998; Barnes et al., 1998; Cevallos et al., 2000), that are generally highly glycosylated. Mucins can be both secreted or membrane-bound proteins. Assembly of mucin macromolecules provide a viscous

material that protects the cell surface, while also presenting an array of specific glycans that are often involved in adhesion.

Toxocara canis, a nematode parasite of dogs, infect humans causing visceral and ocular larva migrans. In noncanid hosts, larvae neither grow nor differentiate but endure in a state of arrested development. Several mucin-like proteins have been identified in the infective larval stage by an EST strategy and constitute good candidates of immune evasion *of T. canis*.

In *S. cerevisiae*, Muc1, a mucin-like protein was shown to be indispensable for pseudohyphal differentiation and invasive growth. This putative membrane-associated mucin contains serine/threonine repeats and shows similarity with mammalian mucin-like protein implicated in the invasion of cancer cells to other tissues.

Not specifically a mucin, but an interesting glycoprotein has been characterized in the phytopathogen *Colletotrichum lindemuthianum*. This glycoprotein, UB31, was immunodetected in the extracellular matrices (ECMs) of all the tested fungi from the *Colletotrichum* species. The UB31 was localized to a thin layer of ECM just outside the appressorial wall (Figure 4). Ultrasonication did not permit to remove UB31 from the substrata suggesting that it may play a role in germlings adhesion to the plant surface (Hutchison et al., 2002). In entomopathogenic fungi, such adhesive mucilage are secreted at the germ tube tip and could play a role in the adhesion but also in the diffusion of secreted enzymes, protection from dessication and perception from host topographical signals (St Leger et al., 1993). Recently, the research group of St Leger constructed ESTs libraries from two pathogenic strains of *M. anisopliae* and from non-pathogenic fungi. They could observe that numerous EST corresponding to mucin proteins were present in the pathogenic fungi, one having significant homology with a mucin-like glycoprotein that mediates invasion by *Cryptosporidium parvum*, a protozoan parasite (Freimhoser et al., 2003; Barnes et al., 1998).

These mucins are heavily glycosylated and could also protect the fungus against the proteolytic activity of its numerous proteases and peptidases excreted to dissolve the insect cuticle, and/or against the proteolytic activity from the insect in response to fungal infection. The specific expression of the LS1-2- and HS-3-genes in the cuticle medium supported such kind of hypothesis. However, the sequence homology between

mucins can be very low, and if the RDA fragments sequence predict numerous proline, serine and threonine residues, they did not contain any of these serine/threonine repeats. The cloning of the complete ORFs should first be performed to confirm that these presumed glycoproteins are mucin-like proteins.

2.4.3. Hypothetical protein

HS-6 and the HS-7 were revealed to be very likely two *Dpn*II fragments from the same gene. Indeed, their respective deduced amino acid sequences showed both 71 % identity and 87 % similarity with an unknown protein from *N. crassa* (Table 2). Northern analysis realized using HS-6 as probe highlighted that the corresponding gene was specifically expressed in the cuticle medium (Figure 17). The cloning of the cDNA could permit the identification of some conserved domains or the further alignment of the deduced amino acid sequence on a known protein.

3. Putative genes specifically or over-expressed in the axenic larvae of L. decemlineata infected by B. bassiana

The aim of this further study was the identification of *B. bassiana* genes whose expression is up-regulated in a natural context of infection. The cDNA RDA, being a PCR-based method, is very sensitive to any kind of contaminations of the RNA sources, and then during the experiment itself. The larvae infected by *B. bassiana* should also being exempt of others micro-organisms, especially, the microbes susceptible to contain poly(A)⁺ RNA. To this aim, a simple and fast procedure to obtain surface-sterilized eggs and the further axenic larvae was established (Figure 19).

In order to obtain genes over-expressed during the infection process, the tester representation should be elaborated from the RNA isolated from the axenic larvae infected by *B. bassiana*, and the driver representation from the mix of RNA isolated from axenic larvae with RNA isolated from *B. bassiana* grown on sterilized larvae (Figure 18).

The difference products obtained appeared as a more diffused smear after each subtraction/amplification cycle with no enriched fragments (Figure 23).

The mix of RNA populations to form the driver representation has been mentioned (Hubank et al., 1999), but never published. The only mixed populations used was the addition of a DpnII fragment from a known abundant transcript in the driver representation of a second cDNA RDA experiment in order to not amplified it again. This revealed perhaps the numerous difficulties encountered for realizing such an experiment. Indeed several facts might explained the failure to enriched some DpnII fragments; the two main hypothesis are:

- (1) RNA from the axenic larvae infected with *B. bassiana* was isolated 1.5 days after their inoculation with the fungal conidia; the speed of penetration of the Colorado potato beetle larvae by our *B. bassiana* strain was estimated to occur between 20 and 25 h (Dr Zimmermann, personal communication). The axenic larvae dying in 3 to 4 days after their inoculation, it was estimated that 1.5 days after inoculation, the fungus was penetrating the insect cuticle or beginning the invasion of the haemocoele. Quantification by real-time PCR showed that only 0.02 % of the RNA isolated from the infected larvae were RNA from the fungus. *B. bassiana* was perhaps not far enough in the pathogenic process to have detectable level of fungal RNA involved in the infection and of larval RNA elicited by the penetration of the fungus.
- (2) At the opposite, the total number of up-regulated genes in both the larvae and the fungus might have been too high for an identification by the cDNA RDA method, and an approach by RT-differential display would have been preferable.

IV. Summary and perspectives

The aim of this research was the identification of genes over-expressed or specifically expressed by the insect pathogenic fungus, *B. bassiana* during infection of the Colorado potato beetle, *L. decemlineata*. Two different approaches for the study of *B. bassiana* gene expression were used: (1) gene expression in an aritficial context of infection, with the culture of the fungus in the presence of its host cuticle as inductor of the pathogenic process, (2) gene expression in a natural context of infection, with *B. bassiana* infecting the larvae of the Colorado potato beetle. In both cases, the method used was the cDNA representational difference analysis (cDNA RDA).

The cDNA RDA has for main advantages the need of low quantities of RNA, an enrichment of the differences between two mRNA populations, and thus, a low number of false positives. Indeed, among the difference products cloned from the two last cDNA RDAs, no false-positives were found. This confirmed the adapted culture conditions for such a study with this sensitive method and the general low-rate of false-positives using cDNA RDA (i.e. in comparison with the differential-display).

Another advantage of the cDNA RDA procedure is the possibility to favour the enrichment of slightly modulated gene or of highly induced gene by varying the stringency conditions of the subtractive hybridisations. In the present work, two cDNA RDAs were performed with the same material as basis, but using different stringency conditions during the subtractive hybridisations. The two low-stringency cDNA RDA allowed the identification of four genes specifically expressed in the cuticle medium culture and two genes over-expressed 2- to 5- fold. The high-stringency cDNA RDA permitted to identify two genes specifically expressed in the cuticle medium, one gene over-expressed 2.5-fold, and two genes over-expressed more than 5-fold in the cuticle medium compared to the poor medium. The high-stringency conditions of the subtractive hybridisations allowed the identification of some genes with a level of overexpression higher than these identified by low-stringency cDNA RDA. However, the low-stringency conditions favoured here the identification of more genes specifically expressed in the cuticle medium, which is also very interesting. Furthermore, only one gene was identified with both cDNA RDAs. From this study, the conclusion would also be that cDNA RDA experiments with different stringency conditions are well complementary.Eleven genes were specifically expressed or over-expressed in the cuticle medium cultures of *B. bassiana*. These genes encode three putative hydrolases (choline sulfatase, serine peptidase, α/β -hydrolase enzyme), three probable transporters (ABC transporter, siderophore transporter, oligopeptide transporter), a presumed metabolic enzyme (pyruvate decarboxylase), a probable V-ATPase, two putative mucin-like proteins and a putative protein, which shares homology with an unknown ORF of the ascomycete *N. crassa*. Grown in the presence of its host cuticle, *B. bassiana* seemed to over-expressed several hydrolases probably involved in the degradation of the cuticle. *B. bassiana* seemed to up-regulate also the expression of genes involved in stress response (i.e., siderophore transporter, choline sulfatase), detoxification (ABC transporter) and transmembrane transport (oligopeptide transporter, V-ATPase). The two putative mucin-like proteins might play a determinant role in pathogenicity.

Some putative pathogenicity determinants of *B*. *bassiana* identified during this PhD opened new perspectives in the research on the pathogenic process of hyphomycetes.

A functional study of the putative siderophore transporter of *B. bassiana* could be first realized in *S. cerevisiae*, because some strains lacking the high-affinity iron uptake system are available.

The probable MDR-type ABC transporter identified in *B. bassiana* should confer resistance to diverse drugs. Thus, the effects of several toxic compounds should be tested in the wild type and deletion/promoter mutants. A parallel Northern blot analysis, using the corresponding cDNA RDA fragment as probe, would enable the confirmation of the transcriptional up-regulation of this presumed ABC transporter by toxicants.

The cDNA sequence of the two putative mucin-like proteins should be cloned. The antisera raised against these two proteins would then be used in *in situ* hybridisation. This experiment could verify the hypothesis of the localization of these putative mucin proteins to the germ tube tip before the penetration of the insect cuticle.

Finally, a differential display approach would be probably more adapted than the cDNA RDA to identified up-regulated gene expression of *B. bassiana* in a natural context of infection. Such a study is indispensable to understand really the host-pathogen interactions during infection by entomopathogenic fungi This thesis marks one supplementary step in our understanding of the mechanisms of fungal pathogenesis in insect, which could further allow the production of more efficient mycoinsecticides and contribute to reductions in chemical pesticides use.

V. Material and methods

1. Materials

1.1. Specified chemicals, consumables and equipment

Bulk chemicals and laboratory articles were obtained from the following companies: Applichem GmbH, Darmstadt Becton, Dickinson & Co., Sparks, USA Carl Roth Gmbh & Co., Darmstadt Mallinckrodt Baker B. V., Deventer, Holland Merck, Darmstadt NeoLab, Heidelberg Roche Diagnostics Gmbh, Mannheim Serva Feinbiochemika Gmbh, Heidelberg

1.1.1. Chemicals 5-Bromo-4-Chlor-3-indolyl-b-D-galactopyranosid (X-Gal) **Diagnostic Chemicals** Lim. Coomassie brilliant blue G250 Serva CompleteTM (Protease Inhibitor Cocktail) **Roche Diagnostics** Dinatrium 3-(4-methoxyspiro {1,2-dioxetan-3, 2'-(5'-chloro) tricyclo [3.3.1.1^{3,7}]decan} -4-yl) phenylphosphat (CSPD) Roche Diagnostics Isopropyl- β -D-thiogalactoside (IPTG) PeqLab Gmbh SYBR Gold Molecular probes Phenylmethylsulfonyl fluoride (PMSF) Sigma 1.1.2. Consumables Biodyne A, B blotting membranes Pall Glass wool, silanized Serva Glass beads (0,5 mm) Serva Immobilon- P (PVDF membrane) Millipore Miracloth Calbiochem MicroSpin columns-300 HR Amersham **Biosciences** ProbeQuant[™]- G-50 micro columns Amersham **Biosciences** Hyperfilm-ECL (RPN 2103) X-ray film Kodak 1.1.3. Equipment Beta-ray counter Tri-carb 1500 Packard Gel dryer (DrygelSr.) Hoefer Scientific Instr. GenePulser (Pulse Controller) **Bio-Rad** Homogeniser Miccra D-8 **ART-Labortechnik**

PhosphoImager	Fuji BAS 1000 Fuji	
Inc.		
Microscope Zeiss Axiophot	Zeiss	
Semi-dry protein blotter apparatus	Pegasus	
Sonifier B15	Branson	
Thermocycler T3-thermoblock	Biometra	
Thermocycler 9600	Perkin Elmer	
Ultracentrifuge L8-70M	Beckman	
UV-crosslinker	Stratagene	
Vac-Man [®] laboratory vacuum manifold	Promega	
Vacuum blotter	Appligene	

1.2. Protein

1.2.1. Antibodies	
Anti rabbit IgG, peroxidase-conjugated Rabbit anti-Pr1 polyclonal antisera	Dianova Dr. Hong Wan
1.2.2. Protein weight standards	
SDS-PAGE Molecular Weight Srandards, Broad Range	Bio-Rad
Precision plus protein [™] standards, all blue	Bio-Rad

1. 3. Reagent kits for methods in molecular biology and enzymes

1.3.1. Reagent kits for methods in molecular biology	
Bio Rad Protein Assay	Bio Rad
Biotin Luminescent Detection Kit	Roche Diagnostics
DNA-free TM Kit	Ambion
Gigapack [®] III gold packaging extract	Stratagene
Lamda DASH [®] II/BamHI Vector kit	Stratagene
SYBR [®] Green Master Mix	Applied Biosystems
Minelute PCR purification kit	Qiagen
Nucleospin plasmid purification kit	Macherey & Nagel
Oligotex mRNA kit	Qiagen
Omniscript reverse transcriptase kit	Qiagen
QIAquick PCR purification kit	Qiagen
Ready-To-Go TM DNA labelling beads (-dCTP)	Amersham Biosc.
Rnase-free Dnase set	Qiagen
RNeasy plant mini kit	Qiagen
Wizard lambda preps DNA purification system	Promega
	=

<u>1. 3. 2. Enzymes</u> Alcaline phosphatase, calf intestinal

Ampli Taq DNA polymerase Biotin High Prime New England Biolabs Applied Biosystems Roche Diagnostics Caylase C3 $DpnII (10 U/\mu I)$ Lysing enzyme E. coli DNA ligase (10 U/ μ I) E. coli DNA polymerase I (10 U/ μ I) Restriction endonucleases Biolabs, Roche Diagnostic Ribonuclease H SUPERase.InTM RNase inhibitor SuperscriptTM II Rnase H⁻ reverse transcriptase Taq DNA polymerase T4 DNA ligase (400 U/ μ I) T4 DNA polymerase (5 U/ μ I)

Sigma New England Biolabs Sigma Invitrogen Invitrogen New England

Invitrogen Ambion Invitrogen Promega New England Biolabs Invitrogen

1.4. Nucleic acids

<u>1.4.1.Plasmid</u> pUC18

New England Biolabs

1.4.2. Primers

All the primers were synthetized by MWG-Biotech AG. The nucleotide code used for the degenerated primers was: Y=C or T, R= A or G, W= A or T, S= G or C, K= G or T, M= A or C, B= no A, V= no T, H= no G, D= no C and N= all.

Name	Sequence (5' - 3')
Pr1.REV	CGCAAGTATGTGTGGGGCTTACA
Pr1.FOR	CGACCACGTTTGACTACGACTCG
ActFunREV	GGNGCIATDATYTTNACYTTCAT
ActFunFOR	ACNAAYTGGGAYGAYATGGA
RTActFunREV	CCAGACTCGAGACCGAGAA
RTActFunFOR	GCTTGAGAAGTCGTACGAGCTT
ActInsREV	GGNGCIATDATYTTDATYTTCAT
ActInsFOR	ATHGTNACNAAYTGGGAYGA
RTActInsREV	CATTTGCTTCCATACCCAAGA
RTActInsFOR	TCCCTTGAGAAGAGCTATGAACTT
R-Bam-24	AGCACTCTCCAGCCTCTCACCGAG
R-Bam-12	GATCCTCGGTGA
R-Bgl-24	ATCTCAGGGGACCTGAGTCAATAG
R-Bgl-12	GATCCTATTGAC
N-Bgl-24	AGGCAACTGTGCTATCCGAGGGAA
N-Bgl-12	GATCTTCCCTCG
P-Bgl-24	ATACGTGCAGGCTGGTTACATCTG
P-Bgl-12	GATCCAGATGTA
J-Bgl-24	ACCGACGTCGACTATCCATGAACA
J-Bgl-12	GATCTGTTCATG
pUC18/DpnII-3	ATGCCTGCAGGTCGACTCTAGA
pUC18/DpnII-4	CACACAGGAAACAGCTATGACCAT

1.4.3.Oligonucleotides	
Set of dATP, dCTP, dGTP, dTTP	Promega
1.4.4.Radiolabelled nucleotide	
Redivue [α - ³² P]-dCTP, (3000 Ci/mmol, 10 mCi/ml) Biosciences	Amersham
1.4.5. DNA and RNA molecular weight markers	
100 pb DNA ladder	New England Biolabs
DNA molecular weight marker IV	Roche Diagnostic
0.24-9.5 kb RNA ladder	Invitrogen

1.5. Bacterial and fungal strains

1.5.1. Escherichia coli

XL1-Blue MRA (P2): Δ(mcrA)183 Δ(mcrCB-hsdSMR-mrr)173 endA1 supE44 thi-1 gyrA96 relA1 lac(P2 lysogen)

XL1-Blue MRF': Δ(mcrA)183 Δ(mcrCB-hsdSMR-mrr)173 endA1 supE44 thi-1 recA1 gyrA96 relA1 lac[F'proAB lacIqZΔM15 Tn10 (Tet^R)]

1.5.2. Beauveria bassiana

The strain of *Beauveria bassiana* used for this work is the strain ARSEF n° 252 obtained from the "USDA-ARS Collection of Entomopathogenic Fungi Cultures", U.S. Plant, Soil and Nutrition Laboratory, Tower Rd., Ithaca, NY,USA. This strain was originally isolated from the Colorado potato beetle, *Leptinotarsa decemlineata*, grown on a rich medium (YPG) and then stored at 4°C.

1.6. Media and stock solutions

1.6.1. Bacterial cultures medium

Luria-Bertani medium (LB): 1% Bacto tryptone; 0,5% Bacto yeast extract, 1% NaCl. LB agar: LB medium plus 2 % (w/v) agar.

1.6.2. Fungal culture media

The pH of all media was adjusted to 5.5 with 1 N NaOH.

- $\begin{array}{l} \text{Basal salts medium 1:0.2 \% (w/v) sucrose, 0.05 \% (w/v) NaNO_3, 0.1 \% (w/v) KH_2PO_4, \\ 0.05 \% (w/v) MgSO_4.7H_2O, 0.2 \, \mu g/ml \ \text{FeSO}_4.7H_2O, 1 \, \mu g/ml \ \text{ZnSO}_4.1H_2O, 0.02 \\ \mu g/ml \ \text{NaMoO}_4.2H_2O, 0.02 \, \mu g/ml \ \text{CuSO}_4.5H_2O, 0.02 \, \mu g/ml \ \text{MnCl}_2.4H_2O. \end{array}$
- Basal salts medium 2:composition identical to the basal salts medium 1, excepted for the concentrations of sucrose and sodium nitrate of 0.01 % and 0.02 %, respectively.

Basal salts medium 2 plus cuticle: basal salts medium 2 plus 0.5 % or 1 % (w/v) of ground (< 0.5 mm) pure cuticle from Colorado potato beetle adults. The cuticle of the Colorado potato beetle was washed and purified according to the protocole of Andersen et al. (1980) using about 200 whole beetles per liter of 1 % (w/v) potassium tetraborate. The pulverized cuticle was added to the previously sterilized medium (20 min at 121°C), and autoclaved 5 min at 115°C.

- Complete medium: composition identical to the basal salts medium 1 excepted for the concentrations of sucrose and sodium nitrate of 1 % (w/v) and 0.2 % (w/v), respectively.
- YPG medium: 0.2% (w/v) Bacto Yeast Extract, 1 % (w/v) Bacto peptopne, 2 % (w/v) glucose.

YPS medium: 0.2% (w/v) Bacto Yeast Extract, 1 % (w/v) Bacto peptopne, 2 % (w/v) sucrose.

YPG/S agar: YPG/S plus 2% (w/v) agar.

Water agar: distilled water plus 2 % (w/v) agar.

1.6.3. Antibiotics stock solutions

Ampicillin: 100 mg/ml in water; filter sterilized; stock at -20°C.

Chloramphenicol: 34 mg/ml in ethanol; store at -20°C.

Streptomycin sulphate: 125 mg/ml in water; filter sterilized; stock at -20° C. Tetracyclin: 5 mg/ml in 100 % ethanol; stock at -20° C.

1.6.4. Buffer and other stock solutions

Denhart's solution: 1 % (w/v) Ficoll 400, 1 % (w/v) PVP, 1 % (w/v) BSA (Sigma, fraction V)

IPTG: 200 mg/ml in water; filter sterilized; stock at -20°C.

10x MOPS buffer: 0.2 M MOPS (pH 7), 50 mM NaOAc, 10 mM EDTA (pH 8).

Northern blot prehybridization buffer: 5x SSC, 5x Denhart's solution, 0.1 % SDS, 100 μ g/ml salmon sperm DNA, 50 % deionised formamide.

2x RNA loading buffer 1: 7 M urea, 2x TBE, 0.01 % bromophenol blue Na salt, 0.05 % xylene cyanol.

10x RNA loading buffer 2: 50 % glycerol, 1mM EDTA (pH 8), 0.25 % bromophenol blue.

20x SSC: 3 M NaCl, 0.3 M Na₃-citrate; adjust pH to 7.

SM (phage buffer): 0.58 % (w/v) NaCl, 0.2 % (w/v) MgSO₄.7H₂O, 50 mM Tris-HCl (pH 7.5), 2 % gelatin.

20x SSC (pH 7): 3 M NaCl, 0.3 M sodium citrate.

50x TAE (pH 7.5): 2 M Tris-acetate; 0.05 M EDTA

- 10x TBE (pH 8.3): 0.9 M Tris-Base, 0.9 M boric acid, 20 mM EDTA.
- TE: 10 mM Tris-HCl (pH 8.0), 1 mM EDTA. X-Gal: 20 mg/ml in dimethylformamid; stock at -20°C.

2. Methods

2.1. Microbiological techniques

2.1.1.Culture of E. coli

The culture media used for growth of E. coli were the Luria-Bertani liquid medium (LB) or containing 2 % (w/v) agar (LBA) (Sambrook et al., 1989). When necessary, sodium salt-ampicillin and hydrochloride-tetracycline were added to the LA or LBA media at the respective final concentration of 60 and 25 μ g/ml.

2.1.2. Electro-transformation of E. coli

Preparation of competent cells

Ten ml of a fresh overnight culture of *E. coli* were transferred in 1 liter of LB medium and cells were grown at 37°C with vigorous shaking to an O.D.₆₀₀ of about 0,7. After chilling on ice for 30 min, cells were centrifuged at 5000 rpm for 15 min at 4°C. The cells pellets were washed once with a total volume of 1 liter of cold sterile water followed by a centrifugation at 5000 rpm for 15 min at 4°C. A second wash was then performed with 0.5 liter of cold sterile water, and centrifugation performed as previously. The pellet was resuspended in 20 ml of 10% cold glycerol, centrifuged as previously and finally resuspended to a final volume of 2-3 ml in cold 10 % glycerol. Aliquots of 50 ml were frozen in liquid nitrogen and stored at -80°C.

Electro-transformation

On ice, 45 μ l of the competent cells were mixed with 40 ng of DNA and incubated for 1 min. The mixture was then transferred to a ice-cold 0,1 cm electroporation cuvette. The Gene pulser apparatus was set to 25 μ F and 1, 25 KV, and the Pulse controller to 200 W. After transformation, the cells were immediately resuspended in 1 ml of LB medium and incubate at 37°C, 130 rpm for 1 h. Cells were then plated on selective medium containing X-gal (80 μ g/ml) and IPTG (40 μ g/ml) for the blue/white screening.

2.1.3. Screening of a phage genomic library

A phage library of the genome of *B*. *bassiana* was constructed by Hong Wan using the Lambda DASH[®]II/BamHI vector kit and the Gigapack[®]III gold packaging extract (Stratagene) according to the instruction manuals.

Plaque lifts

The plaque lifts were performed according to the instruction manual of the Lambda DASH[®]II/BamHI vector kit except for the following points. Positively charged nylon 1,2 μ m Biodyne A membranes (Pall) were used instead of nitrocellulose membranes. After the lifting of the phage plaques on the membranes, the denaturation, neutralization and rinsing steps were not performed by submerging the membranes into the solutions. Instead, the membranes were placed, plaques side up, on Whatman paper soaked in the different solutions.

Plaque hybridization

The prehybridizations were performed by incubating the membranes 1 h at 42°C in a hybridization roller bottle containing in prehybridization solution (48 % (v/v) formamide, 5X SSC, 20 mM Tris-Cl (pH 7,6), 1X Denhart's solution, 1 % (w/v) dextran sulfate, 0,1 % (w/v) SDS). A mix containing 800 μ l water, 200 μ l sonicated herring sperm DNA (10 mg/ml) and 200 ng radiolabelled probe was boiled for 10 min and then placed immediately into ice. This mix was then added to 14 ml of prewarmed fresh prehybridization buffer and used to perform hybridization overnight at 42°C. The washing was performed by incubating the membranes 3 times 10 min in 2X SSC, 0,1 % SDS at room temperature, and then 15 min in 0,2X SSC, 0,1 % SDS at 37°C. The membranes were then put in a plastic wrap and exposed to X-ray film for 1 day at – 70°C with an intensifying screen.

Purification of bacteriophage clones

Positive plaques were excised from the the agarose plate and transferred in a tube containing 500 μ l of SM buffer (0, 58 % (w/v) NaCl, 0,2 % (w/v) MgSO4.7H20, 50 mMTris-Cl (pH7,5), 0,01 % (w/v) gelatin). After the addition of 50 μ l of chloroform, the tubes were incubating for 1 h at room temperature and then stock at 4°C. The phage solutions was then tittered according to the instruction manual of the Lambda DASH[®]II/BamHI vector kit. Plates containing 5000 pfu were prepared and subjected to lifting for a new selection of positive plaques. The screening was performed until pure phage was obtained.

Isolation of Lambda DNA

Isolation of DNA of the purified phage was performed using the Wizard lambda preps DNA purification system and the Vac-Man[®] laboratory vacuum manifold (Promega) according to the instruction manuals.

2.1.4. Culture of B. bassiana

Preparation of conidia stock

Glass tubes with YPG agar medium were inoculated with a -80° C stock of *B*. bassiana conidia, incubated 14 days at 25°C, and then stored at 4°C for up to 6 months.

Solid and liquid cultures of B. bassiana

Five milliliter of sterile distilled water was added to the conidia from the stock at 4°C. The conidial suspension was then pumped and filtered through a glass funnel stuffed by silanized glass wool at one extremity. About 200 μ l of the filtered conidial suspension was used to inoculate Petri dishes with YPG or YPS medium. Plates were incubated for 10 days at 25°C. A final concentration of 5x10⁶ to 5x10⁷ spores/ml was used for the different liquid cultures, which were then incubated at 25°C with agitation (150 rpm in a rotary shaker).

Transfer experiment

About 5 YPS plates were inoculated with conidia from the stock at 4°C and incubated for 10 days at 25°C (Figure 23). The fresh conidia were then used to inoculate 2 Erlen-Meyer flasks containing each 500 ml complete medium with 5.10⁶ spores/ml. After 4 days culture at 25°C, 150 rpm, spores were collected by 10 min centrifugation at 3000xg and 4°C. Spores were rinsed 1 time with cold PBS, centrifuged, resuspended in

basal salt medium 1 and finally filtered through 3 layers of Miracloth. This spores suspension was used to inoculate 2 Erlen-Meyer flasks containing each 250 ml of basal salts medium 1 with a final concentration of 5.10⁷ spores/ml. The flasks were incubated 23 h at 25°C, 150 rpm, to allow the germination of the spores. The germlings were then collected by 10 min centrifugation at 3000xg and 4°C, rinsed with cold PBS and centrifuged again. The germlings were then resuspended in basal salt medium 2 and transferred ina Erlen-Meyer flask containing 100 ml of basal salts medium with or without 0.5-1 % ground cuticle of the Colorado potato beetle. After 3.5 h culture at 25°C with agitation (150 rpm), the young mycelium was centrifuged at 3000xg and 4°C for 10 min, resuspended with cold PBS and centrifuged in the same conditions. The cells pellets were then immediately frozen in liquid nitrogen until used for RNA isolation.

Preparation of pure cuticle from the Colorado potato beetle

Frozen beetles (about 150 adults) were homogenized in 800 ml cold solution of 1 % potassium tetraborate, and immediately rinse several times with the same solution. The ground cuticle was then stirred overnight in 1 % potassium tetraborate at room temperature. The ground cuticle was rinsed several times with distilled water and dried at room temperature. The cuticle was then milled to a fine powder (< 0.5 mm) using a mortar and a pestle, washed several with 1 % potassium tetraborate and rinsed extensively with distilled water. The powder was then allowed to settle several times and any floating material was discarded (the cuticle contains chitin and is also retained at the bottom of the flask when any tegument particles stay in suspension). This operation is repeated several times to obtain, in theory, pure cuticle particles.



Figure 23. Transfer experiment leading to an artificial context of infection by Beauveria brassiana.

The transfer experiment of B. bassiana allowed an increase of the number of spores and, in parallel, a progressively decrease in the concentration of carbon and nitrogen sources to a levelwhich should permit the induction of the infectious process

In the last but one medium, the carbon source concentration (0.2 % sucrose) permit the germination of the spores in 23 hours. The germinated spores were washed and transferred into a firesh medium with hower concentrations of carbon and ninegen sources (respectively, 0.1 % sucrose and 0.02 % softium nitrate) derepressing the synthesis of the protease Pr1, which was shown to be involved in the infections process of Manisoplice and subjected to a general carbon and nitrogen repression mechanism. This last mechan was complemented or not with 0.5-1 % (w/v) of purified ground caticle of the host insect, the Coloado potatobeetle. After 3.5 hours culture, total RNA were isolated from each culture. All the solutions were directly treated with 1:1000 volume of DEPC, or prepared with DEPC-treated water (e.g. for the Tris buffer solution).

2.2.1.RNA isolation

RNA was isolated from germinated spores, young mycelia of *B. bassiana* or from the (infected) larvae using the RNeasy plant mini kit (Qiagen) and according to the recommendations of the manufacturer. In every case, the RNA source was ground in liquid nitrogen using a mostar and a pestle. An on-column DNase digestion was performed with the RNase-free DNase set (Qiagen). After RNA isolation, the integrity of the RNA was checked by gel electrophoresis. One μ l of the diluted SYBR Gold (at 1/100 in DMSO) and the appropriate volume of RNA loading buffer 1 was added to the RNA samples, heated for 3 min at 70°C, chilled on ice and then loaded on a 1 % agarose gel (with TBE buffer).

2.2.2.RT-PCR

Reverse transcription

Reverse transcription was achieved using the Omniscript reverse transcriptase (Qiagen) according to the instructions of the manufacturer.

PCR amplification of the first-starnd cDNA

Five μ l of the reverse transcription was used to perform the PCR amplification. The PCR reactions were performed with 1x PCR buffer, 200 μ M of each dNTP, 0.4 μ M reverse primer, 0.4 μ M forward primer, 2.5 mM MgCl₂ and 2.5 units of Taq polymerase (Promega).

Quantitative PCR

One to 2 μ l of the reverse transcription reaction were used for the further quantitative PCR using the Master Mix (Applied Biosystems) according to the instructions of the manufacturer. The amplification reactions conditions were as following: 10 min at 95°C (initial denaturation and activation of the heat-activated Taq polymerase in the Master mix), and then 40 cycles with denaturation 15 sec at 95°C and 1 min elongation at 60°C. Each reaction was followed by the melting profiles of the amplified DNA via the measurement of the SYBR green fluorescence.

2.2.3. Northern blot analysis

Formaldehyde gel

RNAs were separated on a 0.6 M formaldehyde, 1.2% agarose gel. Twenty μ g RNA in a volume of 4.7 μ l, 1 μ l 10x MOPS, 3.3 μ l deionised formaldehyde, 10 μ l deionised formamide and 1 μ l ethidium bromide (200 μ g/ml) were mixed, denatured 10 min at 65°C and chilled on ice. Two μ l of the RNA ladder 2 were then added to the mix and each RNA sample were rapidly loaded on the gel. After the visualization of the RNA

on a UV transilluminator, the gel was destained overnight in DEPC-treated water at 4°C.

RNA transfer on nylon membrane

The RNAs were then transferred on a nylon membrane positively charged using a vacuum blotter at 40-50 mbar for 45 min. The RNAs were fixed on the membrane by 2 times UV irradiation (10 mJ/cm^2).

Northern blot hybridization

The membrane was prehybridized for 3-4 h at 42°C with 15 ml prehybridization buffer in a roller bottle. The RDA fragments cloned in pUC18 were amplified using the primers pUC18/*Dpn*II-3 and pUC18/*Dpn*II-4 using standard PCR conditions. The PCR products were purified with the PCR purification kit (Qiagen). Fifty ng of each purified PCR product was labelled with $[\alpha$ -³²P]-dCTP using the Ready-To-Go DNA labelling beads (Amersham), and then purified with the Microspin G-25 columns (Amersham) according to the manual of the producer. The radiolabelled probe (1.000.000 cpm, quantified with the Cerenkov radiation) was denatured for 15 min at 37°C after addition of 0.1 volume of 1 N NaOH. The denatured probe was added to 5 ml prewarmed prehybridisation buffer and used for hybridisation overnight at 42°C. The filters were first washed 2 times with a solution containing 2x SSC, 0.1 % SDS at room temperature, and then 2 times with a 0.1x SSC, 0.1 % SDS at 42°C. The membranes were finally washed briefly with 2x SSC, wraped in a plastic wrap and autoradiography or phosphoimager was set up.

2.2.4. cDNA synthesis

DNase treatment

About 300 μ g of the total RNA isolated from the culture in medium with cuticle and from the culture without cuticle were treated in parallel. First, each 300 μ g were concentrated and submitted to an additional DNase treatment using 3 RNeasy mini spin columns, the RNase-free DNase set and the RNA clean-up protocole of the handbook (Qiagen).

mRNA purification

The mRNA were then purified using the Oligotex mRNA mini kit according to the Oligotex mRNA Spin-column protocol of the handbook. Two hundreds fifty μg total RNA were used as starting material. The elution of the columns was performed with 30 μ l of hot OEB buffer (70°C), the eluat was heated for about 2 min at 70°C and used for a second elution. About 4 μ g mRNA could be recovered from the 250 μ g total RNA.

cDNA synthesis

All the enzymes, buffers and the oligo $(dT)_{12-18}$ were purchased from Invitrogen, with the exception of the SUPERase.InTM, the Rnase inhibitor, which was purchased from Ambion, and the 10 mM dNTPs mix from Promega. The protocol was adapted from the SuperScriptTM plasmid system for cDNA synthesis and plasmid cloning instruction manual (Invitrogen). 1.5 μ g mRNA (in 9 μ l) plus 1 μ l (1 μ g/ μ l) oligo(dT)₁₂₋₁₈ were heated to 65°C for 5 min and chilled on ice. To the denaturated mRNA, were then

added 4 µl 5x First-strand buffer, 2 µl 0.1 M DTT, 1 µl 10 mM dNTPs mix and 1.5 µl SUPERase.In (30 units). At this stage, an aliquot of 2 μ l could be mixed with a radiolabelled dNTP (e.g. 1-2 μ Ci of [α -³²P]-dCTP) to perform the "Monitor I" reaction. The mixture was incubated for 2 min at 42°C before the addition of 1.5 µl SuperScript II RT. The reverse transcription was performed for 1h45 min at 42°C, and 15 additional minutes with a progressive increase of the temperature from 42 °C to 50°C. To the 18 μ l of the first-strand cDNA synthesis reaction were added: 93 μ l nuclease-free water, 30 μ l 5x second-strand cDNA buffer, 3 µl 10 mM dNTPs mix, 1 µl E. coli DNA ligase (10 $U/\mu l$), 4 $\mu l E$. coli DNA polymerase I (10 $U/\mu l$) and 1 $\mu l E$. coli RNase H (2 $U/\mu l$). Ten μ l of the reaction could be removed and a radiolabelled nucleotide (e.g.1-2 μ Ci of [α -³²P]-dCTP) added to perform the "Monitor II" reaction. The reaction was incubated 2 to 6 h at 15°C. After completion of the reaction, the cDNA was cleaned up by a phenol:chloroform:isoamyl alcohol (25:24:1) extraction, followed by a cDNA precipitation using 0.5 volume of 7.5 M NH₄OAc and 2.2 volume of cold 100 % ethanol for 30 min at -20°C. After centrifugation for 20 min at 16.000xg and 4°C, the cDNA pellet was resuspended in 20 μ l TE buffer.

2.3. DNA methods

2.3.1. Isolation of genomic DNA from B. bassiana

Spores of *B. bassiana* n°252 were harvested from 10 days old YPG plates and used to inoculate 20 ml liquid YPG medium at a final concentration of 5.10⁶ spores/ml. The culture was incubated 5 days at 25°C on a rotary shaker at 150 rpm. The blastopores were then filtered through 3 layers of Miracloth and used to inoculate 60 ml of YPG medium to a final concentration of 10^7 spores/ml. The culture was incubated for 12 to 13 h, with the conditions mentioned previously, to allow the start of the germination. The germlings were centrifuged at 3000xg for 10 min, rinsed once with distilled water and centrifuged again as previously. The pellet was resuspended in 30 ml SCE buffer (1 M sorbitol, 0.1 M sodium citrate (pH 7), 50 mM EDTA (pH 8)) containing 30 mg of Caylase C3 and 30 mg of Lysing enzyme. The mixture was incubated 20 h at 37°[to ensure that more than 80 % of the spores have been protoplasted. The cells were centrifuged for 15 min at 3000xg and room temperature, rinsed once with cold SCE buffer and then resuspended in 15 ml lysis buffer (20 mM Tris-Cl (pH 8), 25 mM EDTA (pH 8), 0.25 mM NaCl, 1 % SDS) containing 5 mg proteinase K. The mixture was incubated overnight at 55°C or until the solution was cleared. A phenol:chloroforme:isoamyl alcohol (25:24:1) extraction and then a DNA precipitation with 3 M sodium acetate (pH 7) and isopropanol were performed. The DNA pellet obtained was resuspended in TE containing 20 μ g/ml DNase-free Rnase A.

2. 3. 2. Recovery of DNA fragments from low-melting agarose gel

The DNA fragments were recovered from a low-melting agarose gel using the buffer QX1 and the MinElute PCR purification kit (Qiagen) following the instruction of the manufacturer.

2.3.3. Southern blot analysis (Southern, 1975)

DNA transfer on a nylon membrane

The transfer of restricted DNA from agarose gels to a positively charged nylon membrane was performed as following. A depurination of the DNA was accomplished by incubating the gel in 0.25 M HCl for 15 min, followed by a denaturation a 0.5 M NaOH, 1.5 M NaCl solution for 30 min. The pH of the gel was then neutralized in 1 M Tris-HCl (pH 7.5), 1.5 M NaCl solution for 30 min. The transfer of the DNA to the positively charged nylon membrane was performed using a Vacuum Blotter (Appligene) at 50 mbar for 45 min. The DNA was then fixed to the membrane by UV irradiation (2 times cross-linking).

Hybrisation and detection with biotin-labelled probes

All the buffers and solutions were prepared according to the protocole provided by Roche Diagnostics. The prehybridisation of the membrane was performed with 10 ml hybridisation solution (with 50 % formamide) per 100 cm² of the membrane, at 42°C for two hours. The labelling of the probe with biotin was realized with the Biotin-High Prime mix from Roche Diagnostic according to the recommendations. The hybridisation was carried out overnight at 42°C with 10 ml prehybridisation solution containing the denaturated biotin-labelled probe. The membrane was then washed 1 time with 2x SSC, 0.1 % SDS for 30 min at room temperature with gentle agitation. The detection was performed using the Biotin Detection Kit according to the instructions of the manufacturer.

2.3.4. cDNA representational difference analysis (cDNA RDA)

A scheme of the procedure is presented figure 24. The cDNA RDA is a very sensitive technique, thus, big care should be taken to avoid any outside contamination (e.g. use of aerosol-resistant pipette tips, gloves changed often) and cross-contamination during the numerous pipetting operations required for the RDA procedure. All the solutions (e.g. buffers, dNTPs, oligonucleotides) were aliquoted for a single use. All the reagents, water and plastic ware should be RNA/DNA/nuclease free. A detailed laboratory protocol is available from the authors of the article "Optimization of cDNA RDA for the identification of differentially expressed mRNAs" (Pastorian et al., 2000); thus, the technical points, as the centrifugation speeds and times for example, are not presented here. The pair of 12/24-mer primers are listed in the paragraph 1.4.2 of "Materials".

Digestion of the cDNA with DpnII

One to 5 μ g cDNA (from each culture) was digested 30 units of *Dpn*II (New England Biolabs) for 3 hours at 37°C, before one phenol:choloform:isoamyl alcohol, and one chloroform: isoamyl alcohol extractions. The digested DNA was then passed through separated S-300 spin columns (Amersham Biosciences) and precipitated with ammonium acetate for at least 1 h at -20°C. The cDNA pellet was rinsed with 70 % ethanol and resuspended in 20 μ l TE buffer.

Ligation of PCR primers

A core mix, containing 9 μ l 10x ligation buffer, 9 μ g 24-mer primer, 4.5 μ g 12-mer primer, and water to 45 μ l, was prepared. Twenty μ l of the core mix were added to each tube containing the resuspended *Dpn*II-digested cDNA. The tubes were then placed in a preheated 55°C thermoblock and the block was brought to the cold room and allow to cool slowly to a temperature < 10 °C to permit to the 12-mer and the 24-mer to anneal and form cohesive ends complementary to the DpnII sites on the end of the digested cDNAs. A core mix containing the ligase buffer and the T4 DNA ligase (New Englands Biolabs, 400 cohesive end units/ μ l) was prepared and added to each of the cooled tubes. The ligation reaction was performed overnight at 15°C in order to ligate the 24-mers onto the 5'ends of the cDNAs. The next morning, the ligase was inactivated by heating the tubes 10 min at 65°C, and the reactions passed through separated spin columns, as previously. The volume of the eluates was adjusted to 100 μ l. PCR amplification

A PCR master mix was prepared for 12 reactions of 200 μ l, 6 with the ligated cDNA used to form the future driver representation (for example, from the culture without cuticle) and 6 others reactions using the ligated cDNA used to form the future tester representation (for example, from the culture with cuticle). This PCR master mix contained the PCR buffer, the MgCl2 solution, the dNTPs and the 24-mer used during the previous ligation. The master mix is transferred into each of the tubes containing the cDNA, and the PCR tubes are heated at 72°Cfor 1-2 min. Then, the Taq polymerase mix (with the AmpliTaq polymerase, LD from Apllied Biosystems) is added to the preheated tubes and the reactions were incubated 5 min at 72°C to allow the Taq polymerase to fill-in the cDNA ends. The PCR cycling is then as following: (95°C 45 sec/ 72°C 4 min)x 25 cycles and 72°Cfor 10 min. Following the PCR, the 6 PCR reactions from the cDNA population 1 were pooled in 1 tube, and the 6 PCR reactions corresponding to the cDNA population 2 were resuspended in 100 μ l TE.

Removal of the PCR primers by DpnII

Ninety μ l of each of the amplified DNA were digested with 45 units *Dpn*II for 3h at 37°C to completely restrict the PCR primers. A phenol:chloroform:isoamyl alcohol and a chloroform:isoamyl alcohol extraction were performed, following by a purification using the S-300 spin columns and a DNA precipitation with ammonium acetate. The DNA pellets were then resuspended in75 μ l TE buffer. The 2 tubes contained now respectively the so-called tester (from the medium + cuticle) and driver (from the medium – cuticle) representations.

Tester cDNA preparation

One μg of the tester representation cDNA only were ligated with a new 12/24-mer adaptor, according to the same procedure used previously.



Figure 23. Schematic diagram of the cDNA representational difference analysis

Hatched boxes show the oligonucleotides used to generate difference products. The process is shown up to the first difference product. To generate the second and third difference products, the products of the previous round are reintroduced, into the scheme at the tester stage (adapted from Hubank and Schatz (1994)).

Subtractive hybridization

To perform a subtractive hybridisation with a tester:driver ratio of 1:10, 0.5 μ g of the 12/24-mers-ligated tester representation were mixed with 5 μ g of the driver representation in a total volume of 50 μ l. A phenol:chloroform:isoamyl alcohol and then a chloroform:isoamyl alcohol extractions were performed, following by a precipitation using ammonium acetate for at least 2 h at -20°C to coprecipitate the tester:driver cDNAs. The cDNAs pellet was resuspended in 4 μ l hybridisation buffer (30 mM EPPS (pH 8), 3 mM EDTA) and heated in the preheated PCR block for 1 min at 95°C to denature the cDNAs. The denatured cDNA were then immediately transferred to a tube containing 1 μ l 5 M NaCl (preheated at 95°C), mixed well and covered with mineral oil. The cDNAs mixture was then incubated 3 additionnal minutes before allowing the temperature to soak at 67°C. The samples were then incubated for about 20 h at 67°C to allow the cDNAs to hybridise with their complementary strands.

PCR amplification of the subtracted tester cDNAs

Following the hybridisation, the mineral oil was removed and the sample diluted with 45 μ l water. Ten μ l of the sample was then used for the PCR amplification. The PCR was performed in 2 steps. First, the 10 μ l sample were subjected to amplification in 200 μ l reaction via 7 cycles with (95°C 45 sec/ 72°C 4 min). After the 7 cycles, 8 times 1/20th of the sample were transferred in another tube containing fresh PCR mix and polymerase and subjected to 20 further PCR cycles. After the PCR, the 8 tubes were pooled together and cleaned up by phenol:chloroforme:isoamyl alcohol, chloroforme:isoamyl alcohol extractions and precipitation. The difference product 1 was obtained.

Removal of the PCR primers from the DP1

The DP1 was then restricted by 30 units of the DpnII enzyme for 3 h at 37°C and purified as previously mentioned. This "cut DP1" was ligated to a new pair of 12/24mers and used as the tester for a further subtractive hybridisation with a higher tester:driver ratio.

For the second round of subtraction/amplification RDA experiment, a tester:driver ratio of 1:100 was used; for the third round, which used the difference product 2 as tester, a tester:driver ratio of 1:5.000 was used, and for the fourth round, using the difference product 3 as tester, a ratio of 1:25.000 was used. At each RDA round, a new set of 12/24-mer primers was used. In each round of RDA the amount of driver remained constant (5 mg) whiole the amount of tester diminished to 50 ng, 1 ng and 0.2 ng for the second, third and fourth round, respectively. For the third and the fourth round, the number of PCR cycles of the second run (after the 7 cycles) was 25 and 30 cycles, respectively.

For the high stringency RDA experiment, 3 round of subtraction/amplification were performed using the tester:driver ratios of 1:100, 1:800 and 1:10.000 respectively. The amount of driver was also maintained to 5 μ g per subtraction. The protocole used was only slightly modified concerning the dilution of the subtraction reaction and the number of PCR reactions performed.

2.4. Protein methods

2.4.1. Isolation of total protein extract from B. bassiana

The germinated spores or mycelia from *B. bassiana* were sedimented by centrifugation at 5000 rpm for 15 min at 4°C. The pellet was washed once in PBS and store at -80°C or used immediately for protein purification. Sediments were resuspended in lysis buffer (7 M urea, 2M thiorea, 4 % CHAPS, 40 mM Tris-base, 1 % DTT, 1 X Complete) and sonicated 8 times 15 sec at 4°C with 50 % duty cycle. Ultracentrifugation of the samples was then performed at 30.000 rpm at 15 °C for 30 min. The supernatants were transfered into a new tube and the protein concentrations were estimated using the Bradford assay. Protein solutions were aliquoted and stored at -80°C.

2.4.2. Determination of protein concentration (Bradford, 1976)

The protein assay kit (Bio-Rad) was used for the determination of 1-20 μ g protein using BSA as a standard protein. Fifty μ l of different dilutions of the protein solution were mixed with 0.5 ml of the reagent from the kit freshly diluted 5 times in water and filtered. After a period of 5 min to 1 h the OD₅₉₅ was measured and plot versus concentration of standards.

2.4.3. SDS-polyacrylamide gel electrophoresis (Laemmli, 1970)

Proteins samples were separated on 12.5 % polyacrylamide separating gels (375 mM Tris-Cl (pH 8.8), 12.5 % acrylamide-bis-acrylamide (30:0.8), 0.1 % SDS) with a 4.5% stacking gel (125 mM Tris-Cl (pH 6.8), 4.5 % acrylamide-bis-acrylamide (30:0.8), 0.1 % SDS, 0.4 % APS, 0.4 % TEMED). The 2 X protein sample loading buffer was 125 mM Tris-Cl (pH 6.8), 4 % SDS, 10 % b-mercaptoethanol, 10 % glycerol and 0,02 % bromophenol blue. Electrophoresis was done in 1x SDS-Tris-Glycine buffer (33 mM Tris-Cl (pH 6.8), 0.19 M glycine, 0,1 % SDS) at 25 mA.

2.4.4.Coomassie blue staining of gels

After electrophoresis the polyacrylamide gel was soak in the staining solution (45 % methanol, 10 % acetic acid, 0,2 % Coomassie brillant blue R250) for about 30 min at room temperature with gentle shaking. The gel was then soaked 3 times 30 min in destaining solution (5 % methanol, 4,2 % glacial acetic acid) with gentle shaking at room temperature.

2.4.5.Immunoblotting

Protein transfer on the membrane

After electrophoresis, the proteins were transferred to a PVDF membrane using a semidry blotter apparatus. The PVDF membrane was first soaked in methanol for 15 sec and then in the Schafer-Nielson transfer buffer (48 mM Tris-base, 39 mM glycin, 20 % methanol, 10 % SDS) for 10 min. On the blotter were placed respectively, 3 sheets of Whatmann paper soaked in transfer buffer, the gel, the prepared membrane and 3 additional sheets of Whatmann paper soaked in transfer buffer (Sambrook et al., 2001). The transfer was performed at 0,8 mA/cm² for 50 min.

Immunodetection

All the different incubations of the membranes were performed at room temperature with gentle shaking. The membranes were soaked for 1 hour in blocking solution made up of TBS-T buffer (10 mM Tris-Cl (pH 8), 150 mM NaCl, 0.05 % (v/v) Tween 20) and 5 % (w/v) of non-fat dried milk powder. The membranes were then incubated with the primary antibody diluted in 20 ml TBS-T for 1 hour. After washing with TBS-T buffer two times for 10 min, the membranes were incubated with the secondary antibody coupled to horseradish peroxidase (HRP) diluted 20.000 times in TBS-T buffer with 1% (w/v) non-fat dried milk powder for 1 hour. The membranes were finally washed 2 times in TBS-T buffer for 10 min and the detection step performed.

Detection of the HRP conjugated secondary antibody-ECL

The membranes were incubated for 1 min at room temperature in the ECL detection solution (0.068 mM p-coumaric acid (in DMSO), 1.25 mM luminol (in 0.1 M Tris-Cl, pH 8.5), 0.09 ‰ hydrogen peroxide). Once the excess of detection solution removed, the membranes were cover with a plastic wrap and exposed to X-ray films.

2.5. Softwares

Platform	Software	Task
Apple Macintosh	DNA Strider	Sequence editor
	Laser gene	Sequence editor
	C	Mapping of restriction enzyme sites
		Multipe alignments of sequences
	Adobe Photoshop	Image processing
	MacBAS	Density measurement
PC Viewfinder Lite GeneAmp 5700	Image processing	
	GeneAmp 5700	Real-time PCR
www (NCBI)	BLAST	Protein and nucleotide similarity search

VI. References

- Ames, G. F., Mimura, C. S., and Shyamala, V. (1990). "Bacterial periplasmic permeases belong to a family of transport proteins operating from Escherichia coli to human: Traffic ATPases." <u>FEMS Microbiol Rev</u> 6(4): 429-46.
- Andersen, S. O. (1980). "Cuticular sclerotization." In: <u>Cuticle techniques in Arthropods.</u> Miller, T.A., Eds.: 185-217.
- Anderson, T. E., Roberts, D. W., and Soper, R. S. (1988). "Use of *Beauveria bassiana* for suppression of Colorado potato beetle populations in New York State (Coleoptera: Chrysomelidae)." <u>Environmental Entomology</u> 17: 140-145.
- Ardon, O., Nudelman, R., Caris, C., Libman, J., Shanzer, A., Chen, Y., and Hadar Y. (1998). "Iron uptake in Ustilago maydis: tracking the iron path." <u>J Bacteriol</u> 180(8): 2021-6.
- Ardon, O. H., Weizman, R., Libman, J. Shanzer, A., Chen, Y., and Hadar, Y. (1997). "Iron up-take in *Ustilago maydis*: studies with fluorescent ferrichrome analogues." <u>Microbiology</u> 143: 3625-3631.
- Barnes, D. A., Bonnin, A., Huang, J. X., Gousset, L., Wu, J., Gut, J., Doyle, P., and Lipman, D. J. (1998). "A novel multi-omain mucin-like glycoprotein of *Cryptosporidium parvum* mediates invasion." <u>Mol Biochem Parasitol</u> 96:93-110.
- Blow, D. M., Birktoft, J. J., Hartley, B. S. (1969). "Role of a buried acid group in the mechanism of action of chymotrypsin." <u>Nature</u> 221(178): 337-40.
- Boucias, D. G. and Pendland, J. C. (1984). "Nutritional requirements for conidial germination of several host range pathotypes of the entomopathogenic fungus *Nomurea rileyi*." J Invert Pathol **43**: 288-292.
- Boucias, D. G. and Pendland, J. C. (1991). Attachment of mycopathogens to cuticle. <u>The fungal spore and disease initiation in plants and animals.</u> G. T. Cole, Hoch, H. C. (Eds). New York und London, Plenum Press: 101-124.
- Bradfisch, G. A., and Harmer S. L. (1990). "Omega-Conotoxin GVIA and nifedipine inhibit the depolarizing action of the fungal metabolite, destruxin B on muscle from the tobacco budworm (*Heliothis virescens*)." <u>Toxicon</u> **28**(11): 1249-1254.
- Bradford, M. M. (1976). "A rapid and sensitive method for the quantitation of microgramm quantities of protein utulizing the principle of protein-dye binding." <u>Analytical Biochemistry</u> 72: 248-254.
- Bussey, H., Storms, R.K., Ahmed, A., Albermann, K., Allen, E., Ansorge, W., Araujo, R., Aparicio, A., Barrell, B., Badcock, K., Benes, V., Botstein, D., Bowman, S., Bruckner, M., Carpenter, J., Cherry, J.M., Chung, E., Churcher, C., Coster, F., Davis, K., Davis, R.W., Dietrich, F.S., Delius, H., DiPaolo, T., Hani, J. (1997). "The nucleotide sequence of *Saccharomyces cerevisiae* chromosome XVI." <u>Nature</u> 387(6632 Suppl.): 103 - 105.
- Cantwell, B. A., Brazil, G., Murphy, N., and McConnell D. J. (1986). "Comparison of expression of the endo-beta-1,3-1,4-glucanase gene from *Bacillus subtilis* in *Saccharomyces cerevisiae* from the CYC1 and ADH1 promoters." <u>Curr Genet</u> 11(1): 65-70.
- Cantwell, G. E., Cantelo, W. W., and Schroder, R. F. W. (1986). "Effect of *Beauveria bassiana* on underground stages of Colorado potato beetle, *Leptinotarsa decemlineata* (Coleoptarsa: Chrysomelidae)." <u>Great Lakes Entomologist</u> 19: 81-84.

- Cevallos, A. M., Bhat N, Verdon R, Hamer DH, Stein B, Tzipori S, Pereira ME, Keusch GT, Ward HD. (2000). "Mediation of *Cryptosporidium parvum* infection in vitro by mucin-like glycoproteins defined by a neutralizing monoclonal antibody." <u>Infect Immun</u> 68(9): 5167-75.
- Cevallos, A. M., Zhang, X., Waldor, M. K., Jaison, S., Zhou, X., Tzipori, S., Neutra, M. R., Ward, H. D. (2000). "Molecular cloning and expression of a gene encoding *Cryptosporidium parvum* glycoproteins gp40 and gp15." <u>Infect Immun</u> 68(7): 4108-16.
- Charnley, A. K. (1989). Mechanisms of fungal pathogenesis in insects. <u>The biotechnology of fungi for improving plant growth.</u> E. J. M. Whipps and R. D. Lumsden. London, Cambridge University: 85-125.
- Charnley, A. K., and St. Leger, R.J. (1991). "The role of cuticle-degrading enzymes in fungal pathogenesis in insects." In Cole & Moch: 267-286.
- Clarkson, J. M., and Charnley, A.K. (1996). "New insights into the mechanisms of fungal pathogenesis in insects." <u>Trends in Microbiol</u> **4**(5).
- Del Sorbo, G., Andrade, A.C., Van Nistelrooy, J.G., Van Kan, J.A., Balzi, E., and De Waard, M.A. (1997). "Multidrug resistance in *Aspergillus nidulans* involves novel ATP-binding cassette transporters." <u>Mol Gen Genet</u> 254(4): 417-426.
- Di Noia, J. M., D'Orso, I., Aslund, L., Sanchez, D. O., Frasch, A. C. (1998). "The *Trypanosoma cruzi* mucin family is transcribed from hundreds of genes having hypervariable regions." J Biol Chem **273**(18): 10843-50.
- 9. Dujon, B., Albermann, K., Aldea, M., Alexandraki, D., Ansorge, W., Arino, J., Benes, V., Bohn, C., Bolotin-Fukuhara, M., Bordonne, R., Boyer, J., Camasses, A., Casamayor, A., Casas, C., Cheret, G., Cziepluch, C., Daignan-Fornier, B., Dang, D. V., De Haan, M., Delius, H., Durand, P., Fairhead, C., Feldmann, H., Gallon, L., Kleine, K. et al. (1997). "The nucleotide sequence of *Saccharomyces cerevisiae* chromosome XV." <u>Nature</u> **387**(6632 Suppl.): 98 - 102.
- Domek, M. J., Blackman, E. I., Kao, J., Zhang, X. Y., Iwata, F., Seno, K., and Leung, F. W. (1997). "Functional ablation of afferent nerves aggravates dextran sulphate sodium-induced colonic damage in rats." J Gastroenterol Hepatol 12(11): 698-702.
- Domek, M. J., Cantelo, W. W., and Deahl, K. L. (1997). "A meridic diet for the Colorado potato beetle." J Entomol Sci 32: 430-444.
- Drummond, E. C., and Groden, E. (1996). "Insect pests and natural enemies. In: The ecology, economics, and management of potato cropping systems: a report of the first four years of the Maine potato ecosystem project." <u>Maine agricultural and forest experiment station bulletin</u>, Orono, Maine. **843**: 80-118.
- Feng, M., J. Peng, et al. (1994). "Mammalian cell cultivation in space." <u>Microgravity</u> <u>Sci Technol</u> 7(2): 207-10.
- Flikweert, M. T., Van Der Zanden, L., Janssen, W. M., Steensma, H. Y., Van Dijken, J. P., and Pronk, J. T. (1996). "Pyruvate decarboxylase: an indispensable enzyme for growth of *Saccharomyces cerevisiae* on glucose." <u>Yeast</u> 12(3): 247-57.
- Flores, C. L., Rodriguez, C., Petit, T., and Gancedo, C. (2000). "Carbohydrate and energy-yielding metabolism in non-conventional yeasts." <u>FEMS Microbiol Rev</u> 24(4): 507-29.
- Freimhoser, F. M., Screen, S., Bagga, S., Hu, G., and St Leger, R. J. (2003). "Expressed sequence tag (EST) analysis of two subspecies of *Metarhizium anisopliae* reveals a plethora of secreted proteins with potential activity in insect hosts." <u>Microbiology</u> 149: 239-247.

- Gelman, D. B., Bell, R. A., Liska, L. J., and Hu, J. H. (2001). "Artificial diets for rearing the Colorado potato beetle, *Leptinotarsa decemlineata*." J Insect Sci 1(7).
- 5. Goffeau, A., B. G. Barrell, et al. (1996). "Life with 6000 genes." <u>Science</u> 274(5287): 546, 563-7.
- Grula, E. A., Woods, S. P., and Russell, H. (1984). Studies utilizing *Beauveria bassiana* as an entomopathogen. <u>Infection processes of fungi- Conference report</u>. D. W. A. Roberts, J. R. Eds. New York, Rockfeller Foundation: 147-152.
- Haas, H., M. Schoeser, et al. (2003). "Characterization of the Aspergillus nidulans transporters for the siderophores enterobactin and triacetylfusarinine C." <u>Biochem J</u> 371(Pt 2): 505-13.
- Hansson, G. C., Baeckstrom, D., Carlstedt, I. and Klinga-Levan, K. (1994). "Molecular cloning of a cDNA coding for a region of an apoprotein from the 'insoluble' mucin complex of rat small intestine." <u>Biochem Biophys Res</u> <u>Commun</u> 198(1): 181 - 190.
- Hauser, M., Donhardt, A. M., Barnes, D., Naider, F., Becker, J.M. (2000). "Enkephalins are transported by a novel eukaryotic peptide uptake system." J Biol Chem **275**(5): 3037-41.
- Hauser, M., Narita, V., Donhardt, A. M., Naider, F., and Becker, J. M. (2001).
 "Multiplicity and regulation of genes encoding peptide transporters in Saccharomyces cerevisiae." Mol Membr Biol 18(1): 105-12.
- Higgins, C. F. (1992). "ABC transporters: from microorganisms to man." <u>Annu Rev</u> <u>Cell Biol</u> **8**: 67-113.
- Higgins, C. F. (2001). "ABC transporters: physiology, structure and mechanism-an overview." <u>Res Microbiol</u> **152**(3-4): 205-10.
- Hilkens, J., Ligtenberg, M. J. L., Vos, H. L. and Litvinov, S. V. (1992). <u>Trends</u> <u>Biochem Sci</u> 17: 359-363.
- Hohmann, S. (1997). Pyruvate decarboxylases. <u>Yeast sugar metabolism. Biochemistry</u>, <u>genetics</u>, <u>biotechnology</u>, <u>and applications</u>. F. K. Zimmermann, and Entian, K. D., Eds. Lancaster, Technomic: 187-212.
- Holmquist, M. (2000). "Alpha/Beta-hydrolase fold enzymes: structures, functions and mechanisms." <u>Curr Protein Pept Sci</u> 1(2): 209-35.
- Howard, D. H. (1999). "Acquisition, transport, and storage of iron by pathogenic fungi." <u>Clin Microbiol Rev</u> 12: 394-404.
- Hsiao, T. C., Segel, W., and Tang, C. L. (1968). "Apparent aggregation of 28-S ribosomal RNA of *Zea mays*." <u>Biochim Biophys Acta</u> 157(3): 640-3.
- Hubank, M., and Schatz, D. G. (1994). "Identifying differences in mRNA expression by representational difference analysis of cDNA." <u>Nucl Acids Res</u> 22(25): 5640-5648.
- Hubank, M., and Schatz, D.G. (1999). cDNA Representational Difference Analysis: a sensitive and flexible method for identification of differentially expressed genes. <u>Methods in Enzymology</u>. S. M. Weissman, Academic Press. pp 325-348.
- Hunt, D. W. A., Borden, J. H., and Rahe, J. E. (1984). "Nutrient-mediated germination of *Beauveria bassiana* conidia on the integument of the bark beetle *Dendroctonus ponderosae* (Coleoptera : Scolytidae)." J Invertebr Pathol 44: 304-314.
- Hunt, L. M. (1986). "Differentiation between three species of Amblyomma ticks (acari: Ixodidae) by analysis of cuticular hydrocarbons." <u>Ann Trop Med Parasitol</u> 80(2): 245-9.

- Hutchison, K. A., Green, J. R., Wharton, P. S., and O'Connell, R. J. (2002). "Identification and localisation of glycoproteins in the extracellular matrices around germ-tubes and appressoria of *Colletotrichum* species." <u>Mycol Res</u> 106(6): 729-736.
- Hutchison, K. A., Green, J. R., Wharton, P. S., and O'Connell, R. J. (2002). "Identification and localisation of glycoproteins in the extracellular matrices around germ-tubes and appressoria of *Colletotrichum* species." <u>Mycol Res</u> 106(6): 729-736.
- Jacq, C., Alt-Morbe, J., Andre, B., Arnold, W., Bahr, A., Ballesta, J. P., Bargues, M., Baron, L., Becker, A., Biteau, N., Blocker, H., Blugeon, C., Boskovic, J., Brandt, P., Bruckner, M., Buitrago, M. J., Coster, F., Delaveau, T., del Rey, F., Dujon, B., Eide, L. G., Garcia-Cantalejo, J. M., Goffeau, A., Gomez-Peris, A., Zaccaria, P., et al. (1997). "The nucleotide sequence of *Saccharomyces cerevisiae* chromosome IV." <u>Nature</u> 387(6632 Suppl): 75-8.
- Joshi, A. K. and S. Smith (1993). "Construction of a cDNA encoding the multifunctional animal fatty acid synthase and expression in Spodoptera frugiperda cells using baculoviral vectors." <u>Biochem J</u> 296 (Pt 1): 143-9.
- Joshi, L., St Leger, R.J., and Bidochka, M.J. (1995). "Cloning of a cuticle-degrading protease from the entomopathogenic fungus, *Beauveria bassiana*." <u>FEMS</u> <u>Microbiol Letters</u> **125**: 211-218.
- Kaneko, T., Nakamura, Y., Wolk, C.P., Kuritz, T., Sasamoto, S., Watanabe, A., Iriguchi, M., Ichikawa, A., Kawashima, K., Kimura, T., Kishida, Y., Kohara, M., Matsumoto, M., Matsuno, A., Muraki, A., Nakazaki, N., Shimpo, S., Sugimoto, M., Takazawa, M., Yamada, M., Yasuda, M. and Tabata, S. (2001). "Complete genomic sequence of the filamentous nitrogen-fixing cyanobacterium Anabaena sp. strain PCC 7120." <u>DNA Res</u> 8(5): 205 - 213.
- Koh, S., Wiles, A. M., Sharp, J. S., Naider, F. R., Becker, J. M., and Stacey, G. (2002).
 "An oligopeptide transporter gene family in *Arabidopsis*." <u>Plant Physiol</u> 128(1): 21-9.
- Koide, A. and J. A. Hoch (1994). "Identification of a second oligopeptide transport system in *Bacillus subtilis* and determination of its role in sporulation." <u>Mol</u> <u>Microbiol</u> 13(3): 417-26.
- Koltin, Y., Finkler, A., and Ben-Zvi, B. (1987). <u>Fungal infections of plants.</u> G. F. Pegg, and Ayres, P. G. Eds. Cambridge, Cambridge University Press: 334-348.
- Lacey, L. A. and R. L. Chauvin (1999). "Entomopathogenic nematodes for control of diapausing codling moth (Lepidoptera: Tortricidae) in fruit bins." <u>J Econ</u> <u>Entomol</u> 92(1): 104-9.
- Laemmli, U. K. (1970). "Cleavage of structural proteins during the assembly of the heat of bacteriophage T4." <u>Nature</u> **227**: 680-685.
- Lambrechts, M. G., F. F. Bauer, et al. (1996). "Muc1, a mucin-like protein that is regulated by Mss10, is critical for pseudohyphal differentiation in yeast." <u>Proc Natl Acad Sci USA</u> 93(16): 8419-24.
- Leopold, H. and Z. Valter (1970). "[Improvement of the quality of conidia of *Aspergillus niger* strains during citric acid fermentation]." <u>Z Allg Mikrobiol</u> **10**(2): 121-7.
- Lubkowitz, M. A., Barnes, D., Breslav, M., Burchfield, A., Naider, F., and Becker, J. M. (1998). "Schizosaccharomyces pombe isp4 encodes a transporter representing a novel family of oligopeptide transporters." <u>Mol Microbiol</u> 28(4): 729-41.

- Lubkowitz, M. A., Hauser, L., Breslav, M., Naider, F., and Becker, J. M. (1997). "An oligopeptide transport gene from *Candida albicans*." <u>Microbiology</u> **143**(Pt 2): 387-96.
- Markham, P., Robson, G. D., Bainbridge, B. W., and Trinci, A. P. (1993). "Choline: its role in the growth of filamentous fungi and the regulation of mycelial morphology." <u>FEMS Microbiol Rev</u> **10**(3-4): 287-300.
- Nagao, K., Taguchi, Y., Arioka, M., Kadokura, H., Takatsuki, A., Yoda, K., and Yamasaki, M. (1995). "bfr1+, a novel gene of *Schizosaccharomyces pombe* which confers brefeldin A resistance, is structurally unrelated to the ATPbinding cassette superfamily." J Bacteriol 177(6): 1536-1543.
- Nelson, N. and W. R. Harvey (1999). "Vacuolar and plasma membrane protonadenosinetriphosphatases." <u>Physiol Rev</u> 79(2): 361-85.
- Osteras, M., Boncompagni, E., Vincent, N., Poggi, M. C., and Le Rudulier, D. (1998). "Presence of a gene encoding choline sulfatase in *Sinorhizobium meliloti bet* operon: choline-O-sulfate is metabolized into glycine betaine." <u>Proc Natl Acad</u> <u>Sci USA</u> **95**(19): 11394-9.
- Park, Y.-I., Busko, M. L., and Gander, J. E. (1999). "Glycine betaine: reserve form of choline in *Penicillium fellutanum* in low-sulfate medium." <u>App. Environ.</u> <u>Microbiol.</u> 65(3): 1340-1342.
- Parra-Lopez, C., Baer, M. T., and Groisman, E. A. (1993). "Molecular genetic analysis of a locus required for resistance to antimicrobial peptides in *Salmonella typhimurium*." <u>Embo J</u> 12(11): 4053-62.
- Pastorian, K., Hawel, L. 3rd, and Byus, C.V. (2000). "Optimization of cDNA Representational Difference Analysis for the identification of differentially expressed mRNAs." <u>Analytical Biochemistry</u> 283: 89-98.
- Paterson, I. A., Charnley, A.K., Cooper, R.M. and Clarkson, J.M. (1994). "Specific induction of a cuticle-degrading protease of the insect pathogenic fungus *Metharizium anisopliae*." <u>Microbiol</u> 140: 185-189.
- Pekrul, S. a. G., E. A. (1979). "Mode of infection of the corn Earworm (*Heliothis zea*) by *Beauveria bassiana* as revealed by scanning electron microscopy." J Invertebr Pathol **34**: 238-247.
- Perzov, N., Padler-Karavani, V., Nelson, H., and Nelson, N. (2002). "Characterization of yeast V-ATPase mutants lacking Vph1p or Stv1p and the effect on endocytosis." J Exp Biol 205(Pt 9): 1209-19.
- Poprawski, T. J., Carruthers, R. J., Speese, J., Vacek, D. C., and Wendel, L. E. (1997). "Early-season applications of the fungus *Beauveria bassiana* and introduction of the hemipteran predator *Perillus bioculatus* for control of Colorado potato beetle." <u>Biological control</u> 10: 48-57.
- Pronk, J. T., Yde Steensma, H., and Van Dijken, J. P. (1996). "Pyruvate metabolism in *Saccharomyces cerevisiae*." <u>Yeast</u> **12**(16): 1607-33.
- Rudner, D. Z., LeDeaux, J. R., Ireton, K., and Grossman, A. D. (1991). "The spo0K locus of *Bacillus subtilis* is homologous to the oligopeptide permease locus and is required for sporulation and competence." J Bacteriol **173**(4): 1388-98.
- Sambrook, J., Fritsch, E. F., and Maniatis, T. (2001). Molecular cloning: a laboratory manual. Cold Spring Harbor, New York, Cold Spring Harbor Laboratory Press.
- St Leger, R. J., Bidochka, M.J., and Roberts, D.W. (1994). "Isoforms of the cuticledegradingPr1 proteinase and production of a metalloproteinase by *Metarhizium anisopliae*." <u>Archives of biochemistry and biophysics</u> **313**(1): 1-7.

- St Leger, R. J., Butt, T. M., Goettel, M. S., Staples, R. C., and Roberts, D. W. (1989a).
 "Production *in vitro* of appressoria by the entomopathogenic fungus *Metarhizium anisopliae*." <u>Exp Mycol</u> 13: 274-288.
- St Leger, R. J., Butt, T. M., Staples, R. C., and Roberts, D. W. (1989d). "Synthesis of proteins including a cuticle-degrading protease during differentiation of the entomopathogenic fungus *Metarhizium anisopliae*." <u>Exp Mycol</u> 13: 253-262.
- St Leger, R. J., Butt, T. M., Staples, R. C., and Roberts, D. W. (1990). "Second messenger involvement in differentiation of the entomopathogenic fungus *Metarhizium anisopliae*." J Gen Microbiol 136: 1779-1789.
- St Leger, R. J., Charnley, A. K., and Cooper, R. M. (1986c). "Cuticle-degrading enzymes of entomopathogenic fungi : mechanisms of interaction between pathogen enzymes and insect cuticle." J Invertebr Pathol 47: 295-302.
- St Leger, R. J., Charnley, K., and Cooper, R.M. (1986a). "Cuticle-degrading enzymes of entomopathogenic fungi : synthesis in culture on cuticle." <u>J Invert Pathol</u> 48: 85-95.
- St Leger, R. J., Cooper, R. M., and Charnley, A. K. (1986b). "Cuticle-degrading enzymes of entomopathogenic fungi: cuticle degradation *in vitro* by enzymes from entomopathogens." <u>J Invertebr Pathol</u> 47: 167-177.
- St Leger, R. J., Cooper, R. M., and Charnley, A. K. (1987b). "Production of cuticledegrading enzymes by the entomopathogen *Metarhizium anisopliae* during infection of cuticles from *Calliphora vomitaria* and *Manduca sexta*." J Gen <u>Microbiol</u> 133: 1371-1382.
- St Leger, R. J., Cooper, R. M., and Charnley, A. K. (1987c). "Distribution of chymoelastases and trypsin-like enzymes in five species of entomopathogenic Deuteromycetes." <u>Arch Biochem Biophys</u> 258: 123-131.
- St Leger, R. J., Cooper, R. M., and Charnley, A. K. (1993). "Analysis of aminopeptidase and dipeptidylpeptidase IV from the entomopathogenic fungus *Metarhizium anisopliae*." J Gen Microbiol 139: 237-243.
- St Leger, R. J., Durrands, P. K., Charnley, A. K., and Cooper, R. M. (1988a). "Role of extracellular chymoelastase in the virulence of *Metarhizium anisopliae* for *Manduca sexta*." J Invertebr Pathol 52: 285-293.
- St Leger, R. J., Durrands, P. K., Cooper, R. M., and Charnley, A. K. (1988c).
 "Regulation of production of proteolytic enzymes by the entomopathogenic fungus *Metarhizium anisopliae*." <u>Arch Microbiol</u> 150: 413-416.
- St Leger, R. J., Frank, D. C., Roberts, D. W., and Staples, R. C. (1992b). "Molecular cloning and regulatory analysis of the cuticle-degrading-protease structural gene from the entomopathogenic fungus *Metarhizium anisopliae*." <u>Eur J Biochem</u> 204: 991-1001.
- St Leger, R. J., Joshi, L., Bidochka, M. J., and Roberts, D. W. (1996b). "Construction of an improved mycoinsecticide overexpressing a toxic protease." <u>Proc Natl Acad Sci USA</u> 93: 6349-6354.
- St Leger, R. J., Joshi, L., Bidochka, M.J., Rizzo, N.W., and Roberts, D.W. (1996). "Biochemical characterization and ultrastructural localization of two extracellular trypsins produced by *Metarhizium anisopliae* in infected insect cuticles." <u>Appl Environ Microbiol</u> 62(4): 1257-1264.
- St Leger, R. J., Lacetti, L. B., Staples, R. C., and Roberts, D. W. (1990). "Protein kinases in the entomopathogenic fungus *Metarhizium anisopliae*." J Gen <u>Microbiol</u> 136: 1401-1411.

- St Leger, R. J., Roberts, D. W., and Staples, R. C. (1989b). "Calcium- and calmodulinmediated protein synthesis and protein phosphorylation during germination, growth and protease production by *Metarhizium anisopliae*." J Gen Microbiol 135: 2141-2154.
- St Leger, R. J., Roberts, D. W., and Staples, R. C. (1989c). "Novel GTP-binding proteins in plasma membranes of the fungus *Metarhizium anisopliae*." <u>Biochem</u> <u>Biophys Res Com</u> 164: 562-566.
- St Leger, R. J., Staples, R. C., and Roberts, D. W. (1991a). "Changes in translatable mRNA species associated with nutrient deprivation and protease synthesis in *Metarhizium anisopliae*." J Gen Microbiol 137: 807-815.
- Steiner, H. Y., Naider, F., and Becker, J. M. (1995). "The PTR family: a new group of peptide transporters." Mol Microbiol 16(5): 825-34.
- Stergiopoulos, I., Gielkens, M. M., Goodall, S. D., Venema, K., and De Waard, M. A. (2002). "Molecular cloning and characterisation of three new ATP-binding cassette transporter genes from the wheat pathogen *Mycosphaerella* graminicola." <u>Gene</u> 289(1-2): 141-9.
- Stevens, T. H., and Forgac, M. (1997). "Structure, function and regulation of the vacuolar (H⁺)-ATPase." <u>Annu. Rev. Cell Dev. Biol.</u> **13**: 779-808.
- Stover, C. K., Pham, X. Q., Erwin, A. L., Mizoguchi, S. D., Warrener, P., Hickey, M. J., Brinkman, F. S., Hufnagle, W. O., Kowalik, D. J., Lagrou, M., Garber, R. L., Goltry, L., Tolentino, E., Westbrock-Wadman, S., Yuan, Y., Brody, L. L., Coulter, S. N., Folger, K. R., Kas, A., Larbig, K., Lim, R., Smith, K., Spencer, D., Wong, G. K., Wu, Z., Paulsen, I. T., Reizer, J., Saier, M. H., Hancock, R. E., Lory, S., and Olson, M. V. (2000). "Complete genome sequence of *Pseudomonas aeruginosa* PA01, an opportunistic pathogen." <u>Nature</u> 406(6799): 959-64.
- Tetteh, K. K. A., Loukas, A., Tripp, C., and Maizels, R.M. (1999). "Identification of abundantly expressed novel and conserved genes from the infective larval stage of *Toxocara canis* by an expressed sequence tag strategy." <u>Infection and</u> <u>Immunity</u> 67(N°.9): 4771-4779.
- Urban (1999). "An ATP-driven efflux pump is a novel pathogenicity factor in rice blast disease." <u>EMBO J</u>
- Van den Berg, M. A. and H. Y. Steensma (1995). "ACS2, a Saccharomyces cerevisiae gene encoding acetyl-coenzyme A synthetase, essential for growth on glucose." <u>Eur J Biochem</u> 231(3): 704-13.
- Walker, J. E., Saraste, M., Runswick, M. J., and Gay, N. J. (1982). "Distantly related sequences in the a- and b-subunits of ATP synthase, myosin, kinases and other ATP-requiring enzymes and a common nucleotide binding fold." <u>Embo J</u> 1: 945-951.
- Watt, B. A., and Lebrun, R. A. (1984). "Soil effects of *Beauveria bassiana* on pupal populations of the Colorado potato beetle (Coleoptera: Chrysomelidae)." <u>Environmental Entomology</u> 13: 15-18.
- Watts, D. J. (1984). "Protein synthesis during development and differentiation in the cellular slime mould *Dictyostelium discoideum*." <u>Biochem J</u> **220**(1): 1-14.
- Wiebe, M. G., Robson, G. D., and Trinci, A. P. (1989). "Effect of choline on the morphology, growth and phospholipid composition of *Fusarium graminearum*." J Gen Microbiol 135 (Pt 8): 2155-62.
- Wolfger, H., Mamnun, Y. M., and Kuchler, K. (2001). "Fungal ABC proteins: pleiotropic drug resistance, stress response and cellular detoxification." <u>Res</u> <u>Microbiol</u> 152(3-4):375-89.

- Wraight, C. J., van Endert, P., Moller, P., Lipp, J., Ling, N. R., MacLennan, I. C., Koch, N., and Moldenhauer, G. (1990). "Human major histocompatibility complex class II invariant chain is expressed on the cell surface." <u>J Biol Chem</u> 265(10): 5787-92.
- Wright, C. S., Alden, R. A., and Kraut, J. (1969). "Structure of subtilisin BPN' at 2.5 angstrom resolution." Nature **221**(177): 235-42.
- Zacharuk, R. Y. (1970a). "Fine structure of the fungus *Metharizium anisopliae* infecting three species of larval Elateridae (Coleoptera). I. Dormant and germinating conidia." J Invert Pathol 15: 63-80.

Note 1: the red numbers correspond to the references indicated in the tables 1 and 2.

Note 2: Pictures have been found on the following internet sites: http://www.sidney.ars.usda.gov/grasshopper/Handbook/VII/vii 5.htm http://www.entm.purdue.edu/entomology/vegisite/commercial/potatoes.html http://www.biosci.ohio-state.edu/~parasite/glossina.html http://www.insects.tamu.edu/images/insects/fieldguide/bimg191.html http://fireant.tamu.edu http://lancaster.unl.edu/enviro/pest http:://www.biosicherheit.de

VII. Appendixes

1. RDA fragments

<u>LS1-1</u>

GATCGTCTGGCCCTCCTTAAAGCCCTTCTCAGGCACTTTGAACTCCCAGG 50 CGCTGTGAAAGAGCGTCATGGACTTGGAGAAAGCATCGTTGTAGATGAGA 100 CCGGTAAATAGAGAGAACACCGCCATAACCAGGGCGATATAACGCCCGTA 150 200 AATAAATCATGGCCAGCGCAGCAGACAACATGATGACGGCGTGGCCGAAA 250 TCACCAAACATGACGGCAAAAAGAAAAGGGAAAGTCACGATAACCAGCAT 300 GGCGGGGTTGACTTCCTGATAAGTGGCGGTACCGTAAGCGTTGACAATGG 350 TCTGGAAACCTTCGGTAAACTTGTTCGTCTTGAGATATGTGGGAGGCTTT 400 TTGTTGGTGCGAATCTCATTGATGATGGAAGGGACCGAAAGGCCAGCGCG 450 GTTTGTCACATCCTGTAGGGTTGATC 476

<u>LS1-2</u>

50 AATTAGATAATACTCGCTTGGCTACTCAGTATGGCGGGGCAGCCTAGCCA 100 AGTCGCTGGTGCAACGGCACTGCGTGAATGCGCCCGCGACTAAATATTGA 150 CCTTGCAACATCCTCCGGCATTCGGCCTCTTTTCGCGACGCAGCATGGCC 200 250 AGCGGCCAATTTGTCTTGAGCGTCATCATGCCATGAGCGACAGGCTAGGA GTGGTGTTTTACCCGCGATACTGCTTTCACCTGGCCCCGACTGCCAAGGG 300 GTGGTGCTTTCTACGCGTCAAAGACCTATTTACTCTGGAACAGCCAGATG 350 400 ACCGGCGTTACTGCAGGCGAGGGTTTCTATTTCCACCGCAATTTGCCAAT 450 TAAATGGGTTCGCATTGTCGGTGTGATTGTGGCGATTGACAATTTTGCCG 500 GCCTCCGCGCGATGACGATTGACGACAGCAGCGGTGCTTGCATAGAGGTG 550 ATTACCTCGTCGGCCATCCCGACATTATGTCCGGATGGGATC 592

LS2-1

GATCCCATTTTTGGATNTCTGNAGCCCCAAGCCCACCTTTGTCATCAAGT 50 AGGTCACCAACATCATCCAGGCCCTCATCGCCGGCTCCCCCTTTTACAAC 100 GCGCCGAGTAACACGGCCGGTCTGCTCTCCAAGTCTGGCACGCTCTTCT 150 TTCTCTCCTCTATCCGACCCTGGTTGCCATGTCTGAAGTGACGGATTCCT 200 TTNNTGGAAGACCGGTCCTGGTCAAGCACAAGTCCTTTGCCCTTTTCCAC 250 CCTGCTGCATTCCGCCTCGCCCAGATTGCCGCCGACACTCCTGTTCTACT 300 CTTCCAGACGTCCACGTTTTCCCTGATTCTGTACTTCATGGTCGACTTGG 350 AGCGCACCGCTGGGGCCTTCTTCACCTACTGGATC 385

<u>LS2-2</u>

GATCCAATGTACGGNANTACCTCTGCCTACTGCTTCTGGNAACATTCAGN 50 ACACAAACCGTCTACTATGGCGACAAGTGGCTGACTCCCGCCTACAGCAT 100 TCTGCTGCTCCTCTCTACCCAGATGATGGGTCTTGGCTTTTCCGGTCTGC 150 TCCGCCGTTTTGTCATTTATCCTGTCGAAGCTATTTGGCCCAGCATCCTA 200 CCGACTCTCGCTCTAAACCGCGCTCTGCTCGTGCCCGAGAANCGTGAAAC 250 CATTCATGGCTGGTCCATCTCCCGATACAAGTTCTTCTTCATCGTCTTTG 300 CCGCCATGTTTGTCTACTTTTGGATC 326

<u>LS2-3</u>

GATCNCTTTTNTGNTCATCCNAANCCCCTTCGATTTCTACCCAGTAACAG 50 GCCGTGGCCGATGCTGCCTACCTCGCGCAGCACGCCAAGTTTCCTGGGTT 100 TGAACATGCGAATTTGACTTTGGCCAATACGCCATGGATTATTTACGGTG 150 GCTCTTACGCAGGCGCGTATGCGGCCACTACTCGCAAGCTCTACCCTTTT 200 TNCTATTGGGGCGCCATATCGTCGCCGGGTGTCACGAGAGCCGTTTATGA 250 CTACTGGGAGTACAATGAGGCAGCACGTTTATATGCTCCAGGAGATTGCG 300 GACCCAAAATGTCCAACTTGACGCATGTGGTTGACACAGCAATCTTCAGC 350 AGTGATAGGACCAAGCCCAGTGCGATC 377

<u>LS2-4</u>

GATCNCATTTNTNGATCCTTGTNAGCCCGTAAGGCTGAGGCTTGTCGGCC 50 GGGTAAGTAATGTACTTCCAGGGTACCGCGACGAATCATCATCATNCGGA 100 CGGACGGTGCCCTCTCCCGTGTACTCGGCATACACAGTATCGTTGCCCGT 150 CTTCCCCTCGAGATGGGGCATCATGCTGACACCATCCATGGGAAGATAGG 200 GTNTTTTNNTTGACATCAATGAGGTCCCAAATGGTGGGTAGTAGGTCCAG 250 AGTTGAAACATTTTGGGTCACGCGATGAGGCTTGAACCACTTGGGGTAGT 300 TGACCAGCAGAGGCACTCGTACAGACGACTCAAAGTAGCTCATCCTGTAC 350 CAGAGACCACGCTCGCCGAGCATGTCGCCGTGGTCGCCGCTAAAGATGAC 400 AATGGTATCTTCAGCCAGACCAGCATCGTCCAGGGTTTGAAGAAGCTTGC 450 CAATGCAGTCATCAACGTAGCTGACAGAGCCGTNGTAAGCACGCTTGGCA 500 CGCTTTGATC 510

<u>HS-1</u>

<u>HS-2</u>

GAT×ATGTCGGAAG×CCTCTGCCTACTGCTTCTGGAACATTCAGGACACA 50 AACCGTCTACTATGGCGACAAGTGGCTGACTCCCGCCTACAGCATTCTGC 100 TGCTCCTCTCTACCCAGCATGATGGGTCTTGGCTTTTCCGGTCTGCTCCG 150 CCGTTTTGTCATTTATCCTGTCGAAACTATTTGGCCCA×CATCCTACCGA 200 CTCTCGCTTTAAACCGCGCTCTGCTCGTGCCCGAGAAGCGTGAAACCATT 250 CATGGCTGGTCCATCTCCCGATACAAGTTCTTCTTCATCGTCTTTGCCAC 300 CATGTTTGTCTACTTTTGGATC 322

<u>HS-3</u>

GATCCATGACCTATTAAGTTGCTATCTACTATTAATTGATACTAAGAGCC 50 AAGCCGTGTAAGATTTGGCAGTGCGCTGGGCAAAATATTGACCAAATTTC 100 ACTTCTGAAATAGAAATACTGCAATGTCGAAAATGGACAATCGGCACGAG 150TAGATTATCGAGCATGGCCGCCCCGCCACCTTTTTTTTAAACGACAGTT 200 CCGTGAACCTGCTTCTTTTGTAGATGTTGATATCTCTCCACATCCAGGT 250 GCAACCCCAAGCAACGGCCAAGAGGCAGACACTGGTCGTGAGCATGTATC 300 GCTGCGAGGCAGAGTAAGCTTCTGAGATGCCGATGCGGATAGGGGTGCCC 350 GGTGCATATGAAAGCTGGGTCGGAAGCTTGGCGTAGATGGCATCAATAGG 400 GGCATCGGATGGGAGGCGTTTAATGAGCTCGTTCTTGAAAGTACCGGTCC 450 AAATCGCGCCCGAGATGGTAGAGCCAATCGCACCAATACTGGCAAAG 500 AGATC 505

HS-4

GATCTCGAGAAAAGTCNTTCTACGGNGGAGCAGCTTGTCAAAGACGCGGG 50 ATACAGTGCTGATAGCTCGACCGCGCTTCTAAACTACGTCGGCCTCCAGA 100 AGAGTGGCTCCGACAATGAGTCGAAGCAAAGAAAAGGATGGCCCAATCAA 150 CTGCGCAAGCGAGCNGCGCAGCTCAATGATGGCTATAGCTGGTTCTACCA 200 GACATGCAGCCAATGGGGCTTTTTCATCTCGGGATC 236

<u>HS-5</u>

GATCCATGACCTATTAAGTTGCTATCTACTATTAATTGATACTAAGAGCC 50 100 AAGCCGTGTAAGATTTGGCAGTGCGCTGGGCAAAATATTGACCAAATTTC ACTTCTGAAATAGAAATACTGCAATGTCGAAAATGGACAATCGGCACGAG 150 TAGATTATCGAGCATGGCCGCCCCGCCACCTTTTTTTTAAACGACAGTT 200 CCGTGAACCTGCTTCTTTTGTAGATGTTGATATCTCTCCACATCCAGGT 250 GCAACCCCAAGCAACGGCCAAGAGGCAGACACTGGTCGTGAGCATGTATC 300 GCTGCGAGGCAGAGTAAGCTTCTGAGATGCCGATGCGGATAGGGGTGCCC 350 GGTGCATATGAAAGCTGGGTCGGAAGCTTGGCGTAGATGGCATCAATAGG 400 GGCATCGGATGGGAGGCGTTTAATGAGCTCGTTCTTGAAAGTACCGGTCC 450 AAATCGCGCCCGAGATGGTAGAGCCAATCGCACCACCAATACTGGCAAAG 500 AGATC 505

<u>HS-6</u>

GATCCTGGGTGACGGACGAAACGTCGACAATACGCAGACCAGAGCCGTAA 50 TTAGCCTGGTATGAGAGCCCGTTAATGACATACTGGTTGTGGTCGATGGA 100 CTTGGCCGGGCTCTTGTACAGGCCGGTAAAGACGGGCTTTGTCAGGTCAG 150 AGACGTCGACAATATACGTCGTCGTCTTTTGATC 184
2. genomic DNA fragment of *Beauveria bassiana* containing LS1-2

GGCTAATCAAAACCAAAGGGATAGATTCAATCAATTTATTAAAGATGGAT	50 100
	150
	200
	250
	200
	300
	300
	400
	450
	500
	550
	600
ALLILGILGGULAILULGALAITAIGILLGGAIGGGAILGAGULLGLAGA	650
AGUGALAALAGGUUUGGUGUGUGUAGGUTAAUGUAUATAUGAUTAUGTUG	700
	/50
GICAAGCAGCICAGGGIGGAGAAGAIGGCIGCCAICCGCGGGACGGCGGA	800
GGAAAIGAGGCICIGGGCCAAGAGGGGCGCIIIICGGAGGGGCGIGCICG	850
AGAAGCCCIGGGICGIGCCGGACAAGACGCIICGCAAGIGCCGIAGGGAG	900
GCGGAGCGGTCAGAGGCCGAGATGGAGAGAAGAAGAAGCGCCTCAAGGC	950
IGCGGCTTACGIGCAAAAGGGAGACGAGACAAATAGGAGACAGGATGCGG	1000
IGGAGGAAAGACGCAGCAAGCCGAGCAGAGAAGGAGIIGAGGIICIGAAA	1050
GCCCTGGCAAGCTCCAACGGAGGCTATAACGCTCTAGGGCTTTGAGGGAA	1100
AGTTGTATAATATTATGATTGACGGGACTCGTGACGCCCCCAGACGAATT	1150
TGATGGGACTAGGATGGGACTTGGCATCTACTGATACCCCGTTGGATAAT	1200
TCACAATAGAAATTGAAACTGCAATCTCCTTTTTTGCTTTTATTTCCATA	1250
TTGAATCCCCACGGACGGTTTAATTTAATAAAGTGTGGCAACTATCTCTG	1300
ACGCATACATAGCTATAGCCTATCGCGGAATCTGCCTTTCCGAGCCGCTA	1350
CCATCGCCTAGGTGCCTAGTTCCACGGAAGGTGCGGTAGCGACGACATAC	1400
GAATTGTGCATCCTTTCGAACCCCGCGCAAGCAATTCGTCTCGACTCTTG	1450
CAAACCTAAAATCCATCCTATCCACTCCCTATCATACCTCGAAAAGCCTT	1500
TCTATTAGTAGTCGCAGCAACAGGGGCAGGAAAAAGGGTTTGTTT	1550
GCAACCAGGGGTCTGCCTCTCCGGGCCGAAAAGCAAGACGCCACGCCATT	1600
CTCCGCCAGGTTGACTATTTTGGACCCTGTTGTCCCGGCCCCAGCCCCGA	1650
AAACTTTTCTGTAAGTGACAGGTTTTTTTTTATACCGAGTATGGATCTCGT	1700
CATTATGTGCTGCCCACATTCTTTGCGTATTATCGCGTGGATCCTGGTCG	1750
GCGGGGGGTTTGGGACTACCACCATGGTGAGACTCGTTGTTCTGCCCTCG	1800
ACTGGAATGGGGATCTATTTCGTACACTTGGGCTGCCACGAGGGGCTTGG	1850
CCCATAACCGCGGATCATCCATCGTCTTCATATATGGGCAATTGGCTGCT	1900
GTTTGGGGAGATTGTCTTATTTCCTGTTCTGAAGTGATCGCATATCTCGC	1950
TGTACTTGATAATTTCTGTCCGTGAGAGGCAGATTAGAAGAGCAGCCATG	2000
CGTTTCTCTACCGTGTTATTGGGTTCCGAGGCGCTGACATTGATCAAAGC	2050
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GCTCCGACTTTTACCTTTTGTCCTGATGACGAACTGAACCCTTTCAATAT	2200
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TCTACAACGTGTCGAATACTGTCGTAGTGACGACCACCATAACCGACACA	2300
GCTACCATCTGTTACCTCCCGGAGCTTACGAGCGTCACGCCGCAGACTCA	2350
TGGTGTCTTAAATACTGAACTTGGCGAACTTGGCCAACCAA	2400
CTCTGCTAACTGACGTTATCCCTACCCGGTCATCTCTACCAACCTTTGCA	2450
AGCACTCTGTCTGGAACCACTGCAAAGACTACCCCGGAATCTGGATCGTC	2500
CAACTTCGATTCTGGATCCGAATTC 2525	

3. Actin gene regions from Beauveria bassiana and Leptinotarsa decemlineata

primers	Alignment of the actin gene sequences amplified using the degenerated primers ActfunFOR/REV for <i>B. bassiana</i> and the ActinsFor/Rev for <i>L. decemlineata</i> .	
	Tecoration 'Decoration #3': Box residues that differ from B. bassiana 2.	
B. bassiana 1B. bassiana 2L. decemlineata 1L. decemlineata 2	79 GAA-GGT 34 GAA-AGT 75 GAA-AGT 35 GAA-ATC	
B. bassiana 1B. bassiana 2L. decemlineata 1L. decemlineata 2	 CACTT CGCCGCT AACT CTTTT ADT CT GGT GGT ACT A CCAT GT A CCCT GGT CT T GCC GAC CGT A T GCA GGA GGA GGA GA T ACT A T T CT T GCT CT T CT	
B. bassiana 1B. bassiana 2L. decemlineata 1L. decemlineata 2	 se ccacccccccccccccccccccccccccccccccccc	
B. bassiana 1B. bassiana 2L. decemlineata 1L. decemlineata 2	 49 AGCTT CCT GACGGT CA AGT CATCACCATT GGCAA CGAGCGT TT CCGCACCCCCGAGGGT CT CT TCCAGCCT CCGT TC CGGT CT CGAGT CT GGCGGT AT 49 AGCTT CCT GACGGT CATCACCATT GGCAA CGAGCGT TT CCGCACCCCCGAGGGT CT CT TCCAGCCT CCGT TC CGGT CT CGAGT CT GGCGGT AT 40 AGCTT CCCGATGGGT CATCACCATT GGT ACGGAA CGAGCGT TC CGGT CCCCCGAGGCT CT TCCAGCCT CCGT TC CGGT CT CGAGT CT GGCGGT AT 40 AGCTT CCCGATGGGT CATCACCATT GGT ACGGAA CGAGCGT TC CGGT CT CCAGCCT CCGT TC TCCAGCT CT TCCAGCT CT TCCAGGT CT CGAGT CT GGCGGT AT 40 AGCTT CCCGATGGGT CATCACCATT GGT ACGGAA CGAA C	
B. bassiana 1B. bassiana 2L. decemlineata 1L. decemlineata 2	 arcerregreacarcaageageageageageageageageageageageageage	
B. bassiana 1B. bassiana 2L. decemlineata 1L. decemlineata 2	29 CAT CGCCCGT GT CG ACAT GGCT GGT CGT GAT CCT ACCG ACT ACCT GAT GAA GAT CCT CGCT GGT GGT TACGCCTT CT CCACCGCCGCGGGGGGGGGG	
B. bassiana 1B. bassiana 2L. decemlineata 1L. decemlineata 2	i) CCCTCTACGCCT CTGGCCGTACCACCGGTATCGTCCTCGACTCTGGTGATGGTGACCCCACGTTGTCCCCATCTACGAGGGTTTCGCTCTTCCTCACGC 4) CCCTCTACGCCTCTGGCCGTACCACCGGTATCGTCCTCGACTCTGGTGATGGTCACCCACGTTGTCCCCATCTACGAGGGTTTCGCTCTCCTCACGC 30 CTCTGTATGCTTCTGGTCGTACTGGTATTGTGCTGGTGATGGTGTTTCTCCCACGTTGTCCCATCTACGAGGGTTTCGCTCTCCTCACGC 30 CTCTGTATGCTTCTGGTCGTACTGGTATTGTGCTCTGGAGATGGTGTTTCTCCCACGTTGTCCCTCACGCC 30 CTCTGTATGCTTCTGGTCGTACTGGTGTTTGTCCCCCCCACGTTGTCACGAGGGTTTCCCTCACGC 30 CTCTGTATGCTTCTGGTCGTACTGGTGTTTGTCCCCCCCC	
B. bassiana 1B. bassiana 2L. decemlineata 1L. decemlineata 2	 94 GCCCCCATCAACCCCAAGTCCAACCGTGAGATGATGTCTTTGAGACGTCAACGCCCCCGCCTTCTACGTCTCAAGGCCGTCCTCT 94 GCCCCCATCAACCCCAAGTCCAACCGTGAGATGATGTCTTTGAGACGTCAACGCCCCGGCCTTCTACGTCTCCATCCA	
B. bassiana 1B. bassiana 2L. decemlineata 1L. decemlineata 2	1 ACEMAACT GGGAGEACAT GGAGAAGATTT GGCACCACCCTTCT ACAACGAGCT CCGT GT CGCCCCGGGAGGAGCACCCCGGT CCTTCT CACCGAG A. TAGTAACTAGGAACATTGGGAGAAGATTEF GGCACCACCCCTTCT ACAACGAGCT CGCTGT GGCAGCGGGGGGGGGG	

4. Abbreviations

ABC transporter	ATP-binding-cassette transporter
ATP	adenosine triphosphate
b	base
bp	basepair
BSA	bovin serum albumin
CDNA	complementary DNA
Da	Dalton
DEPC	diethyl pyrocarbonate
DNase	deoxyribonuclease
DNA	deoxyribonucleic acid
DNTP	deoxynucleoside triphosphate
DP	difference product
EDTA	ethylenediaminetetraacetic acid
EST	expressed sequence tag
σ	oram/oravity
5 HS RDA	high stringency representational difference analysis
HS_"r"-gene gene containi	ing the DnnII-fragment HS-"x" from the high stringency
RDA	ng the Dphil-Jrugment 115- x Jrom the high stringency
k	kilo
	Luria Partani madium
	Luna-Dertain incurum
LS KDA LS1 or LS2 "y" conclusions	some containing the Duril fragment "y" from the low
LSI- OI LS2- X -gene	gene containing the Dpini-nagment x^{2} from the low
М	stringency RDA experiment n°1 or n° 2.
M	molar
μ	
MDR	multidrug resistance
MRNA	messenger ribonucleic acid
NBD	nucleotide binding domain
OD	optical density
OPT	oligopeptide transporter
ORF	open reading frame
PAGE	polyacrylamide gel electrophoresis
POT	proton oligopeptide transporter
PCR	polymerase chain reaction
pfu	plaque forming unit
PTR	peptide transporter
RDA	representational difference analysis
RNA	ribonucleic acid
RNase	ribonuclease
Rpm	revolution per minute
RT(-PCR)	reverse transcription
SDS	sodium dodecyl sulfate
TCA cycle	tricarboxylic acid cycle
ТМ	transmembrane spanning domain
Tris	tris-(hydroxymethyl)-aminomethane
UV	ultraviolet
v/v	volume to volume
w/v	weight to volume