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Molecular biology of the entomopathogenic fungus *Beauveria bassiana*:

Insect-cuticle degrading enzymes and Development of a new selection marker for fungal transformation

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Molecular biology of the entomopathogenic fungus *Beauveria bassiana*:

Insect-cuticle degrading enzymes and Development of a new selection marker for fungal transformation

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I. General Introduction

II. Characterization of insect cuticle-degrading enzymes from *B. bassiana*

IV. Materials and Methods

I. General Introduction

Insecticide resistance and the demand for reduced chemical inputs in agriculture have provided an impetus to the development of alternative forms of pest control. Biological control offers an attractive alternative or supplement to the use of chemical pesticides. Microbial biological control agents are naturally occurring organisms and perceived as being less damaging to the environment. Furthermore, their generally complex mode of action makes it unlikely that resistance could be developed to a bio-pesticide. Biological pest control agents includes viruses, bacteria, fungi, and nematodes. The use of microorganisms as selective pesticides has had some notable successes.

1.1. Presently used bio-insecticides

1.1.1. Entomopathogenic viruses

The insect pathogenic viruses belong to of the family Baculoviridae (BV). BV includes two genera (*Nucleoplyhedrovirus*, NPV and *Granulovirus*, GV) of arthropod-specific pathogens and the majority of the baculoviruses are infectious only for insect species within the order Lepidoptera, with no adverse effect on members of other orders. In addition, most of the baculoviruses exhibit a very narrow, mostly single-species, host range. Target specificity makes them good candidates for use in integrated pest management systems (Romanowski, 2002).

Up-to-date, the most successful case of using baculoviruses as bio-control was the exploitation of the multinucleocapsid nucleopolyhedrovirus of *Anticarsia gemmatalis* (AgMNPV) for the control of the velvetbean caterpillar in soybean in Brazil. In the season 2001/2002, the treated area with AgMNPV (tradename Coopervirus®) was over 1,550,000 ha (more than 11% of the soybean cultivated area in the country) and the use of the AgMNPV in Brazil has generated substantial economical, ecological, and social benefits (Moscardi *et al.,* 2002). In addition, the multinucleocapsid nucleopolyhedrovirus of *Spodoptere frugiperda* (SfMNPV), which is the principal pest of maize and sorghum, has been developed as a bioinsecticide in Mexico and Central America (Williams, 2002); a single nucleocapside nucleopolyhedrovirus of *Helicoverpa armigera* (HaSNPV) has been developed as a commercial pesticide for

the control of the cotton bollworm in China. The genome of the HaSNPV has been entirely sequenced and several genetically modified HaSNPVs have been constructed and tested in the laboratory and in the field (Sun *et al.,* 2002). However, the ingestion of a lethal dose by the target insect and the length of time required to kill or prevent the damage of the crop are the major limitations for their extensive usage in agronomic systems.

1.1.2. Entomopathogenic bacteria

The best example of bacterial insecticides that have been investigated in detail belong to the genus *Bacillus*. Four species have been used as insecticides commercially: *B. thuringiensis, B. popilliae, B. moritai and B. sphaericus*. *B. thuringiensis,* the most successful biological control agent produced entomocidal toxins: the α- and β-exotoxins and δ-endotoxin. The latter, also called insecticidal crystal protein (ICP), is synthesized during sporulation, and accounts for the commercial value of *B. thuringiensis* as biological pest control agent (Zhong, 2000). *B. thuringiensis* var. *israelensis* (*Bti*) was first discovered in 1976 and has proven to be highly pathogenic for many aquatic Diptera. *Bti* has been successfully utilized for control of mosquitoes and black flies worldwide. Additionally, *Bti* has an excellent safety record and resistance has not been found probably due to its complex mode of action involving synergistic interaction between up to four proteins. Furthermore, several toxin genes from *B. thuringiensis* have been used in transgenic crops such as cotton, corn and soybean (Khachatourians, 1986).

1.1.3. Entomopathogenic nematodes

Entomopathogenic nematodes of the families *Steinernematidae* and *Heterorhabditidae* together with their symbiotic bacteria, *Xenorhabdus* and *Photorhabdus*, respectively, represent a unique biological control agent. The nematode vectors the entomopathogenic bacteria within their gut into the insect host, where the bacteria multiply and produce a wide range of toxins and hydrolytic exoenzymes that are responsible for the death and bioconversion of the insect larvae into a nutrient soup, that is ideal for nematode growth and reproduction. The nematodes reproduce until the nutrient supply becomes limiting at which time they develop into infective juveniles, which are recolonized by the symbiotic bacterium (Forst and Clarke, 2002). The nematode-bacterium complex kills insects rapidly (48hr). they do not form intimate, highly adapted, host-parasite relationship that

could be found in the majority of other parasites used for biological control and therefore can exploit a wide host range of insect species. The target insects of EPN include the black vine weevil in nurseries and landscape, cranberry girdler in cranberry bogs, Diaprepes root weevil in citrus, fungus gnats in nurseries, sciarid flies in mushrooms, Western flower thrips in the greenhouses, hunting billbugs in turfgrass, white grubs in turfgrass, and mole crickets in turfgrass. The proper nematode species must be used against the target insect as all nematode species are not effective against a given target insect (Stock, 2002).

EPNs and their associated symbiotic bacteria have been considered to be a save approach to pest control. They have been proved to be not harmful to humans and other vertebrates. EPNs can be easily mass-produced using conventional fermentation technology, are exempt from registration requirements in many countries and therefore several formulations of EPNs are available on the market for the control of soil and cryptic pests in North America, Europe, Asia, and Australia.

1.1.4. Entomopathogenic fungi

Entomopathogenic fungi were among the first organisms to be used for the biological control of pests. More than 700 species of fungi from around 90 genera are pathogenic to insects. Most are found within the deuteromycetes and entomophthorales. some insect-pathogenic fungi have restricted host ranges, for example, *Aschersonia aleyrodis* infects only scale insects and whiteflies, while other fungal species have a wide host range, with individual isolates being more specific, for example, *Metarhizium anisopliae* and *Beauveria bassiana*(Fig. 1.1). The commercial produced entomopathogenic fungi and their targeted hosts are shown in Table 1.1.

Fungal species such as *M. anisopliae* and *B. bassiana* are well characterized in respect to pathogenicity to several insects and they have been used as agents for the biological control of agriculture pests worldwide. In Colombia, about 11 companies offer at least 16 products based on the entomopathogenic fungi *B. bassiana*. These products are used not only in the coffee crop but also in other crops such as cabbage, corn, bean, tomato, potato. They are also used to treat public disease vectors (e.g., flies and mosquitoes) (Florez, 2002).

Fig. 1.1. Biological control of insect pest (alfalfa weevil). Left and Center, Adult insect killed by *Beauveria bassiana*. Right, Healthy adult insect (control).

Table 1.1. Entomopathogenic fungi in commercial and experimental production (adapted from Khachatourians, 1986)

Fungus	Targets	Product
Beauveria bassiana	Colorado potato beetle Coding moth European corn borer Pine caterpillar	Boverin Mycotrol®
Culicinomyces clavisporus	Mosquito larvae	
Hirsutella thompsonii	Citrus rust mite	Mycar
Metarhizium anisopliae	Spittle bug Sugarcane frog hopper	Metaquino Meta-Sin®
Nomuraea rileyi	Lepidopteran larvae	
Verticillium lecanii	Aphids Coffee green bug Greenhouse whitefly thrips	Vertalec

1.2. The entomopathogenic deuteromycete *B. bassiana*

The filamentous fungus *Beauveria bassiana* belongs to a class of insect pathogenic deuteromycete (imperfect fungus). The different *Beauveria* strains are highly adapted to particular host insects. A broad range of *B. bassiana* species have been isolated from a variety of insects worldwide that are of medical or agricultural significance. An interesting feature of *Beauveria* is the high host specificity of many isolates. Hosts of medical importance include vectors for agents of tropical infectious diseases such as the tsetse fly *Glossina morsitans morsitans*, the sand fly *Phlebotomus* that transmits *Leishmania,* and the bugs of the genera *Triatoma* and *Rhodnius*, the vectors of Chagas' disease. Hosts of agricultural significance include the Colorado potato beetle, the codling moth and several genera of termites. Furthermore, the high level of persistence in the host population and in the environment provides long-term effects of the entomopathogenic fungi on pest suppresson.

In China, *B. bassiana* is applied against the European corn borer *Ostrinia mubilalis*, pine caterpillars *Dendrolimus* spp., and green leafhoppers *Nephotettix* spp.. In the Soviet Union, *B. bassiana* is produced under the trade name Boverin for control of the Colorado potato beetle *Leptinotarsa decemlineata* and the codling moth *Laspeyresia pomonella*.

1.2.1. The life cycle of *B. bassiana*

B. bassiana has a dimorphic mode of growth. In the absence of the specific insect host *Beauveria* passes through an asexual vegetative life cycle that includes germination, filamentous growth and the formation of sympoduloconidia (Fig. 1.2B). In the presence of its host insect, *Beauveria* switches to the pathogenic life cycle. The conidiospores germinate on the surface of the cuticle and the germinated hyphal tubes penetrate the insect's integument directly. When having penetrated the cuticle, the fungus alters its growth morphology to a yeast-like phase and produces hyphal bodies, which circulate in the haemolymph and proliferate by budding (Fig. 1.2A). Following the death of the host, fungal growth reverts back to the typical hyphal form (the saprotrophic stage). The ability to convert to the yeast-like phase may be a prerequisite for pathogenicity.

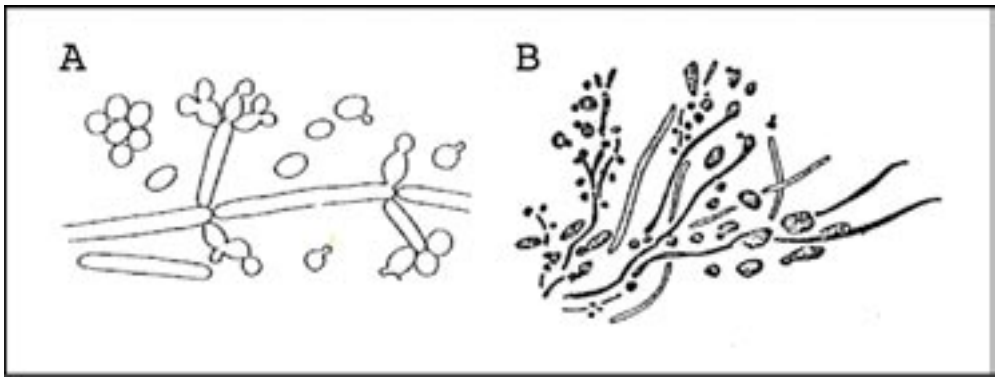


Fig. 1.2. Dimorphic growth mode of B. bassiana. (A) yeast.like parasitic phase when infecting susceptible species. (B) Saprobic phase shows filamentous hypha.

1.2.2. The infection process

In contrast to bacteria and viruses that pass through the gut wall from contaminated food, fungi have an unique mode of infection. They reach the hamocoel through the cuticle or possibly through the mouth parts. Ingested fungal spores do not germinate in the gut and are voided in the faeces. The death of the insect results from a combination of factors: mechanical damage resulting from tissue invasion, depletion of nutrient resources and toxicosis (Fig. 1.3).

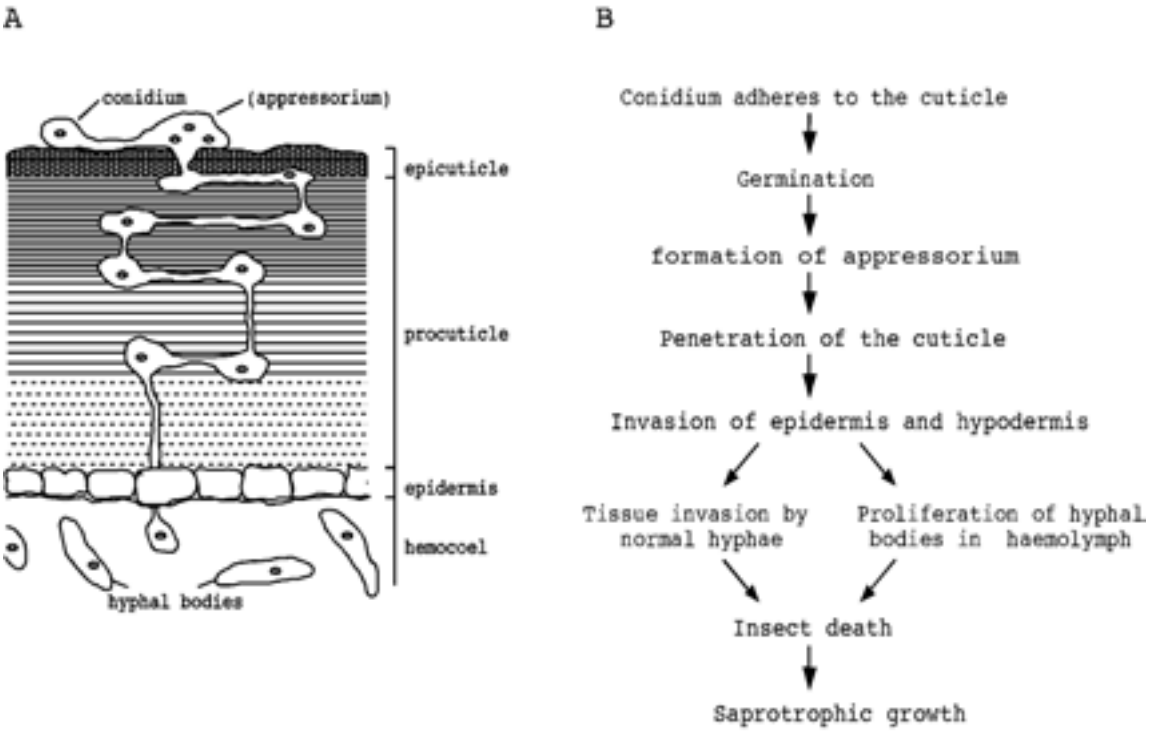


Fig. 1.3. The infection process of *B. bassiana*. (A) Structure of insect cuticle and mode of penetration. According to Clarkson and Charnley (1996). (B) Diagram of the infection steps. (C) Scanning electron micrograph showing appressorium has formed at the base of the hair socket. According to Charnley (1996).

1.2.2.1. Adhesion and germination of conidia

Attachment of a fungal spore to the cuticle surface of a susceptible host represents the initial event in the establishment of mycosis. For most entomopathogenic fungi host location is a random event and attachment is a passive process with the aid of wind or water. It was found that dry spores of *B. bassiana* possess an outer layer composed of interwoven fascicles of hydrophobic rodlets. This rodlet layer appears to be unique to the conidial stage and has not been detected on the vegetative cells. The adhesion of dry spores to the cuticle was suggested to be due to non-specific hydrophobic forces exerted by the rodlets (Boucias *et al.,* 1988). In addition, lectins, a kind of carbohydrate binding glycoproteins, have been detected on the conidial surface of *B. bassiana*. It was also suggested that lectins could be involved in binding between conidia and the insect cuticle. The exact mechanisms responsible for the interaction between fungal spores and the cuticle remain to be determined (Latge, 1988).

After the pathogen reaches and adheres to the host surface, it proceeds with rapid germination and growth which are profoundly influenced by the availability of nutrients, oxygen, water, as well as pH, and temperature, and by the effects of toxic host-surface compound. Generally, fungi with a broad host range germinate in culture in response to a wide range of nonspecific carbon and nitrogen sources. Entomopathogenic fungi with restricted host range appear to have more specific requirements for germination (St Leger, 1989a).

1.2.2.2. Formation of an infection structure

Entomopathogenic fungi invade their hosts by direct penetration of the host cuticle. The cuticle has two layers, the outer epicuticle and the procuticle. The epicuticle is a very complex thin structure that lacks chitin but contains phenol-stabilized proteins and is covered by a waxy layer containing fatty acids, lipids and sterols (Hackmann, 1984). The procuticle forms the majority of the cuticle and contains chitin fibrils embedded into a protein matrix together with lipids and quinones (Neville, 1984). Protein may account for up to 70% of the cuticle. In many areas of the cuticle the chitin is organized helically giving rise to a laminate structure.

In common with many entomopathogenic fungi, *B. bassiana* conidia germinate on the host surface and differentiate an infection structure termed appressorium. The

appressorium represent an adaptation for concentrating physical and chemical energy over a very small area so that ingress may be achieved efficiently (Fig. 1.3C). Thus, formation of the appressorium plays a pivotal role in establishing a pathogenic interaction with the host. Appressorium formation may be influenced by host surface topography and biochemical investigations indicate the involvement of the intracellular second messengers Ca^{2+} and cyclic AMP (cAMP) in appressorium formation (St Leger *et al.,* 1991).

1.2.2.3. Penetration of the cuticle

Pathogenic fungi need to penetrate through the cuticle into the insect body to obtain nutrients for their growth and reproduction. Entry into the host involves both enzymic degradation and mechanical pressure as evidenced by the physical separation of lamellae by penetrated hyphae. A range of extracellular enzymes that can degrade the major components of insect cuticle, including chitinases, lipases, esterases and at least four different classes of proteases, have been suggested to function during the fungi pathogenesis. The production of cuticle-degrading enzymes by *M. anisopliae* during infection structure formation on *Calliphor vomitoria* and *Manduca sexta* has been investigated by biochemical and histochemical analysis both *in vivo* and *in vitro*. Among the first enzymes produced on the cuticle are endoproteases (termed PR1 and PR2) and aminopeptidases, coincident with the formation of appressoria. N-Acetylglucosaminidase is produced at a slow rate as compared to the proteolytic enzymes. Chitinase and lipase activities were not detected (St Leger *et al.,* 1989b). Although the complex structure of the insect cuticle suggests that penetration would require the synergistic action of several different enzymes, much of the attention has focused on the cuticle-active endoprotease as a key factor in the process.

1.2.2.4. Production of toxins

There is considerable circumstantial evidence from deuteromycete pathogens for the involvement of fungal toxins in host death. The action of cytotoxins is suggested by cellular disruption prior to hyphae penetration. Behavioural symptoms such as partial or general paralysis, sluggishness and decreased irritability in mycosed insects are consistent with the action of neuromuscular toxins (Charnley, 1984). *B. bassiana* and *M. anisopliae* produced significant amounts of toxic compounds within their hosts. For example, the toxins Beauvericin, Beauverolides, Bassianolide and Isarolides

have been isolated from *B. bassiana* infected hosts (Hamill, 1969; Elsworth, 1977); toxins Destruxins (DTXs) and Cytochalasins have been isolated from *M. anisopliae* infected hosts. The toxins have shown to have diverse effects on various insect tissues. DTX depolarizes the *lepidopteran* muscle membrane by activating calcium channels. In addition, function of insect hemocytes can be inhibited by DTX (Bradfisch, 1990). Presumably, there are still many toxins that remain to be isolated from parasized insects and except DTXs, their relevance to patogenicity remains to be established.

1.2.3. Host defense systems

In order to prevent invasion by fungi, insects have evolved various defense mechanisms. The defensive arsenal of insects contains both passive structural barries, such as the cuticle, and a cascade of active responses to pathogens that gain access to the hemocoel. This active responses include melanization, cellular reactions, humoral reaction to recognize the non-self pathogen, and production of protease inhibitors.

Melanization: the oxidation of phenolic compounds to dihydroxyphenylalanine, typified by the production of brown or black melanic pigments, is a common feature of the response of many insects to fungal infection. Melanin may partially shield cuticle from enzymatic attack or may be toxic to fungi. However, such protection is incomplete. The investigation from St Leger (1988) indicates that melanization is primarily an effective defence against weak or slow growing pathogens, but is ineffective against more virulent fungi.

Cellular reactions: once the cuticle and epidermis have been breached the invading fungus is faced with the defence systems of the hemolymph. The responses to mycopathogens within the haemocoel include phagocytosis, encapsulation and nodulation. However, the effect on fungal elements is uncertain. With the arbitrary injection method, Bidochka (1987) found that haemocytes of the migratory grasshopper, *M. sanguinipes*, encapsulate viable conidia of *B. bassiana*, however they fail to suppress conidial germination within the nodule. It was suggested that the production of toxins and extracellular proteases by *B. bassiana* could trigger the evadation of encapsulation.

Humoral reactions: in response to fungal challenge, insects elicit an acquired humoral "immunity" to subsequent infection. Recognition of "non-self" is critical to the initiation of the hemocytic defense reaction and this selective response in insects depends on a specific chemical recognition on part of the hemocytes. Serum and hemocyte cell membrane-bound lectins have been found in many insects (Mello, 1999). They could play a role in immune defense reactions since they agglutinate pathogens as well as fungi (Mello, 1999). Thus, insect serum agglutinin may function as opsonic mediating the enhanced attachment of granulocytes to the hyphal bodies (Pendland *et al.,* 1988).

Production of protease inhibitors: host-produced protease inhibitors, which inhibit cuticle-degrading enzyme activities of pathogens, may contribute to insect defence systems. Such compounds have been isolated from the serum of *Anticarsia gemmatalis* larvae which were resistant to infection by *Nomuraea rileyi* (Boucias *et al.,* 1987).

1.3. Aspects of using entomopathogenic fungi as bio-control agents

The advantages of using fungi as insecticides are:

(1) Their high degree of specificity for pest control. Fungi can be used to control harmful insect pests without affecting beneficial insect predators and non-harmful parasites.
(2) The absence of effects on mammals and thus the reduction of the hazards normally encountered with insecticide applications, such as pollution of the environment.
(3) The lack of problems caused to insect resistance and prolonged pest control.
(4) A high potential for further development by biotechnological research.
(5) High persistence in the environment provide long-term effects of entomopathogenic fungi on pest suppression.

However, there are also a number of constraints on the use of fungi as insecticides:

(1) 2-3 weeks are required to kill the insects whereas chemical insecticides may need only 2-3 hours.
(2) Application needs to coincide with high relative humidity, low pest numbers and a fungicide free period.

(3) Due to the high specificity additional control agents are needed for other pests.
(4) Their production is relatively expensive and the short shelf life of spores necessitates cold storage.
(5) The persistence and efficacy of entomopathogenic fungi in the host population varies among different insects species, thus insect-specific application techniques need to be optimised to retain long-term impacts.
(6) A potential risk to immunodepressive people

1.4. Genetic engineering of entomopathogenic fungi

A more widespread use of fungi for bio-control will depend on improvements of wild-type strains by combining characteristics of different strains and mutants.. Two type of improvements may be considered: (i) improvement the efficacy of the insecticide, e.g., by reducing the dose necessary to kill the insects, by reducing the time to kill the pest or decreasing crop damage caused by the pest by reducing the feeding time; (ii) expanding the host range. Essential for the development of a hypervirulent strain is a complete understanding of the remarkable pathology of fungi infections.

Molecular biology provides the necessary tools for dissecting the mechanisms of pathogenesis and in the longer term for producing recombinant organisms with new characteristics. Initial development towards these goals has occurred with *M. anisophliae* and to a much lesser extent with *B. bassiana* (Hegedus, 1991). Genetic transformation systems, which are an essential part of modern fungal research, and is necessary for the experimental manipulation of virulence genes *in vitro* and *in vivo,* have been established (Goettel and St Leger, 1990). The success of utilizing these procedures depend on the availability of selectable transformation markers. transformation techniques have been used to isolate specific pathogenic genes, investigate virulence determinants of *M. anisophliae*, and to produce a strain with enhanced virulence. Unravelling the molecular mechanisms of fungal pathogenesis in insects will provide the basis for the genetic engineering of entomopathogenic fungi.

1.5. The aims of this thesis

As described above, the entomopathogenic fungus *B. bassiana* is attracting increased attention as a potential tool for biological control of insect pests. Understanding the mechanisms of fungal pathogenesis in insects will provide a rational basis for strain selection and improvement. The research of this thesis is undertaken with a *B. bassiana* strain, ARSEF 252, which is highly pathogenic for the Colorado potato beetle, *Leptinotarsa decemlineata*. The aim of this thesis is to evaluate the contribution of individual enzymes to pathogenesis of *B. bassiana252*. In addition, in order to help in the manipulation of pathogenic genes in entomopathogens or even to introduce heterologous pathogenic genes designed to improve their potential for biocontrol, we also interested in optimizing the transformation procedures which is suitable for *B. bassiana*252. Therefore, a new dominant selection marker for fungal transformation is developed. The outcome of these two independent studies will be presented and discussed separately in two sections.

II. Characterization of insect cuticle-degrading enzymes from *B. bassiana*

2.1. Introduction

2.1.1. The mechanism of cuticle degradation by endoprotease

Metarhizium anisopliae is by far the best studied entomopathogenic fungus and several virulence factors involved in the disease process have been identified (Clarkson and Charnley, 1996). A subtilisin-like protease, termed PR1 has been cloned and characterized (St Leger *et al.,* 1992). PR1 is synthesized as a large precursor containing an 18-amino acid signal peptide and an 89-amino acid propeptide. The mature protein (28.6 kDa) contains 281 amino acid residues. The sequence shows considerable similarities with other enzymes of the subtilisin subclass of serine endoproteases. In particular, the serine, histidine, and aspartate residues that comprise the active site of these proteases are conserved in PR1. PR1 possesses 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 possesses a secondary specificity for extended hydrophobic peptide chains with the active site recognizing at least five subsite residues. This relative nonspecificity accounts for its activity against a range of proteins.

By immunogold electron microscopy, it was shown that PR1 is secreted by the appressorium and penetrating hyphea within the cuticle. For *M. anisopliae*, PR1 appears to be a pathogenicity determinant by virtue of its ability to extensively degrade the cuticle and its production at high levels by the pathogen in situ during infection (St Leger *et al.,* 1987). Furthermore, addition of multiple copies of *pr1* under the control of a constitutive promoter increases the virulence of the transformants (St Leger *et al.,* 1996). The mechanism of cuticle degradation by PR1 in *M. anisopliae* was suggested as follows: (1) PR1 is adsorbed to the cuticle via nonspecific electrostatic forces; (2) the active site comes into contact with any part of the accessible cuticle protein chains and under appropriate conditions, e.g., temperature, splits susceptible peptide bonds thus relaesing cuticle proteins; (3) solubilized proteins are further degraded until a chain length of around 5 is obtained (St Leger *et al.,* 1987). The progression of knowledge regarding the major pathogen protease, PR1, followed a course that may serve as a model for research on other entomopathogenic fungi. Furthermore, the characterization of PR1 also aided in

unravelling additional factors that contribute to pathogenicity. In addition to PR1, another endoprotease, trypsin-like protease (PR2), has also been characterized from *M. anisopliae*, but the role of PR2 is not clear (St Leger, 1996).

2.1.2. The function of phospholipases in cuticle penetration

Since lipids represent major chemical constituents of the insect cuticle, enzymes capable of hydrolyzing these compounds, such as phospholipases, could be expected to be involved in the cuticle disruption processes that occur during host invasion. Phospholipases are a heterogeneous group of enzymes that are able to hydrolyse one or more ester linkages in glycerophospholipids. The action of phospholipases can result in the destabilization of membranes, cell lysis and release of lipid second messengers (Ghannoum, 2000). These enzymes are categorized according to the location of the ester link that is cleaved (Fig. 2.1). Although phospholipase B (PLB) refers to an enzyme that can remove both *sn*-1 and *sn*-2 fatty acids, this enzyme also has lysophospholipase-transacylase activity.

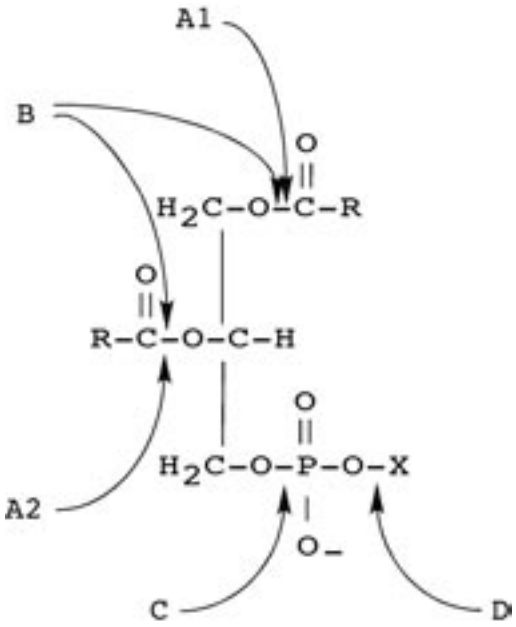


Fig. 2.1. Sites of action of phospholipases. A1, A2, B, C and D indicate cleavage sites of the corresponding phospholipases (PLA1, PLA2, PLB, PLC and PLD).

Extracellular phospholipases have been implicated as pathogenicity factors for bacteria, rickettsiae and protozoa, The type of phospholipase involved in virulence varies with the organism. For example, *C. perfringens* (Alape-Giron, 2000*)* secretes a phospholipase C (PLC), whereas *T. gondii* secretes a phospholipase A (PLA). The importance of these enzymes, especially PLB, for virulence has so far only been verified in medically important fungi. PLB was secreted by different clinically important fungal species such as *Candida albicans* (Mukherjee, 2001), *Aspergillus fumigatus* (Burch,1996*)* and *Cryptococcus neoformans* (Cox, 2001). The role of PLB in the pathogenicity of entomopathogenic fungi remains to be determined, even in the best-studied species *M. anisopliae*.

2.1.3. General remarks

The great diversity of fungi, and the many routes by which they have achieved their success as pathogens, render it imprudent to consider *M. anisopliae* or any other fungi as an overall model for pathogenicity. The general validity of the "protease concept" remains to be proved. Nevertheless, given that the proteinaceous cuticle is a barrier to all entomopathogenic fungi, the involvement of proteases in the infection process is likely to be ubiquitous.

There is a surprising degree of species and strain variability observed in the production of insect cuticle-degrading enzymes. In *M. anisopliae*, variability was not limited to the quantitative levels of enzymes produced, but extended to the patterns of enzyme expression on different growth media. Fungal strain variability in cuticle-degrading enzyme production may be directly related to variability in virulence. It is also possible that these enzymes may be involved in host range determination.

2.1.4. Experimental plan:

So far there have been no detailed investigations of the enzymes involved in the infection processes of *B. bassiana*. Because of the strong evidence supporting the role of proteases (PR1 and PR2) and phospholipase B in fungal pathogens and the correlation observed in *M. anisopliae* and *C. albicans*. An initial characterization of these potential virulence determinants in *B. bassiana* was undertaken. The following experimental work was performed:

- Cloning of the genes encoding PR2 and PLB from *B. bassiana.*
- Sequence analysis of the genes encoding PR2 and PLB.
- Partial recombinant expression and the development of antisera against PR1 and PLB.
- Examination of the expression of PR1 and PLB in cuticle cultures.

2.2. Results

2.2.1. Cloning of the genes encoding PR2 and PLB from *B. bassiana* 252

Initial experiments by A. Leclerque indicated the presence of two *p*r2 homologues and two *plb* homologues in *B. bassiana.* Degenerate primers complementary to conserved regions within fungal *pr2* and *plb* genes were used for PCR amplification from the *B. bassiana* genome. Two amplicons of different size from each gene were cloned and sequenced. Their homology to other fungal *pr2* and *plb* genes was confirmed by alignments with the related genes. The results indicated that there are two isotypes of the *pr2* gene (designated *try1* and *try2*) and two isotypes of the *plb* gene (designated *plb1* and *plb2*) in *B. bassiana* (A. Leclerque, unpublished).

Plasmids containing the *pr2* and *plb* amplicons were obtained from A. Leclerque and the cloned fragments were amplified by PCR. The PCR products were used as probes for screening a phage genomic DNA library of *B. bassiana* in order to isolate phage clones harbouring the full length of *try1, try2* genes and *plb1, plb2* genes, respectively. A total of 20,000 plaques were screened at high stringency and 11 *try1* clones, 4 *try2* clones, 3 *plb1* clones and 4 *plb2* clones were identified. One phage clone of each gene was further purified and designated TA4, TB3, PA1 and PB3.

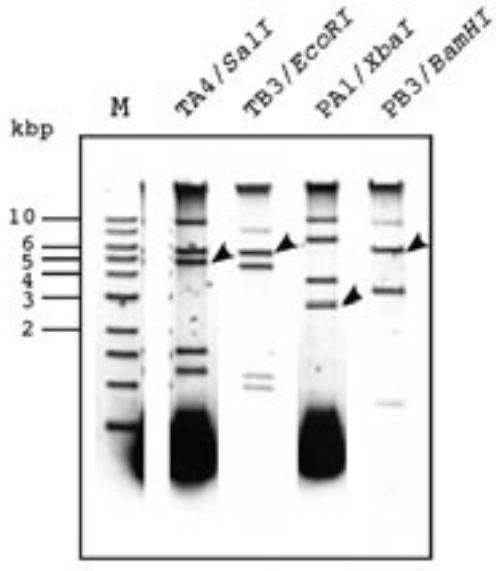


Fig. 2.2. Restriction pattern of phage clones.
1 µg of DNA from the phage clones TA4, TB3, PA1 and PB3 was digested with *Sal I, EcoR I, Xba I* and *BamH I,* respectively, and separated on an 0.8% agarose gel. The presence of the respective fungal genes try1, try2, plb1 and plb2 in the restricted fragments was proved by Southern Blot analysis. Positive bands are indicated by arrowheads. M, 1 kb DNA ladder.

Sal I restricted fragments from the clone TA4, *Eco*R I restricted fragments from the clone TB3, *Xba I* restricted fragments from the clone PA1, and *Bam*H I restricted fragments from the clone PB3 were subcloned into a pUC18 vector and sequenced. Thefragments selected for sequencing are indicated in Fig. 2.2. In addition, fragment/ fragments obtained by *Not* I restriction from all four clones was/ were cubcloned into pBSSKII+ vector to acquire sequence data derived from overlapping clones to ensure that the subcloned DNA fragments were contiguous.

2.2.2. Sequence analysis of the *pr2* genes

Nucleotide sequence and deduced amino acid sequence of the *pr2* genes

The *try1* gene has an ORF of 813bp which is interrupted by three introns. The intron locations were inferred by disruption of consensus trypsin sequence and supported by the presence of consensus 5' and 3' splice sites which are characteristic of fungal introns (Gurr *et al.,* 1987). The protein encoded by the *try1* gene comprises 271 amino acid residues (28.1 kDa). The *try2* gene has an ORF of 738bp and no obvious intron splice sites were found. The encoded protein contains 245 amino acid residues (25.8 kDa).

A signal-peptide cleavage site was predicted for both TRY1 and TRY2 peptide sequences according to the "(-3, -1) rule" of Von Heijne (1986), indicating that the encoded enzymes are preproenzymes which are subjected to post-translational cleavage.

Homology comparison and secondary structure of the *pr2* genes

The predicted peptide sequences of *try1* and *try2* show 22.4% identity. Furthermore, they contain all of the most conserved amino acid positions common to other trypsin-like proteins, and possess a putative histidine, asparagines and serine catalytic triad that is indicative of a serine protease (Fig. 2.3).

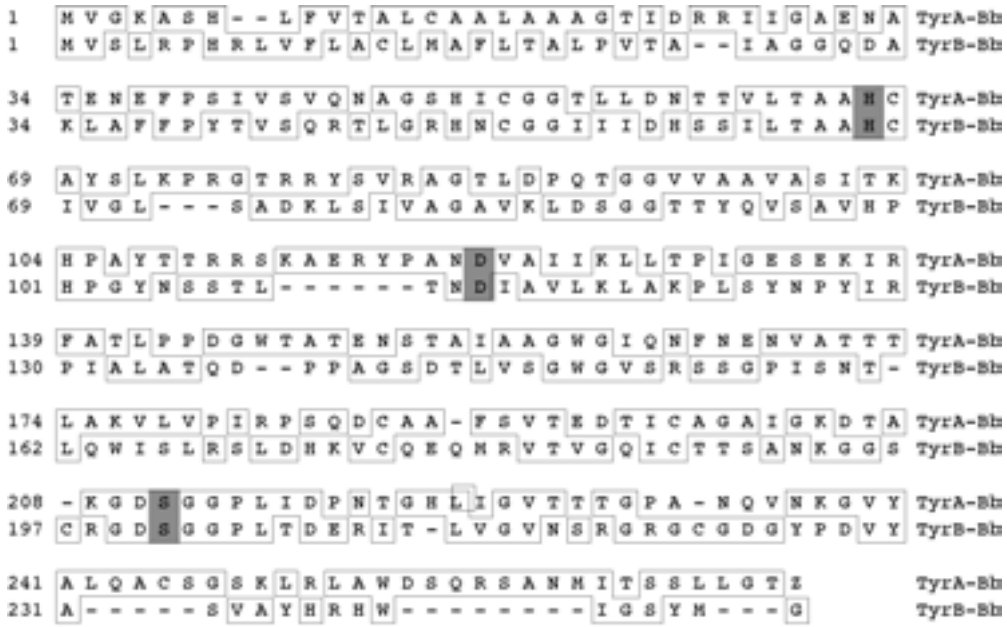


Fig. 2.3. Amino acid sequence alignment of the proteins encoded by *try1* and *try2* from *B. bassiana*. Putative His-Asp-Ser catalytical triad is shaded.

Database searches using the BLASTP programe showed that both sequences have high homology with other serine proteases of the trypsin subclass found in the database as indicated by phylogenetic analysis (Fig. 2.4). Interestingly, the two trypsin-like genes are related to two different genes. The deduced amino acid sequence of *try1* showed high homology to the trypsin-like proteases from fungi, e.g., 41% identity to *Trichoderma harzianum*; 41% identity to *Metarhizium anisopliae*; 37% identity to *Phaeosphaeria nodorum*; and 36% identity to *Fusarium oxysporum*. In contrast, the deduced amino acid sequence of *try2* showed the highest homology to the trypsin-like proteases from insects, e.g., 41% identity to that of *Drosophila melanogaster*; 39% identity to *Anopheles darlingi*; 39% identity to *Stomoxys calcitrans*; 37% identity to *Aedes aegypti*; and 37% identity to *Anopheles gambiae.*

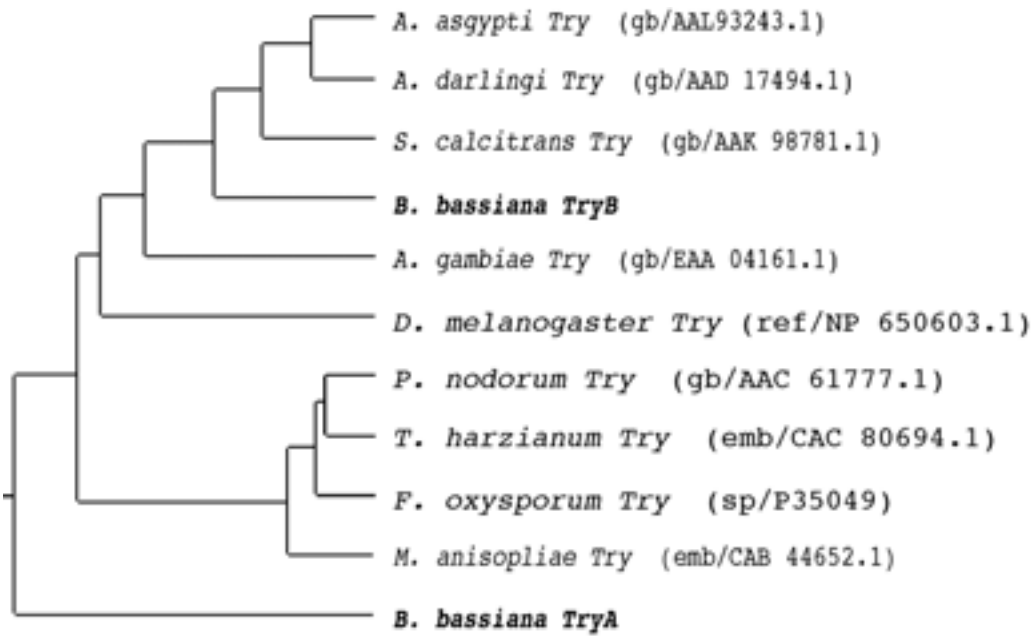


Fig. 2.4. Phylogenetic tree of trypsins inferred from amino acid sequence alignments. A phylogenetic tree was constructed for trypsin-like genes using the Multiple Sequence Alignment program of the lasergene package.

2.2.3. Sequence analysis of the *plb* genes

Nucleotide sequence and deduced amino acid sequence of the *plb* genes

The ORF of the *plb1* gene was found to be 1980 bp in size encoding a 660-amino-acid protein with a predicted molecular mass of 70.9 kDa. The *plb2* gene has an open reading frame of 2007 bp, encoding a predicted protein of 669 amino acid residues with a molecular mass of 72.5 kDa. The presence of two introns in both the *plb1* and the *plb2* gene was confirmed by RT-PCR with primers flanking the splice sites.

Several potential N-glycosylation sites, indicated by a Asn-X-Ser/Thr motif, were identified in the predicted protein sequence of both PLB1 and PLB2. Furthermore, three amino acid residues essential for the catalytic function of lipolytic enzymes were found in both PLB1 (^{138}Arg, ^{177}Ser, ^{429}Asp) and PLB2 (^{143}Arg, ^{182}Ser,

^{436}Asp) in accordance with the following motifs: SGGGXRA(M/L), GLSG(G/S) and D(S/G)G(E/L)XXXN.

Homology comparison and secondary structure of the putative PLBs

The deduced amino acid sequence of PLB1 was highly homologous to that of PLB2 (57% identity). Comparison of the putative *B. bassiana* PLBs (PLB1 and PLB2) with other proteins in the database (using the BLASTP program) revealed high homology to known fungal PLBs from *S. cerevisiae* (40% and 38%), *P. chrysogenum* (46% and 43%), *N. crassa* (44% and 43%), *C. albicans* (38% and 35%), *S. pombe* (34% and 29%) (Fig. 2.5).

Unlike the PLBs of the nonpathogenic fungi, *S. cerevisiae* and *S. pombe*, *B. bassiana* PLB1 and PLB2 were characterized by the absence of a hydrophobic COOH terminus which may signal for the addition of a glycosylphophatidylinositol (GPI) anchor (Lu, 1995).

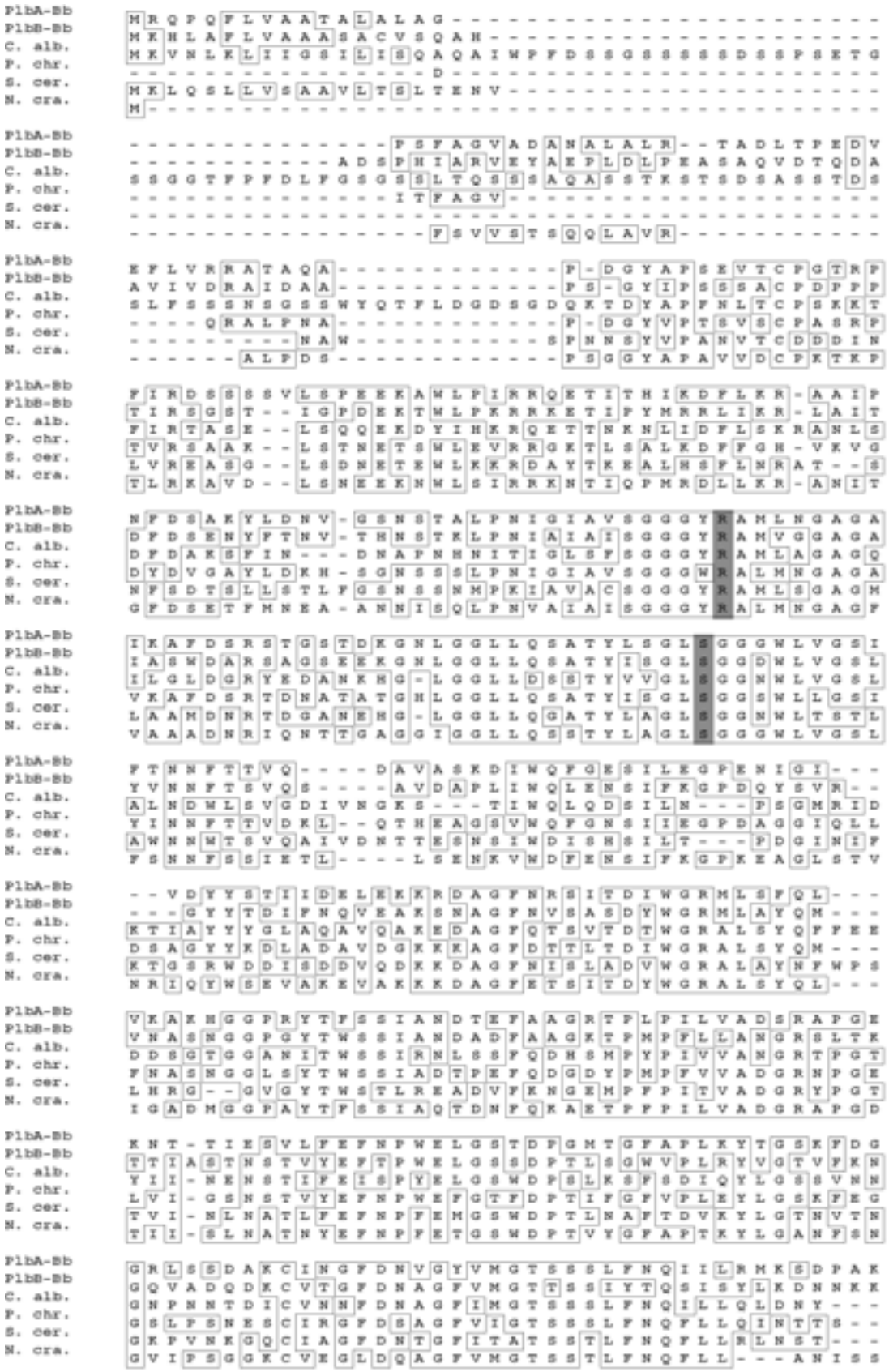

PlbA-Bb
PlbB-Bb
C. alb.
P. chr.
S. cer.
N. cra.

Fig. 2.5. Sequence alignment of fungal PLBs. The amino acid sequences predicted by *B. bassiana* PLB1 and PLB2 were aligned with PLBs from *C. albicans* (C. alb.; gb/ AAF08980.1), *P. chrysogenum* (P. chr.; WISS-PROT: P39457), *S. cerevisiae* (S.cer.; ref/ NP_013721.1), *N. crassa* (N.cra.; gb/ AAC03052.1), and *S. pombe* (S. Pom.; ref/ NP_593196.1). Identical amino acids are boxed. Shaded areas indicate residues of putative importance for catalysis.

2.2.4. Functional studies of two enzymes involved in the infectious process

2.2.4.1. Heterologous expression of the fragments encoding antigenic determinants of PR1 and PLB2

In order to investigate the significance of cuticle-degrading enzymes and their formation in the course of the infectious process, antisera against peptides derived from PR1 and PLB2 were raised. Part of the cDNAs encoding PR1 (obtained from A. Leclerque) and PLB2, which display high antigenicity were chosen as antigens, and cloned in frame into the multiple cloning sites of the vector pQE40 generating the plasmids pQE-PR1 and pQE-PLB2 (Fig. 2.6). The gene encoding Dehydrofolate reductase (DHFR) and a hexahistidine tag, harboured by pQE40, was located at the 5' end of the cloning site. The hybrid gene encodes the corresponding fusion protein. The expression of the fusion protein is under the control of the T5 promoter that is fused to the *lacZ* operon. The generated fusion proteins were termed DHFR-PR1 and DHFR-PLB2, respectively.

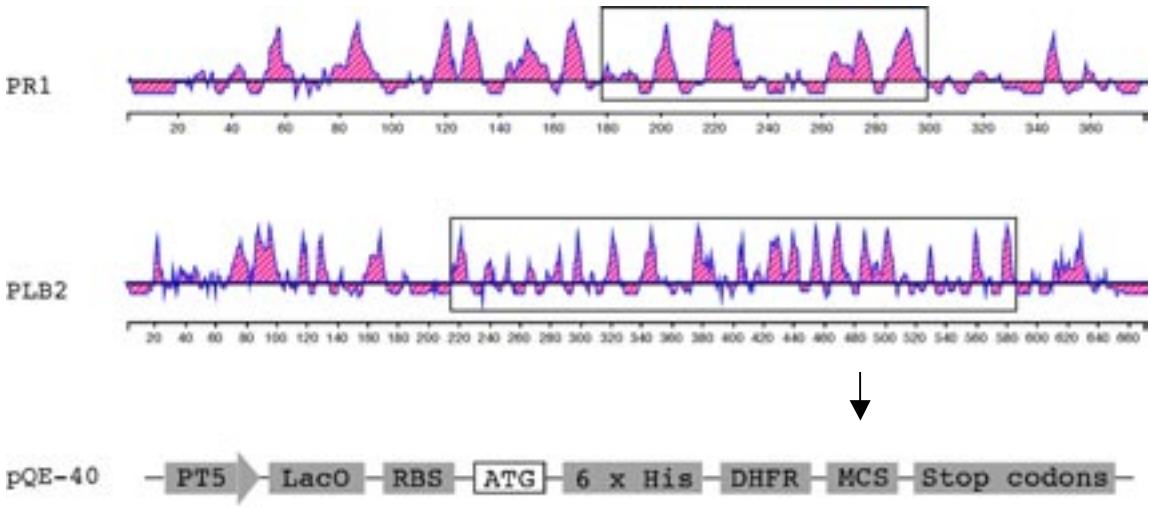


Fig. 2.6. Cloning the DNA fragments encoding the PR1 and PLB2 antigen in the pQE-40 expression vector. The antigenicity of the PR1 and PLB2 amino acid sequences was obtained using the Lasergene program package. Domains with high antigenicity correspond to shaded areas above the baseline. DNA sequence encoding the regions with strong antigenicity (boxed) were selected for bacterial expression using the vector pQE40.

2.2.4.2. Purification of the fusion proteins and raising of antisera

The plasmids pQE-PR1 and pQE-PLB2 were transformed into the *E. coli* strain SG13009 (Qiagen). A helper plasmid pREP4 contained in the recipient strain encodes the *lacZ* repressor, thus the expression of the fusion protein was regulated by the addition of IPTG which can induce the *lacZ* promoter. The fusion protein which was present mainly in a soluble form was purified under denaturing conditions on a Ni-NTA agarose column (Fig. 2.7). The fusion proteins of about 39kDa for DHFR-PR1 and 70kDa for DHFR-PLB2 were visible on Coomassie-stained SDS-PAGE gels and their identity was confirmed by Western blot analysis using an anti-DHFR antiserum. The proteins were excised from the gel and used to raise antisera in rabbits.

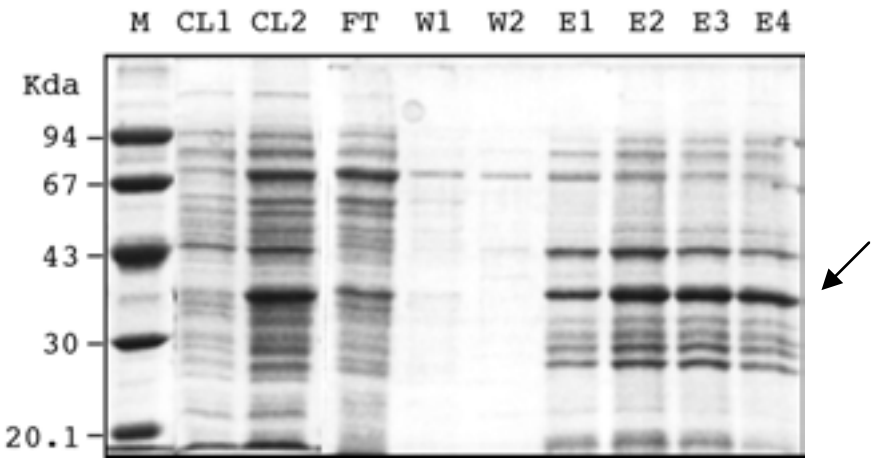


Fig. 2.7. Purification of DHFR-PR1 using a Ni-NTA agarose column under denaturing conditions. Fractions were visualized by Coomassie blue staining after separation on a 12.5% SDS-PAGE gel. M, protein marker; CL1, cleared lysate of noninduced control cells; CL2, cleared lysate of IPTG (1 mM) induced cells; FT, flow-through; W1-W2, first and second washing fractions; E1-E4, serial eluates. The arrow indicates the PR1 fusion protein band.

2.2.4.3. Insect cuticle as an inducer of PR1 and PLB2 production

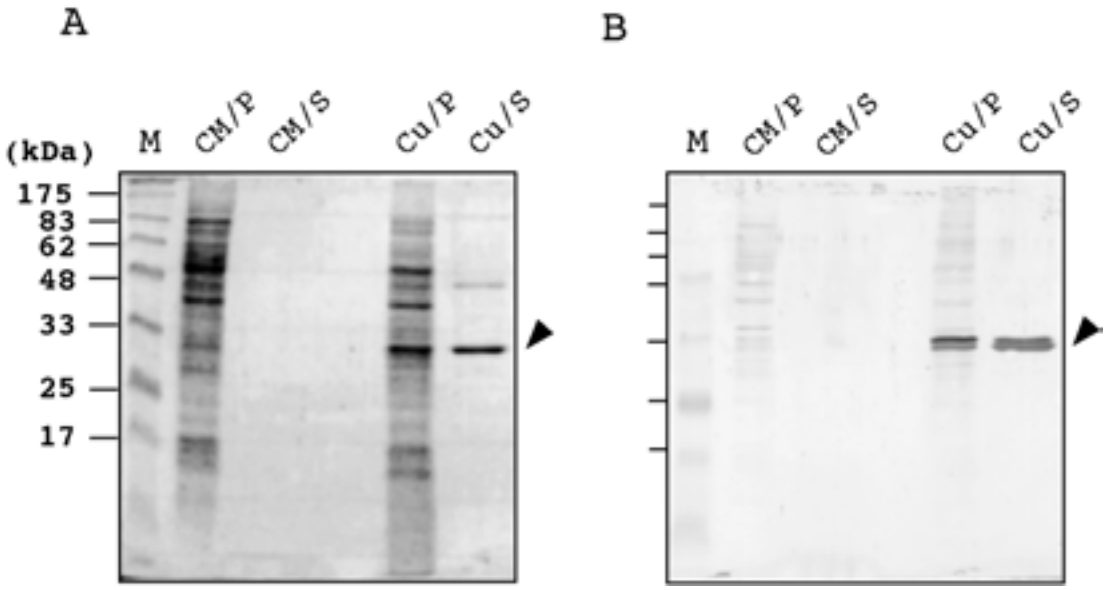
Previous results from *M. anisopliae* have shown that production of PR1 is transcriptionally modulated by carbon catabolite and nitrogen metabolite repression. Further induction is obtained in poor media by the addition of insect cuticles. Therefore, two kinds of media were prepared: (1) Cuticle medium (Cu), 0.8%(w/v) of pulverized insect cuticle was added in basal salt medium as the sole carbon and nitrogen source; (2) Complete Medium (CM) containing sucrose and nitrate as the carbon and nitrogen source.

B. bassiana was grown for 12 hours in complete medium and cuticle medium following the medium transfer steps introduced in Materials and Methods. Gene expression and enzyme synthesis was detected by RT-PCR and Western blot analysis, respectively.

Production of PR1 by *B. bassiana*

From the Coomassie-stained gel (Fig. 2.8A) it can be seen that the total array of protein production in *B. bassiana* has been altered in cuticle medium as compared to that in the complete medium. A band corresponding to the predicted size of PR1 was clearly detected in the mycelium fraction as well as in the extracellular protein fraction when *B. bassiana* was grown in cuticle medium (Fig. 2.8B). In contrast, PR1 could not be detected extracellularly and intracellularly when the cells were grown in the complete medium. Synthesis of PR1 occurs rapidly upon cuticle induction. The protein can be detected in cells and the supernatant of a culture inoculated in cuticle medium after four hours (data not shown).

Corresponding results at the transcriptional level were obtained by RT-PCR analysis. As shown in Fig. 2.8C, *pr1* mRNA was highly expressed in *B. bassiana* grown in cuticle medium. Formation of *pr1* mRNA could not be detected after growth in complete medium.



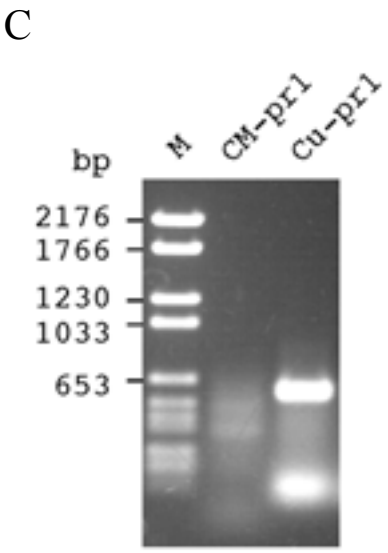


Fig. Fig. 2.8. PR1 production by *B. bassiana*. Mycelium was grown in complete medium (CM) and cuticle medium (Cu) for 12 hours. The proteins from mycelium (P) and supernatants (S) were extracted and separated on a 12.5% SDS-PAGE gel followed by: (A) Coomassie analysis for estimation of protein production; (B) Immunoblot analysis using the antisera against PR1. Arrowhead indicates the PR1 band. M, protein marker. Panel (C): Transcription of *pr1* mRNA detected by RT-PCR analysis. Total RNA was isolated from mycelium harvested from different growth media. The RT-PCR reactions were carried out using the same amounts of RNA. M, DNA marker VI (Roche).

Production of PLB2 by *B. bassiana*

By immunoblot analysis using the PLB2 antisera, a polypeptide fragment that corresponds in size to PLB2 (70 kDa) was detected in *B. bassiana* culture grown in complete medium and cuticle medium, respectively. The highest amount of PLB2 was produced by cells grown in cuticle medium (Fig. 2.9A). Secretion of PLB2 to the culture medium was not detected under the same experimental conditions.

The transcription of the *plb2* gene was detected by RT-PCR analysis. As shown in Fig. 2.9B, *plb2* mRNA was expressed in the cells independent of growth conditions.

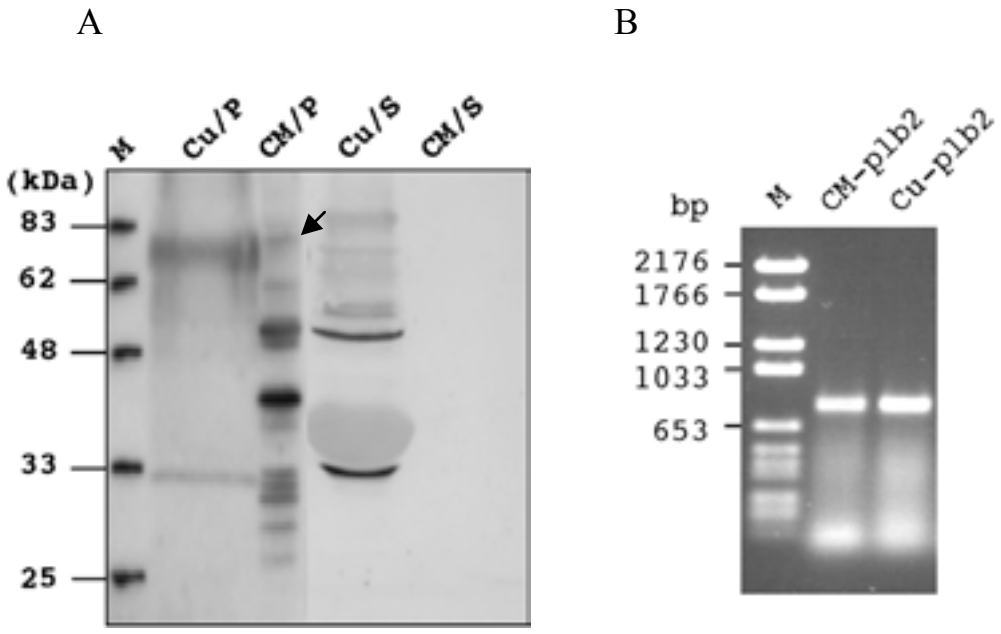


Fig. 2.9. (A) PLB2 production by *B. bassiana*. Mycelium was grown in cuticle medium (Cu) or complete medium (CM) and the proteins from mycelium (P) and supernatants (S) were extracted and separated on a 10% SDS-PAGE gel followed immunoblot analysis with anti-PLB2 antisera., arrow indicates the PLB2 band. M, protein marker. **(B) *plb2* mRNA transcription detected by RT-PCR.** Total RNA was isolated from mycelium harvested from different growth medium. The same amounts of RNA was applied for each RT-PCR reactions. M, DNA marker VI (Roche).

2.3. Discussion

2.3.1. Production of proteases by *B. bassiana* 252

Insect pathogenic fungi produce an array of enzymes capable of degrading protein, chitin, and lipid components of the insect cuticle (St Leger *et al.,* 1986a). However, a convincing role in pathogenesis has been established only for the serine protease PR1. The activity of PR1 is a prerequisite for successful penetration of the host by *M. anisopliae* (St Leger *et al.,* 1989b).

From the cDNA sequence and protein structure, it has been shown that PR1 resembles the powerful serine endoprotease proteinase K, but PR1 is far more effective than proteinase K in degrading insect cuticle, indicative of pathogenic specialization. The higher activity of PR1 could come from the positively charged residues (His17, Arg18 and Arg20) located on the surface of the protease PR1, that are absent from Proteinase K. The presence of this charged domain might allow electrostatic interactions with negatively charged groups in the insect cuticle (St Leger, 1992).

The study of PR1 from *M. anisopliae* has proven that PR1 expression is regulated by both carbon catabolite and nitrogen metabolite repression and is specifically induced by cuticular proteins after carbon and nitrogen starvation. The level of PR1 was induced tenfold within 24h of contact with cuticle and the regulation was found to be at the transcriptional level (Paterson *et al.,* 1994). Data from this work show that although mycelium growing in complete medium containing carbon and nitrogen sources can synthesize different proteins, the production of PR1 is simultaneously repressed by high levels of nutrients (e.g., sucrose). Under these conditions expression of PR1 was not detectable at both transcriptional and translational level. In *B. bassiana,* the production of PR1 seems to be regulated in a similar way as in *M. anisopliae*. PR1 was found to be exclusively expressed under the cuticle-induced conditions, as judged by immunoblot analysis using an anti-PR1 antiserum.

A protein band migrating slightly slower than PR1 was detected by Western Blot analysis, but its identity is not determined. Several possibilities can be considered: it may arise from protein degradation, crossreaction of the antibody possibly related to the infectious process as suggested by St Leger (1989) or posttranslational modification.

2.3.2. Trypsin-like serine proteases

Another major endoprotease produced in vitro on insect cuticle is the trypsin-like serine protease PR2. The expression of this enzyme has been detected in *M. anisopliae*. The PR2 protease has been purified and its regulation has been investigated in both *M. anisopliae* and *B. bassiana* (Cole, 1993 and St Leger, 1987). But biochemical characterization of PR2 has only been performed in *M. anisopliae*. Two isoforms of PR2 have been identified from *M. anisopliae*, however, the unique, specific functions of PR2 in pathogenesis has not yet been elucidated. St Leger (1996) determined the location of PR2, using immunogold labeling, to be at the *M. anisopliae* cell wall during growth through insect cuticles. This indicates that the PR2 proteins might have a role in degrading extracellular proteins as a complement to PR1 and other enzymes. PR1 is produced as a pro-enzyme, requiring processing before it is active. Thus, PR2 could also play a role in the activation of PR1.

Cloning data from this work showed that *B. bassiana* possesses two genes encoding PR2-like serine proteases of the trypsin family. The sequences of these two genes, designated *try1* and *try2*, have been determined. Interestingly, the predicted amino acid sequence of *try1* showed highest homology to the trypsin-like proteases from fungi, whereas the predicted TRY2 protein showed highest homology to the trypsin-like proteases from insects. Considering the experimental conditions and previous results obtained under the same conditions, the possibility of contamination was ruled out. Similar observations have been made regarding trypsin-like proteases from the bacterium *Streptomyces griseus* which showed close homology to mammalian trypsin-like proteases. It was hypothesized that a gene transfer might have been taken place from a mammal to a bacterium (Olafson, 1975). However, this suggestion could later be ruled out using phylogenetic analysis of sequence data accumulated from different organisms (Rypniewshi, 1994). The structure similarity of TRY2 protease from *B. bassiana* to insects enzymes might allow the fungal cells to evade host “non-self” recognition and thus might represents one of the important virulence determinants.

Like PR1, the trypsin-like proteases PR2 is also controlled by multiple regulatory mechanisms which include carbon and nitrogen metabolite repression/ derepression as well as induction by a range of proteins. Based on the model system *M. anisopliae*, St Leger (1988) found that the regulation of PR1 and PR2 is not identical; although PR1 was not detected under carbon/nitrogen repression

conditions, low levels of PR2 were produced. It was found that the soluble protein bovine serum albumin (BSA) represses production of PR1, and at the same time stimulates synthesis of PR2 in poor medium. In addition, PR2 production could be more tightly regulated by nitrogen than carbon availability (Paterson, 1993). This type of protein regulation is not uncommon. There are examples of fungal proteases which are induced by any protein substrate, as observed in *N. Crassa* (Drucker, 1975) and *Candida spp.* (Ross, 1990). These proteases are produced when the fungus is starved for either carbon, nitrogen or sulphur sources. Some fungal proteases appear to be regulated by derepression alone, for example, in *Schizophyllum commune* (Willick *et al.,* 1984) and many *Aspergillus* species (Hanzi *et al.,* 1993).

2.3.3. Expression of phospholipase B in *B. bassiana* 252

Two PLB-encoding genes were cloned from *B. bassiana* and their sequences were determined. They displayed 57% identity at the amino acid level. Three PLB-encoding genes in *S. cerevisiae* (Witt *et al.,* 1984) and two PLB homologues in *C. albicans* (Takahashi *et al.,* 1991) have been isolated. The multiple PLB homologues from these fungi display high homology to each other, but the significance of this genetic redundancy is unclear.

Potential N-glycosylation sites were identified in both PLB1 and PLB2. All characterized fungal phospholipases have been glycosylated. Chen *et al.,* (2000) found that deglycosylation of the protein resulted in almost total loss of enzyme activity. This indicates that N-linked carbohydrates are important for the catalytic function of the protein. The immuno blot assay in this work with an antibody against PLB2 produced a broad band corresponding to a mass of around 70 kDa. The slightly heterogeneous migration of PLB2 upon SDS/PAGE separation has also been observed in *C. neoformans* (Chen *et al.,* 2000) and other fungal PLBs (Oishi *et al.,*1999). It is supposedly due to heterogeneity of the carbohydrate moiety linked to the enzyme.

A hydrophobic carboxy-terminal region was identified in PLBs from non-pathogenic fungi, such *as S. cerevisiae, S. pombe, P. notatum* (Caro *et al*., 1997; Gerber *et al.,* 1992). These COOH-terminal regions have been hypothesized to contain conserved sequence motif that is a signal for the addition of a glycosylphosphatidylinositol (GPI) anchor. Proteins modified with a GPI anchor may be transiently tethered to the

plasma membrane or ultimately cross-linked to the insoluble glucan component of the cell wall (Lu *et al.,* 1995). Release of proteins associated with the plasma membrane would require the action of a GPI-specific phospholipase. Therefore, the GPI anchor may serve to regulate the release of the enzyme to the surroundings. The absence of a GPI anchor in *B. bassiana* PLBs that is also observed in the *C. albicans* PLBs, could cause the PLBs from these organisms to be secreted directly thereby enhance the virulence of pathogenic fungi.

The high amounts of PLB2 synthesised from *B. bassiana* grown on insect cuticle suggests that this enzyme is likely to be involved in the early steps of host invasion. Unlike PR1, production of PLB was not tightly regulated by carbon/ nitrogen repression mechanisms. The transcription of PLB mRNA was detected by RT-PCR in both mycelial samples harvested from rich medium and cuticle-induced medium. Although PLB2 synthesis is highest in the mycelium grown in cuticle-induced medium, the enzyme is also detected in cultures grown on rich medium. Some unspecific bands were detected by immunoblot analysis and this unspecific banding maybe because the synthesized antisera was not pure enough.

2.3.4. Function of cuticle-degrading enzymes during fungal infection processes

The high-level production of proteases during infection process implies an important function for these enzymes. Aside from the proteolytic degradation of the cuticular barrier, other possible roles include the utilization of host proteins for nutrition, the destruction of antifungal proteins of the host, and the release of amino acids for amine production which could elevate the pH to produce better growth conditions. Other effects may be indirect, for example, the proteolytic activation of toxin precursors.

The results obtained from this work are consistent with the idea that a major function of the extracellular proteases is to make nutrients available from the cuticle. The pathogenic process involving enzyme production and penetration of host cuticle occurs only when it is necessary to establish a nutritional relationship with the host. It is probable that the synthesis of proteolytic enzymes and phospholipases occurring in cultures containing host cuticle may also occur when the pathogens are infecting living insects.

A highly reliable method to categorically demonstrate the involvement of a protein in pathogenicity is through genetically engineered null mutants. The multiplicity of cuticle-degrading enzymes provides a major challenge with respect to establish which particular enzyme has which function in adapting to a new environment or in pathogenicity. The exact role of individual cuticle-degrading enzymes in pathogenicity could be assessed by disruption of multiple genes in combination with site-directed mutagenesis experiments. An increased understanding of these virulence factors could ultimately lead to the generation of genetically engineered strains more suited for insect pest control.

III. Development of a new dominant selection marker, *sorR*, for fungal transformation

3.1. Introduction

The filamentous fungi are a diverse group of eukaryotic microorganisms with a number of properties which make them important both scientifically and economically. The economic importance can be illustrated by the large variety of products that are made by filamentous fungi, such as organic acids (e.g. citric acid), antibiotics (e.g. penicillin and cephalosporin) and numerous industrial enzymes (e.g. glucoamylase, cellulase, alpha-amylase, proteases, lipases, etc.). Filamentous fungi are also used as food products (e.g. cheese, mushrooms), food additives (e.g. the meat extender "Quorn") and condiments (e.g. soy sauce). Furthermore, entomopathogenic fungi have been widely used for the biological control of either insect pests or plant pathogenic fungi in agriculture and forestry.

A severe, negative economic influence of filamentous fungi is their detrimental effect on crop yield. Plant pathogenic fungi cause devastating crop losses all over the world. In the medical field, fungal diseases have become increasingly important. *Candida albicans* and some fungal pathogens, although normally existing as a harmless commensal, can cause significant morbidity and mortality in patients immunodepressive due to AIDS, organ transplantation, or chemotherapy.

In addition to their economic importance, genetic and biochemical studies of filamentous fungi have contributed to the development of concepts about biosynthetic pathways and gene-enzymes relationships. The " one-gene-one-enzyme" hypothesis was based on work done with the filamentous fungus *Neurospora crassa* (Beadle *et al.,* 1941). Filamentous fungi have several interesting biological properties such as a complex life cycle, cell differentiation, highly regulated metabolic pathways and efficient secretion of proteins and they offer many of the complexities of a eukaryote with the ease of manipulation of a microorganism which make them attractive as a model for basic biological research. (Timberlake and Marshall, 1989)

The importance of fungi in medicine, industry, agriculture, and science has led to intensive investigation for many years with the aim of understanding and controlling

their detrimental and beneficial activities. Genetic engineering of filamentous fungi provides the ultimate tools for this aim in novel ways. Genetic transformation of fungi started from the experiments with *S. cerevisiae* (Hinnen,1978), *A. nidulans* (Yelton,1984) and *N. crassa* (Case,1980) which are the traditional favourites of the fungal geneticist. A great deal of information was accumulated concerning the nature of transformation events and selection procedures. Shortly thereafter, representatives from all major fungal classes have been shown to be amenable to transformation.

The application of recombinant DNA techniques and the development of transformation systems for filamentous fungi initially was hampered by the lack of useful selectable markers and/or the availability of the appropriate recipient. This was overcome by the isolation and application of homologous and heterologous selection markers and the development of selection systems (Goosen,1980). Two types of selection marker have been used: (1) auxotrophic selection markers, (2) dominant selection markers.

3.1.1. Auxotrophic selection markers

Transformation using auxotrophic selection markers depends on the availability of auxotrophic mutants. The wild-type gene, in plasmid-borne form, complements corresponding auxotrophic mutations of both homologous and heterologous recipients and therefore provides a selectable marker. Several genes have been used as auxotrophic selection markers for transformation studies, e.g., *N. crassa pyr-4* gene, *Aspergillus nidulans amdS, trpC, pyrG, niaD,sC* and *argB* genes. Such transformation systems have been developed for a number of filamentous fungi, such as, *N.crassa* (Buxton and Radford, 1984), *A. nidulans* (Johnstone, 1985) and *Penicillium chrysogenum* (Sanchez , 1987). An overview of commonly used auxotrophic markers is given in table 3.1.

3.1.1.1. **General introduction of commonly used auxotrophic selection marker**

For *P.chrysogenum*, relief of an auxotrophic requirement for tryptophan has been achieved by transformation with the wild-type *trpC* gene that, similarly to the *N.crassa trp-1* gene (Schechtman and Yanofsky, 1983) or the *A. nidulans* (Yelton *et al.,* 1983) and *A. niger* (Kos,1985) *trpC* genes, encodes a trifunctional polypeptide

that contains the GAT, IGPS and PRAI activities of the tryptophan biosynthetic pathway (Sanchez, 1986).

The *pyrG* gene has been used successfully as a selectable marker in filamentous fungi. It encodes the orotidine-5-monophosphate decarboxylase enzyme which is required for the synthesis of uracil, providing the precursor of the three pyrimidine trinucleotides as well as for the synthesis of di- and polysaccharides. The cell with wild-type *pyr* gene is uridine prototroph but unable to grow in the presence of 5-fluoroorotic acid (5-FOA) which can be converted to 5-fluorouracil, a toxic compound to the cells whereas the *pyr(-)* strain confers resistance against 5-FOA. *pyrG* homologs have been identified in a number of other species as well, including *A. nidulans* (*pyrG*) (Oakley and Rinehart,1987), *A. niger* (*pyrG*) (Wilson & Carmona,1988), *Candida albicans* (*ura3*) (Losberger and Ernst,1989), and *Schizosaccharomyces pombe* (*ura4*) (Grimm and Kohli,1988).

The *niaD* gene encodes nitrate reductase which is a key enzyme involved in the first step of nitrate assimilation. The *niaD* gene is required for growth when nitrate is the sole nitrogen source. The *niaD* (+) strain is nitrate prototroph and sensitive to chlorate whereas the *niaD* (-) strain is resistant to chlorate. The chlorate is not itself toxic, but is rendered toxic by conversion to chlorite as a result of the catalytic action of nitrate reductase.

The *sC* gene, which encodes an ATP sulfurylase that catalyses the activation of inorganic sulfate to produce adenosine-5'-phosphosulfate (APS), has been developed as a selectable marker for the transformation of *A. fumigatus* (De Lucas and Dominguez, 2001) and *A. niger* (Buxton and Gwynne, 1985). In filamentous fungi, expression of the *sC* gene is required for the assimilation of sulfate as a source of sulfur. *sC* (-) mutants are unable to utilize sulfate as sulfur source. Instead they require reduced sulfur (e.g., methionine) as sulfur source. In addition, they are resistant to selenate, a metabolite that resembles sulfate but becomes toxic after reduction inside the cell.

Table 3.1. Auxotrophic selectable markers commonly used for transformation of filamentous fungi

marker	Isolated species	Encoded function	Phenotype	Reference
argB	*Aspergillus nidulans*	L-ornithine carbamoyl-transferase	Arginine synthesis	John,1984
trp-1	*Neurospora crassa*	tryptophan synthase	Trytophan synthesis	Kim,1988
NiaD	*Aspergillus nidulans*	Nitrate reductase	(+)Nitrate utilization (-)Chlorate resistance	Malardier, 1989
pyrG	*Aspergillus nidulans*	Orotidine-5'-phosphate decarboxylase	(+)Uridine prototrophy (-)5-FOA resistance	Kronstad, 1989
sC	*Aspergillusfu migatus*	ATP sulfurylase	(+)Sulfate utilization (-)Selenate resistance	De Lucas, 1998

3.1.1.2. Auxotrophic selection markers used for counterselection

Among auxotrophic selection markers, the *pyrG, niaD* and *sC* genes are suitable for developing a gene transfer system in genetically poorly characterized fungal species since they can be both selected and counterselected. They are also particularly useful for genetic manipulation strategies, such as site-specific gene inactivation, gene-replacement experiments by introduction of several genes in an otherwise unaltered genetic background. For example, in the *pyr*/5-FOA system, positive selection for the *pyr* marker yields clones that are uridine prototroph, counterselection of *pyr* (-) clones that are uridine auxotroph can be performed in the presence of 5-FOA. The *nia*/chlorate and *sC*/selenate system can be used in a similar way.

3.1.1.3. Characters of auxotrophic selectable markers

Auxotrophic selectable markers have several advantages in transformation experiments. First, they can be used to direct chromosomal integration of plasmids to homologous sites. Second, selections against auxotrophic strains are frequently very

effective, leading to low backgrounds of growth. Third, complementation by a single copy of a plasmid is usually complete. However, one possible disadvantage in using auxotrophic markers is that only strains carrying the auxotrophic mutation can be transformed. For many fungal organisms the isolation of auxotrophic mutants is very difficult and in some cases, e.g., industrially important filamentous fungi, plant pathogenic fungi, a change in the genetic background may not be desirable. In such cases, filamentous fungal transformation based on dominant selection markers is therefore of particular value.

3.1.2. Dominant selectable markers

A number of filamentous fungi are sensitive to several antibiotics such as phleomycin/ bleomycin and hygromycin B. The genes, either mutant fungal genes such as benomyl resistant β-tubulin (*benA*) (May *et al.,* 1985) or bacterial antibiotic-resistance genes placed under the control of the fungal promoters, that produce resistance to such compounds can be used as dominant selectable markers. With dominant selectable markers both wild-type and mutant strains can be transformed. A list of dominant markers which are utilized is given in Table 3.2. Several of these markers are "broad-host range" markers which can be employed in different fungal species.

3.1.2.1. General introduction of commonly used dominant selection markers

The *A. nidulans amdS* gene, which encodes acetamidase, has been of particular value in devising transformation systems for other fungi. Many other species have a limited ability to utilize acetamide as a nitrogen or carbon source. Thus, expression of the *amdS* gene permits conversion of acetamide to acetate and ammonia and provides a direct selection for transformants by their ability to grow on acetamide (Hynes and Davis,1986).

Bialaphos is a nonselective herbicide produced by *Streptomyces hygroscopicus*. This compound is a unique tripeptide composed of two L-alanine residues and an analogue of glutamic acid known as phosphinothricin (PPT) (Anzai,1987). Following cleavage of the two alanines from PPT, this substance is a potent inhibitor of glutamine synthetase in plants. Inhibition of glutamine synthetase by PTT results in a rapid accumulation of ammonia, which leads to death in the plant cell. The *bar* gene encodes phosphinothricin acetyltransferase (PAT), which converts PPT into a

nonherbicidal acetylated form by transferring the acetyl group from acetyl-CoA onto the free amino group of PPT. This phosphinothricin resistance gene has been used in the transformation of plant and filamentous fungus as a dominant selectable marker (Avalos and Geever,1989).

Table 3.2. Dominant selectable markers commonly used for transformation of filamentous fungi

Marker	Isolated species	Encoded function	Phenotype	Reference
amdS	*A. nidulans*	Acetamidase	Acetamide utilization	Kelly *et al.,* 1985
bar	*S. hygroscopicus*	Phosphinothricin acetyltransferase	Phosphinothricin resistance	Avalos, 1989
benR	*N. crassa*	mutated β-tubulin	Benomyl resistance	Orbach, 1986
ble	*S. hindustanus*	Phleomycin binding protein	Phleomycin resistance	Mattern, 1988
cbxR	*U. maydis*	Mutated iron-sulfur protein subunit of succinate dehydrogenase	Carboxin resistance	Keon *et al.,* 1991
G418R	*E. coli*	Geneticin/kanamycin phosphotransferase	Geneticin resistance	Arnau *et al.,* 1988
glpR	*E. coli*	Mutated EPSP synthase	Glyphosate	Stalker, 1985
hph	*E. coli*	HygromycinB phosphotransferase	Hygromycin B resistance	Queener, 1985
mpaR	*C. albicans*	Mutated inosine-monophosphate dehydrogenase (IMPDH)	Mycophenolic acid resistance	Köhler *etal.,* 1997
oliC	*A. nidulans*	Mutated mitochondrial ATP synthase subunit 9	Oligomycin resistance	Ward *etal.,* 1988
sul1	*E. coli*	Dihydropteroate synthetase	Sulfonamide resistance	Carramolin, 1989

Benomyl is an agricultural fungicide which binds to the tubulin heterodimer and inhibits microtubule assembly. The *A. nidulans benR* gene encodes a mutated β-tubulin gene which was cloned from a benomyl-resistant strain. The mutation responsible for benomyl resistance comes from a single phenylalanine to tyrosine change at amino acid position 200 which decreases the binding affinity of benomyl for the β-tubulin molecule (Jung *et al.,* 1992). *benR* has been widely used as a dominant marker in transformation experiments of *A. niger* and *A. nidulans* (Orbach,1986).

The *ble* gene, which confers resistance to phleomycin/ bleomycin, has been used for transformation of *A. fumigatus* and *A. nidulans*. Bleomycin acts by intercalating into DNA, which leads to degradation of DNA. The Ble protein binds to bleomycin with high affinity and thus prevents it from interacting with DNA (Gtignol and Durand, 1988).

Carboxin, a systemic fungicide highly active against basidiomycetes, has been widely used to control several fungi. The compound inhibits respiration by preventing the oxidation of succinate to fumarate by inhibiting the tricarboxylic acid cycle enzyme succinate dehydrogenase (*Sdh*). This enzyme consists of two subunits, an iron sulfur protein (Ip) and a flavoprotein (Fp) which, together with two other integral membrane proteins (ubiquinone-binding proteins), make up Complex II (succinate-ubiquinone reductase) of the respiratory electron transport chain. Although the precise mechanism of action is unclear, carboxin appears to prevent the transfer of electrons from succinate to ubiquinone by preventing reoxidation of the non-haem iron redox centers of the Ip subunit. The *cbxR* gene, encoding a mutated Ip subunit of succinate dehydrogenase, conferred dominant resistance to carboxin (Keon *et al.,* 1991)

The hygromycin B phosphotransferase (*hph*) gene of *E. coli* is one of the most frequently used dominant selection marker. Hygromycin B (HmB) is an aminoglycoside antibiotic produced by *Streptomyces hygroscopicus*, which inhibits protein synthesis in prokaryotic and eukaryotic cells by interfering with translocation and causing misreading (Gonzalez and Jimenez, 1978). The protein encoded by the *hph* gene phosphorylates the hygromycin B molecule, which results in complete loss of biological activity (Pardo and Malpartida, 1985).

Mycophenolic acid (MPA) is a specific inhibitor of inosine monophosphate dehydrogenase (IMPDH). IMPDH is the key enzyme in the *de novo* biosynthesis of guanidine monophosphate (GMP), catalyzing the NAD-dependent oxidation of inosinate (IMP) to xanthylate (XMP). This conversion of IMP to XMP is the rate-limiting step in the biosynthetic pathway of guanine nucleotides. The mutant IMPDH gene, conferring MPA resistance, has been developed as a dominant selectable marker in *C. albicans.*

3.1.2.2. Characters of dominant selectable markers

All of these dominant markers have the distinct advantage of not requiring the presence of a particular mutation in the recipient, but rather require that the organism be sensitive to the applied selective pressure and that the cloned gene can be expressed in the recipient and relieve the selection. Providing that these conditions are met, virtually any fungal species should, in principle, be transformable with the selectable markers that are currently in use.

3.1.3. Utilization limits of the current available selectable markers

Although in theory there are a fairly large number of selection markers available for fungal transformation system, in practice it is difficult to generate strains with successive transformation events. The possible reasons might include:

(i) Among different fungal species there are large variations in sensitivity to various antibiotics. Some species are even naturally resistant to a broad range of commonly used antibiotics, making the successive selection difficult or not feasible. e.g., *B. bassiana* 252 strain which was investigated in this work is naturally resistant to the compounds phosphinothricin, phleomycin, oligomycin and sulphomylurea precluding use of the related selectable marker genes (A. Leclerque, personal communication). The wild type entomopathogenic fungus *M. anisopliae* can utilize acetamide and is resistant to high concentration of the antibiotics geneticin, hygromycin B, oligomycin, and phleomycin (Bernier *et al.,*1989). Although mutations in genes encoding β-tubulin have been shown to be a mechanism of resistance to benomyl in normally sensitive fungal species, such as *S. cerevisiae*, *S. pombe*, *N. crassa*, and *A. nidulans* (Thomas,1985; sheir-Neiss, 1978; Orbach,1986; Huffaker,1987), *C. albicans* is highly resistant to this tubulin binding agent precluding the use of *benR* as a selectable marker.

(ii) Random and multi-copy genomic integration of some selectable marker genes (e.g. *amdS, glpR* and *benR*) also limits their usage for targeted genomic insertion which is necessary for the analysis of gene structure, function, and regulation. Goettel (1990) found that, when the entomopathogenic hyphomycete *M. anisopliae* was transformed to benomyl resistance using the *benR* gene from *A. nidulans* as a selectable marker, only transformants with multiple copies of the *benR* gene had appreciable resistance. The reason might be that there is not sufficient homology between the *benR* gene and the β-tubulin gene of the recipient strain.

(iii) Utilization of certain selectable marker genes might impair the physiological characters of the host strain. Ruiz-Herrera (1999) analyzed the pathogenicity of chitin synthetase disruptants of *Ustilago maydis* obtained with the carboxin-resistance or the hygromycin-resistance cassettes. They found that only chitin synthetase mutants obtained by gene disruption with the carboxin resistance cassette lost their virulence to maize seedlings. This did not occur when chitin synthetase was disrupted by the hygromycin resistance cassette. They proposed that the modified subunit of succinate dehydrogenase existing in the carboxin-resistant mutant affected the normal activity of the enzyme, handicapping the growth and pathogenicity of the mutant.

(iv) Some selectable markers are probably species-specific. Santos *et al.* (1995) found that at least 11 *Candida* species read the CUG codon as serine instead of leucine, rendering potential transgenic resistance genes and/ or reporter genes nonfunctional in these fungi. The *oliC* marker, based on an oligomycin-resistant mutated ATP synthase subunit 9 gene of *A. nidulans*, requires the formation of a functional oligomycin-resistant ATP synthase complex and it does not function as a selectable marker in other fungal species. The equivalent resistance gene from other fungi has to be found and isolated for transformation (Ward, 1988).

(v) For some selectable markers, the success of their usage for selection purposes is also correlated with their mode of action. When the *bar* gene was used as selectable marker for plant transformation, the ammonia metabolism was disturbed under the selection pressure of phosphinothricin. Because ammonia is mainly produced during reactions linked with photosynthetic electron transport, its accumulation is higher in plants exposed to light than in those kept in the dark. Reduced accumulation of ammonia will lead to slow cell death.

The selection efficiency can also be interfered by the medium composition. e.g., The *amdS* marker can be used only in strains that have no requirements for nitrogen-containing compounds, which interfere with the *AmdS* selection . Goshorn and Scherer (1989) found that exogenous application of guanine to minimal medium acts as a competitive inhibitor of MPA and prevent selection. When *niaD* was used as a selectable marker for the transformation of *A. nidulans*, chlorate is much more toxic to wild-type strains with some nitrogen sources than with others. A concentration of chlorate at least thirty times greater is required to bring about a corresponding level of toxicity when urea is the nitrogen source than when L-glutamate is. Chlorate is even not toxic on certain carbon sources (Cove, 1976). For the *B. bassiana* isolate 252 , the selection efficiency of *niaD*/chlorate system was also dependent on the different nitrogen sources. Chlorate was more toxic to the wild type strain when glutamate and aspartic acid was used as the sole nitrogen and carbon source (personal observation). In addition, geneticin (G418) can offer selection pressure on wild type *pichia pastoris* in YPD medium but not in minimal medium (personal observation).

To construct a strain containing multi-deletion mutations of a number of independent genes, one has to use different selectable markers. Availability of such markers in a given system limits the construction of a multi-gene deletion strain. Therefore, in order to carry out repeated selection rounds, the development of new dominant selectable markers would provide useful tools for functional analysis of different genes via transformation in a single strain. In the following section, the development of a new dominant selectable marker, *sorR*, for fungal transformation based upon the expression of *E. coli* acytel-CoA carboxylase (ACC) which confer resistance to soraphen A will be described.

3.1.4. *sorR*, a new dominant selectable marker for fungal transformation

The myxobacteria, a group of Gram-negative eubacteria that are micropredators or saprophytes in soil encironments, have been found to be a rich source of novel bioactive compounds. Among these compounds, many showed antifungal activity, with a variety of mechanisms of action. For example, myxothiazol, stigmatellin and the aurachins are electron transport inhibitors (Kunze, 1994); gephyronic acid is an inhibitor of eukaryotic protein synthesis equaling cycloheximide in efficiency (Sasse, 1995); disorazol probably is an inhibitor of eukaryotic RNA-polymerase I (Irschik, 1995).

Strains of *Sorangium cellulosum* are slow growing, cellulolytic members of the myxobacteria that have been found to produce a diverse range of natural products. These include sorangicin that inhibits the initiation of transcription in eubacteria (Irschik *et al.,* 1987), epothilones A and B that have antitumor and cytotoxic activity (Gerth *et al.,* 1996a), and the antifungal compounds jerangolid A and ambruticin (Gerth *et al.,* 1996b) and soraphen A (Gerth *et al*, 1994). Soraphen A is active against a wide range of fungal species, especially plant pathogenic fungi, and was developed as an agricultural fungicide until it was discovered that it is a weak teratogen.

3.1.4.1. Basic characterization and biosynthesis of soraphen A

Soraphen A is produced by *Sorangium cellulosum* strain So ce26. Elucidation of the structure of soraphen A demonstrated that it is a macrocyclic polyketide containing an 18-membered lactone ring with an unsubstituted phenyl side ring (Fig. 3.1). The molecular mass of soraphen A is about 520kDa. Its structure is shown in Fig. 3.1 (Bedorf *et al.,* 1993). Gene cloning and sequence analysis of the complete soraphen A biosynthetic gene cluster revealed that a modular type I polyketide synthase (PKS) is responsible for the synthesis of soraphen A. Two ORFs encode large, multifunctional type I PKSs that together make up the soraphen synthase. One gene, sorA, encodes a putative PKS with three biosynthetic modules, while the second, sorB, encodes five modules. Examination of the soraphen A structure suggests that these eight PKS modules are required for the synthesis of the soraphen A macrolide ring and that other post-PKS activities such as O-methylation might also be necessary. (Ligon, 2002).

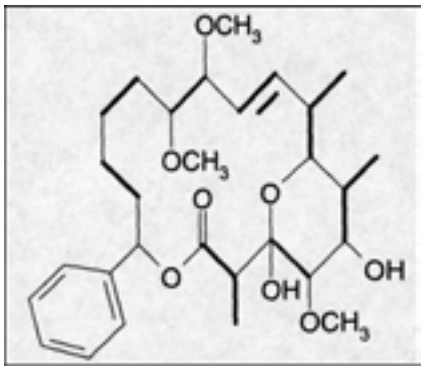


Fig. 3.1. The chemical structure of soraphen A (according to Bedorf *et al.,* 1993).

3.1.4.2. Antimicrobial spectrum and the mechanism of action of soraphen A

The growth of a broad spectrum of yeast and hyphomycetes is inhibited by low concentrations of soraphen A (Table 3.3). Strains which appeared resistant to the fungicide in the agar diffusion test were sensitive to soraphen A at higher concentrations in liquid medium. Only *S. pombe* was completely resistant. In contrast, prokaryotes were not sensitive to soraphen A (Gerth, 1994).

Table 3.3. Antifungal spectrum of soraphen A

Organism	Diameter of inhibition zone (mm)	MIC (µg/ml)
Altenaria solani	27	no
Pythium debaryanum	45	0,1
Ceratocystis ulmi	23	0,5
Botrytis cinerea	30	0,15
Ustilago zeae	10	4,0
Mucor hiemalis	30	0,03
Rhizopus arrhizus	10	4,0
Candida albicans	25	0,06
Nematospora coryli	35	0,05
Saccharomyces cerevisiae	20	1,0
Debaryomyces hansenii	29	0,4
Rhodotorula glutinis	21	1,0
Schizosaccharomyces pombe	0	>100

Previous studies showed that growth inhibition by soraphen A can be counteracted by the addition of fatty acids to the medium (Pridzun, 1995). This suggested that soraphen A inhibits a step in the fatty-acid synthesis pathway. Additional biochemical investigation demonstrated that soraphen A inhibits the activity of fungal acetyl-CoA carboxylase (ACC), the key enzyme of fatty-acid biosynthesis, and therefore disrupts the synthesis of fatty acids (Vahlensieck, 1994). Although soraphen A is a potent inhibitor of fungal acetyl-CoA carboxylase, the corresponding enzymes of plants and bacteria are insensitive to this fungicide.

Soraphen A acts directly at the enzyme level and does not, for example, interfere with the expression of the *acc* gene. However, the enzymatic step of ACC which is blocked by soraphen A has not been identified.

3.1.4.3. Acetyl-CoA Carboxylases

ACC is a biotin-containing enzyme, which catalyzes the ATP-dependent carboxylation of acetyl-CoA to form malonyl-CoA. This reaction is the initial and committed step in fatty acid synthesis, malonyl-CoA formed by this reaction provides the two-carbon units from which long-chain fatty acids are synthesized by the fatty acid synthase complex and by malonyl-CoA-dependent elongation processes (Harwood *et al.,*1990). In fungi, malonyl-CoA also acts as a precursor for the synthesis of polyketides (Hopwood, 1986) and some melanin polymers (Bell and Wheeler, 1986). ACC has been found in all forms of life except the archae (which have isoprenoid lipids in place of lipids based on fatty acids). In common with other biotin-dependent enzymes, ACC has two enzyme functions (Knowles, 1989; Samols ,1988): a biotin-carboxylase (BC) activity which catalyses the carboxylation of protein-bound biotin with bicarbonate and a carboxyl transferase (CT) activity which transfers the activated carboxyl group from carboxy-biotin to acetyl-CoA. A third component of ACC, the biotin carboxyl carrier protein (BCCP), carries the essential biotin prosthetic group covalently bound to a lysine residue proximal to its carboxyl terminus (Fig. 3.2).

Although enzymes that use biotin as a carboxyl carrier contain highly conserved regions (Samols, 1988), the organization of the components of these enzymes is not the same in all organisms. Two types of ACC have been identified:

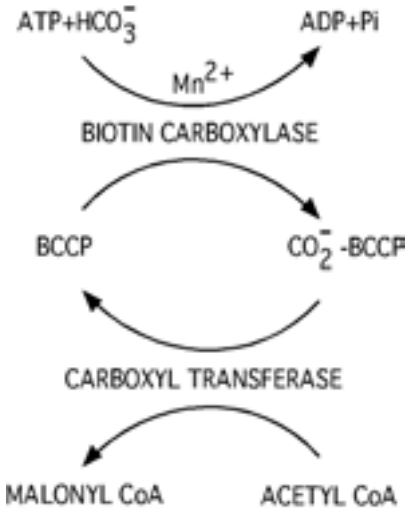


Fig. 3.2. The enzymatic reactions catalysed by ACC.

(i) Prokaryotic ACC contains the three functional domains located on separate subunits. This type of ACC is found in bacteria and most plant chloroplasts.
In *E. coli*, the BC, CT and BCCP domains of ACC reside on four distinct proteins, which are the products of separate genes. These four polypeptide chains function together in an enzymatic complex (Table 3.4). The genes encoding the subunits of E. coli ACC are called *accA* (CT α subunit), *accB* (BCCP), *accC* (BC), and *accD* (CT β subunit). *accC* and *accB* form one operon, whereas *accA* and *accD* are not linked to each other or to accCB (Li and Cronan, 1992). In *E. coli*, the funtional ACC consists of a dimer of two 49-kDa BC monomers, a dimer of two 17-kDa BCCP monomers, and a CT tetramer containing two CT α and two CT β subunits.

Table 3.4. Subunits and genes of *E. coli* ACC

Subunit	Encoding gene	Gene length (bps)	Protein MW (kDa)
Biotin carboxylase (BC)	accC	1350	49.4
Carboxyltransferase, α subunit (CT-α)	accA	960	35.1
Carboxyltransferase, β subunit (CT-β)	accD	918	33.2
Biotin carboxyl carrier protein (BCCP)	accB	471	16.7

(2) Eukaryotic ACC contains three enzyme components located on a multifunctional polypeptide derived from the expression of a single gene (Al-Feel, 1992; Gornicki,1994), This type of ACC is found in mammals, fungi and plant cytosols. Fungal ACC is assembled from four identical peptide subunits and tetramer formation is required for enzymatic activity. (Sumper and Riepertinger,1972). In vertebrates, ACC forms multimers composed of identical subunits (Lopez-Casillas, 1988). The molecular weights of eukaryotic single-subunit ACCs range from 240 to 260 kDa. They represent a class of unusually large enzymes.

The distinct structure of the ACC enzymes of eukaryotes and prokaryotes may be the reason for their different sensitivity to soraphen A. Depending on the resistance of *E. coli* ACC to soraphen A and their relatively small size, the coding genes can be used as a new selection marker for fungal transformation after being fused to the functional fungal promoter. This new dominant selectable marker is designated *sorR* in this work.

3.1.4.4. The strategy of utilizing *sorR* selectable marker for fungal transformation

The goal of this work is to develop a new selectable marker, *sorR*, for the transformation assay of *B. bassiana 252* strain. In order to achieve efficient expression of the *sorR* gene in the recipient, each of the *E. coli acc* genes will be fused with the promoter of the *acc* gene cloned from *B. bassiana 252*.

Since the constructed *sorR* selectable marker consists of four expression cassettes, these could be used, separately or together, for transformation of *B. bassiana 252*. When combined with other selectable markers suitable for this strain (e.g., *pyr, hph*), successive transformations could be applied for the sequential disruption of intended genes via recombinational inactivation strategies. Thereby, the role and functional interaction of different genes in the pathogenicity of *B. bassiana 252* can be analyzed according to the phenotype of different mutants.

3.1.5. The focus of this part of work

The aim of this work is to develop a new dominant selectable marker for *B. bassiana 252* transformation based upon the expression of *E. coli* ACC in fungus which confers resistance to soraphen A. By testing the growth of the transformants, several important questions can be addressed: can the *E. coli acc* genes be expressed in fungi? Can the four ACC subunits assemble to an active enzymatic complex? Does the *E. coli* ACC render fungi resistance to soraphen A? If the *sorR* selectable marker is suitable for transformation of *B. bassiana 252* strain, it might be used for transformation of other soraphen A-sensitive filamentous fungi as well.

In addition, a mutated form of the *acc* gene from *B. bassiana252,* which is resistant to soraphen A, could also be used as selection marker. Therefore, the gene encoding ACC in *B. bassiana* 252 need to be cloned and sequenced. This alternative approach will be proceeded in parallel with the development of the *sorR* selection marker in case the *E. coli acc* genes will not function as expected in fungus.

In order to achieve the above mentioned goals, the following experimental work was performed:

- Construction of a λ phage genomic DNA library of *B. bassiana 252*
- Cloning and sequence analysis of the *B. bassiana 252 accB1* gene
- Determination of the transcriptional initiation site of *accB1 gene*
- Construction of transformation vectors to identify the core promoter sequence of *B. bassiana accB1* gene
- Construction of an expression plasmid containing *E. coli acc* genes and determination of the ability of *E. coli* ACC to confer soraphen A resistance when *Pichia pastoris* was used as the recipient for ACC.

3.2. Results

3.2.1. The sensitivity of *B. bassiana 252* to Soraphen A

A prerequisite for the use of Soraphen A resistance as a selection marker in *B. bassiana* transformation is the sensitivity of the host strain to this drug. The sensitivity was examined, As shown in Fig. 3.3, at the concentration of 0.2µg/ml of soraphen A, colony formation was reduced to 50% compared to control without soraphen A after one week; the colony formation was reduced by around 25% of control after two weeks and there is no obvious inhibitory effect after three weeks. At the concentration of 0.5µg /ml and 1µg/ml of soraphen A, colony formation was inhibited by 95% and 100% as compared to the control, respectively, during the whole testing period.

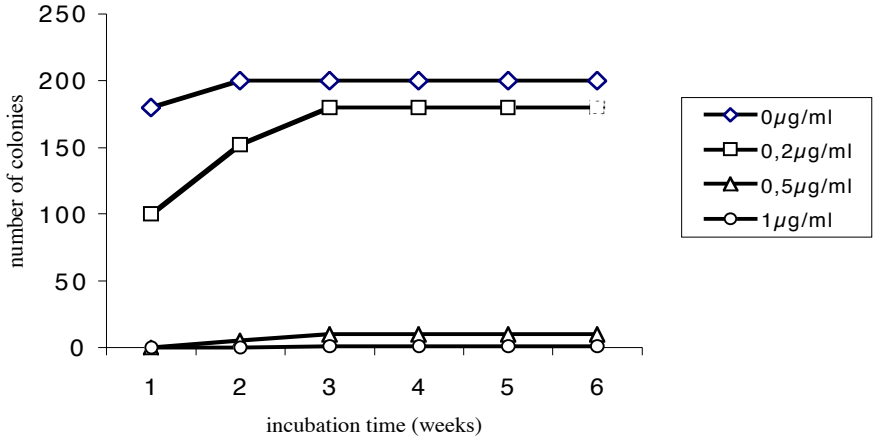


Fig. 3.3. Sensitivity of *B. bassiana* 252 to different concentrations of soraphen A on YPD medium.

3.2.2. Construction of a *B. bassiana 252* genomic library

In order to facilitate cloning and characterization of *B. bassiana 252* genes, a genomic library was constructed. The bacteriophage vector Lambda DASH® II (Stratagene) predigested with restriction enzyme *Bam*H I ,which accommodates inserts ranging from 9 to 23 kb, was used as the vector to construct the genomic library of *B. bassiana* 252.

Isolation of high molecular weight chromosomal DNA from *B. bassiana* 252

High molecular weight chromosomal DNA from *B. bassiana* 252 was isolated using a procedure described by Pfeifer and Khachatourians (1993) with modifications. The procedure is described under Materials and Methods. Analysis of the isolated DNA by gel electrophoresis confirmed that the DNA was not degraded during the isolation procedure. The DNA migrated as a distinct band with a size larger than 50kbp. The isolated genomic DNA was used for the construction of a lambda genomic library.

DNA fraction by partial cleavage with *Mbo I*

To obtain a truly random library, the genomic DNA was fractionated by partial digestion with the restriction endonuclease *Mbo* I which produces *Bam*H I-compatible cohesive ends. An analytical time course of the *Mbo* I digestion was performed to determine the optimal time point for generating fragments with a size of 9 to 23 kbp (Fig. 3.4). The optimal time point was estimated to be between 45 and 60 minutes (lane 7 and 8). Therefore, a digestion time of 50 minutes was chosen for the library construction.

Construction of phage genomic library of *B. bassiana* 252

Library construction was performed following the instruction manual of Lambda DASH® II /*Bam*H I vector kit (Stratagene) and the detailed steps are described in Materials and Methods. The size of this genomic library was determined to be $1.5x10^6$ plaque forming unit (pfu)/µg of genomic DNA, which offers more than 99% probability of isolating a particular gene from a fungal genome.

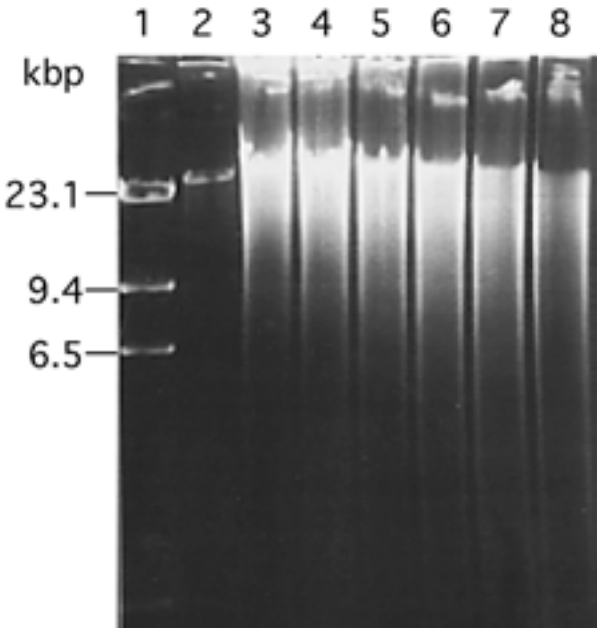


Fig. 3.4. Partial cleavage of high molecular weight genomic DNA with *Mbo* I. Digested DNA were separated by electrophoresis overnight at 20V in a 0,8% agarose gel and then stained with ethidium bromide. lane1, marker, lambda/HindIII ; lane 2, undigested DNA, lane 3 to 8, DNA was digested with MboI for 5,10, 20, 30, 45, 60 min, respectively.

3.2.3. Isolation of the *accB1* gene from *B. bassiana 252*

Degenerate oligonucleotide primers for PCR were designed from regions of similarity within the *acc* genes of *A. nidulans, S. cerevisiae, S. pombe, U. maydis*, and *A. thaliana*. Two sets of primers were designed in the BC and CT domains of the *acc* gene, respectively, and use of the primers from the BC domain resulted in detectable PCR products. A 880-bp fragment was amplified from *B. bassiana* 252 genomic DNA using the degenerate oligonucleotides BCF1 and BCR2 designed from the highly conserved amino acid sequences DQYVEVPGG and VEHPTTEMV at amino acid positions 107-115 and 378-386, respectively, in the *A. nidulans acc1* gene. This PCR fragment was cloned into the plasmid vector pCR®-Blunt II-TOPO and the sequence was determined. The amino acid sequence derived from the nucleotide sequence, in the reading frame predicted from the flanking primer sequence, was compared with the corresponding region of *acc* genes from other

fungi with BLAST searches of Genbank sequences. The sequence displayed a high degree of identity at the amino acid level, indicating that it belonged to an internal coding region of the *B. bassiana acc* gene.

The BCF1-BCR2 PCR fragment was used as a probe to screen the phage genomic DNA library of *B. bassiana* 252 by in situ plaque hybridization. From about 10,000 plaque, two phage clones (λBbacc1 and λBbacc2) that hybridized strongly to the probe were isolated. Restriction enzyme analysis showed that the clones contained overlapping DNA inserts and were about 17 kb and 19 kb in length, respectively. Restriction fragments generated from these clones by digestion with *Bam*H I and *Sal* I were subcloned into a pUC18 vector and sequenced. Both DNA strands were sequenced by extension of specific primers on double stranded templates. Sequence data derived from overlapping clones ensured that the DNA fragments were contiguous. The full-length gene, designated *accB1*, together with the upstream and downstream flanking region were sequenced (Fig. 3.6A).

3.2.4. Sequence analysis of *B. bassiana* ACC*B1* gene

The *accB1* gene has an open reading frame (ORF) of 6801 bp and encodes a protein containing 2232 amino acid residues with a calculated molecular mass of 258 kDa. Sequence analysis of the *accB1* gene indicated the presence of two small introns near the 5'-end (Fig. 3.6A).

3.2.4.1. Amino acid sequence homology

At the amino acid level, the overall sequence identity between *accB1* and the homologous gene from *A. nidulans, S. cerevisiae, S. pombe* and *U. maydis* is 69%, 59%, 57% and 50%, respectively. Within the BC and CT domains, the sequence identity between *B. bassiana* and the other homologus was around 80-90% in some regions.

The putative ATP-binding site, found in all ACC proteins so far identified, is located at amino-acid residues 295-314. The highly conserved biotin-binding motif MKM is located at amino-acid residues 783-785, which represents the region of biotin carboxyl carrier protein. In addition, a putative carboxybiotin-binding site located at amino-acid residues 1685-1720 and an acetyl-CoA binding site at residues 1961-

1980 have been assigned by aligning with the homologous genes from other organisms (Fig. 3.6B).

The sequence alignment allowed the determination of the order of the enzyme domains within *B. bassiana accB1*. These domains were the same as those found in other fungal and yeast *acc* genes. Beginning with the NH2-terminus they have the following order: BC-BCCP-CT (Fig. 3.6B).

3.2.4.2.Transcription initiation site of *accB1* gene

The transcriptional initiation site of *accB1* was investigated by primer extension and rapid amplification of cDNA 5'-ends (5'RACE)-PCR experiments. *B. bassiana* 252 total RNA was used as template. ^{32}P-labelled oligo nucleotide PE1 and PE2 that is complementary to the *accB1* sequence from nucleotides -1 to +24 and -188 to -218 were used as reverse primers for primer extension. Only the primer PE2 gave a primer-extended product. This product was about 200 bp in size indicating an 5'-untranslated leader mRNA sequence of about 400 nucleotides.

Because of the existence of introns in the eukaryotic genome, the location of the transcription initiation site was confirmed using 5'RACE-PCR assay. The gene-specific primers PE3, that is complementary to the *accB1* sequence from nucleotides +67 to +91, and PE1 were used as the reverse primers. The first strand of cDNA was synthesized with primer PE3 followed by addition of a homopolymeric dA-tail. dT anchor primer and PE3 were used in the first round of PCR. In a second round of PCR, PE1 was used as reverse primer instead of PE3. A cDNA fragment, of around 420 bp in length, was obtained after the second round of PCR (Fig. 3.5B) and cloned into the plasmid vector pCR®-Blunt II-TOPO for sequencing. The cDNA sequence obtained by 5'-RACE revealed that the transcriptional initiation site of mRNA was located 380bp upstream of the ATG start codon and that no additional introns were present at the 5' end of the *accB1* gene (Fig 3.5).

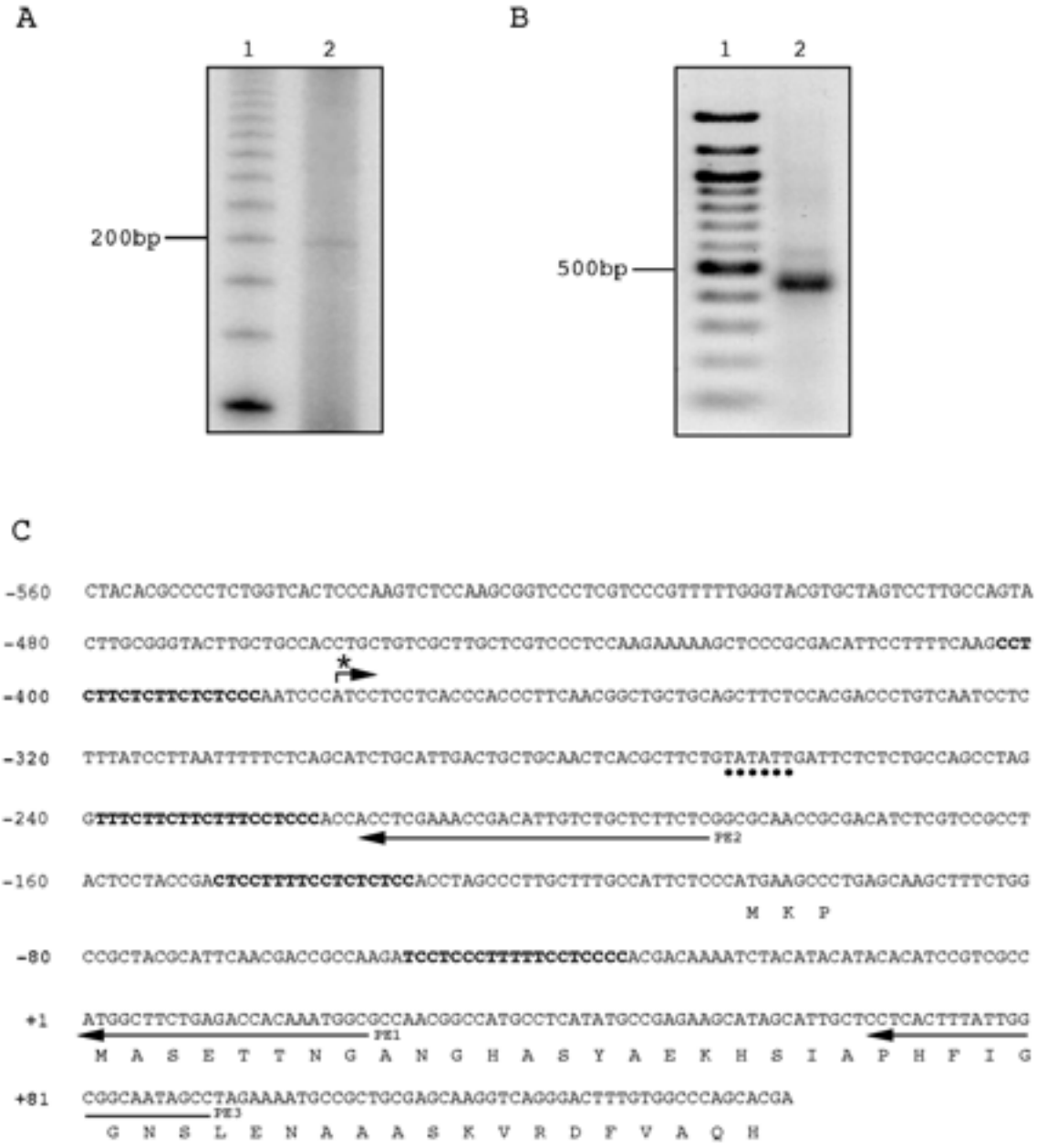


Fig. 3.5. Determination of the transcriptional initiation site of *accB1*. A. Primer extension analysis. Lane1, 25bp DNA ladder. Lane 2, γ-^{32}P end-labelled primer PE2 (indicated in panel C) was extended by reverse transcriptase. **B. 5'RACE**. Lane1, 100bp DNA ladder. Lane 2, amplification of the upstream region of *accB1* mRNA with primer PE1 (indicated in panel C). **C. The upstream sequence of *accB1*.** CT-rich regions are typed in bold; a putative TATA box is marked with a dashed line; PE2, PE1 and PE3 are primers used in primer extension and 5'RACE assay; the transcriptional initiation site is indicated with an arrow plus asterisk.

3.2.4.3.Introns in the *accB1* gene

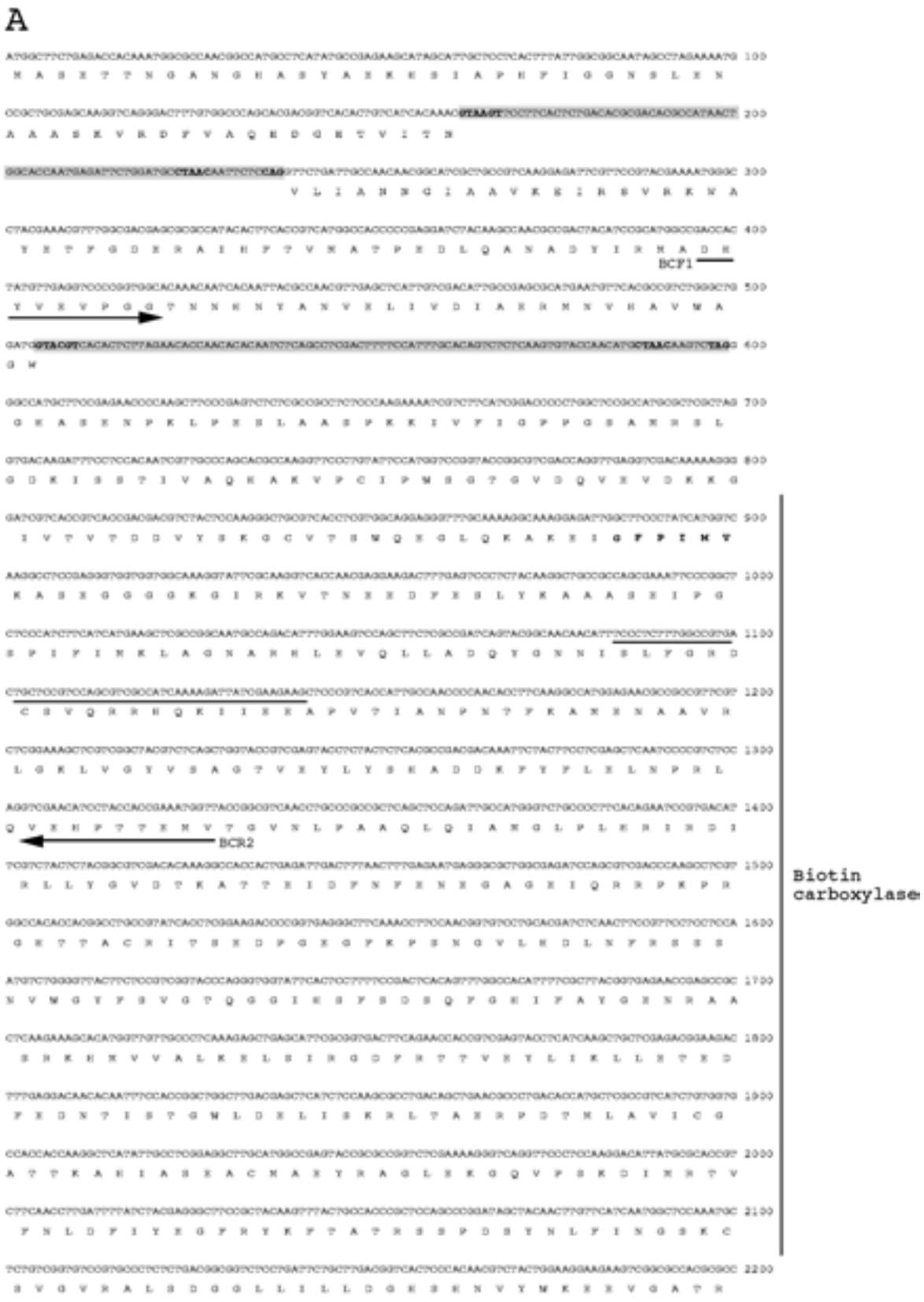
To confirm the putative splice sites in the *accB1* gene, RT-PCR was performed using primers that flanked the putative introns. The corresponding cDNA was cloned and sequenced. Two introns have been mapped in *accB1* to positions +163 and +505, relative to the +1 ATG. They are 76 bp and 95 bp in length, respectively (Fig. 3.6A). The 5' and 3' splice sites are in agreement with the consensus sequences for introns of filamentous fungi. Sequences resembling the "lariat formation" site (GTRAY, Gurr *et al*., 1987) have also been identified within the intron regions.

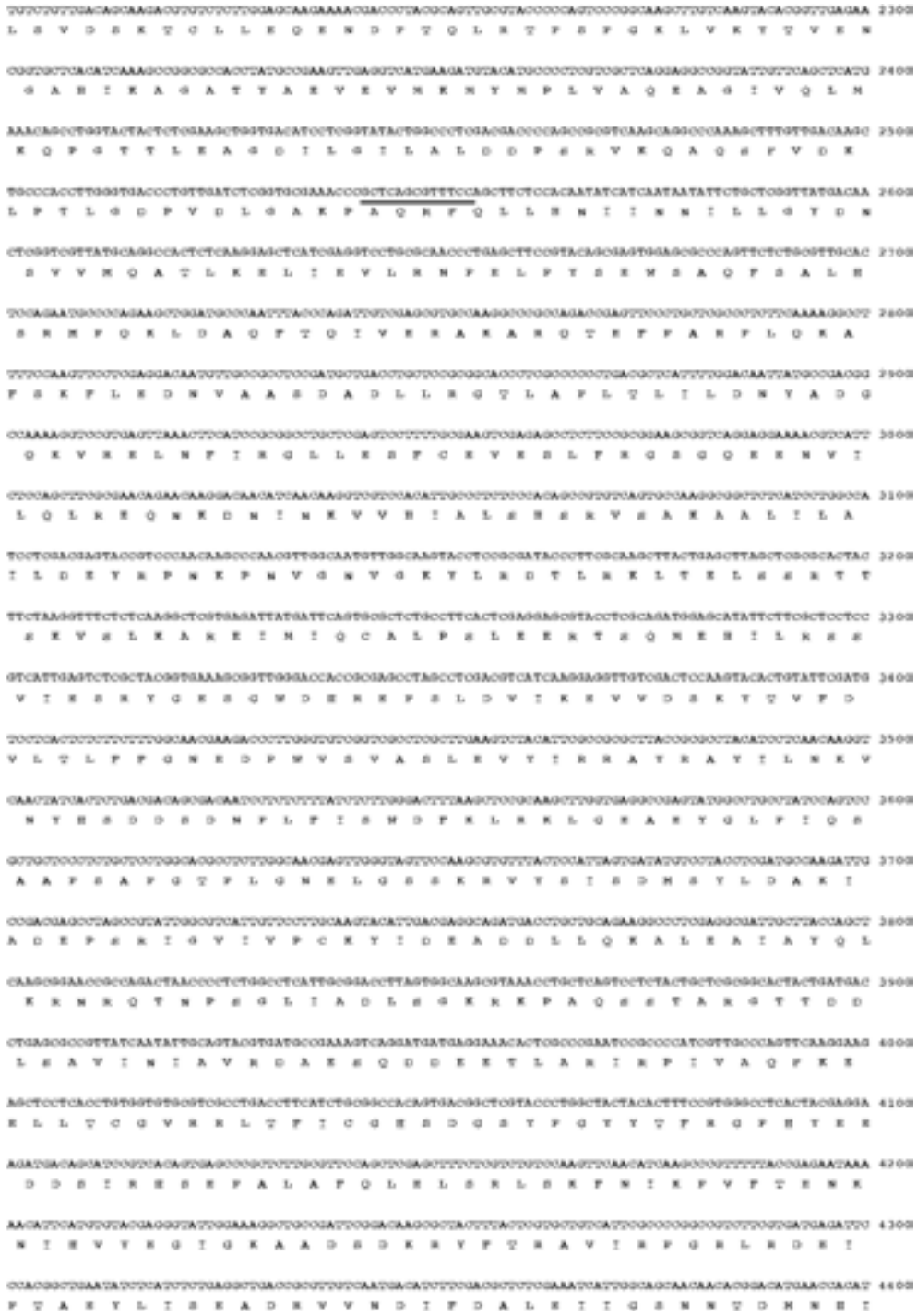
3.2.4.4. The upstream region of *acc*B1

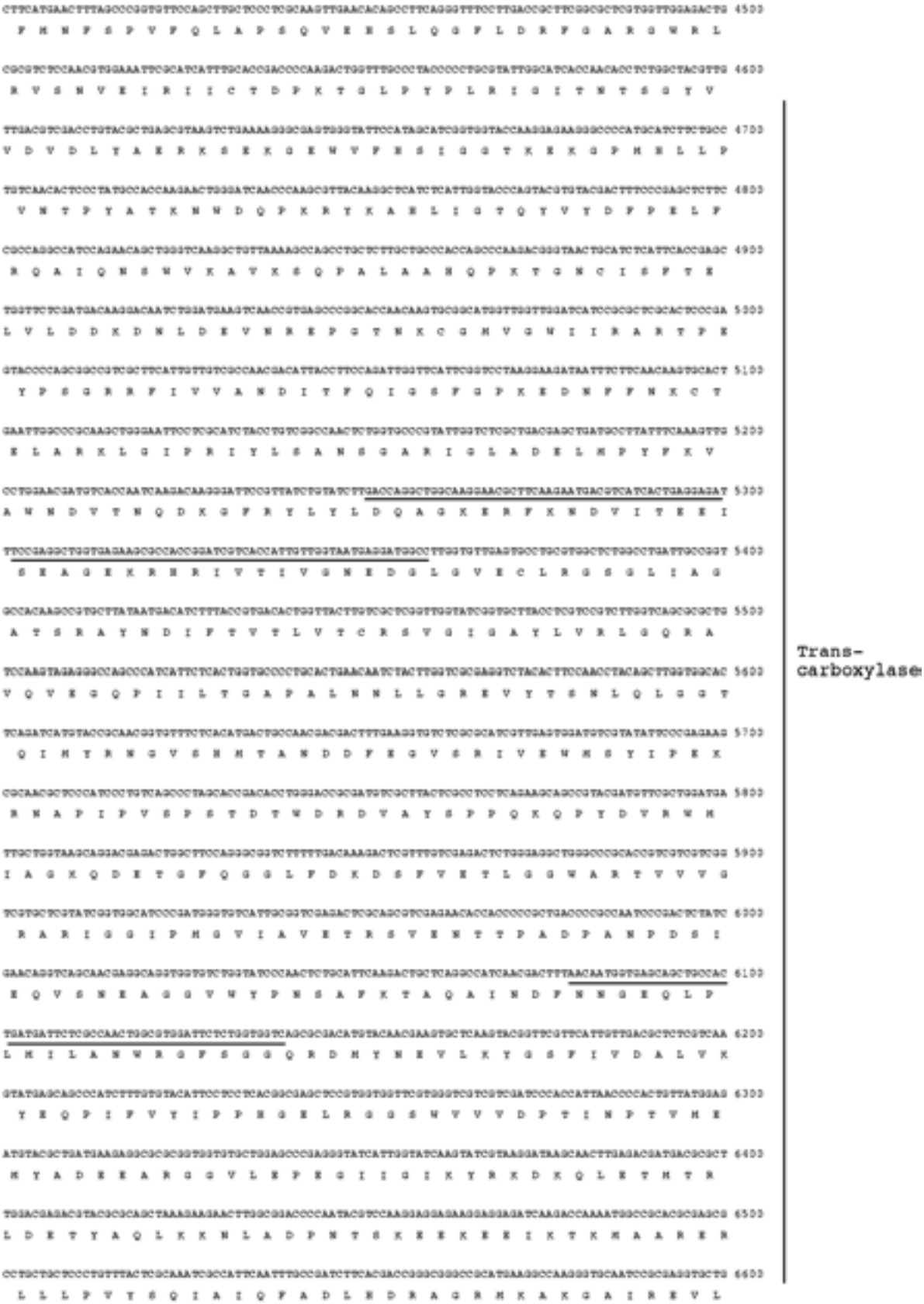
The sequence of the 3.7 kbp upstream region of *accB1* was determined. The ATG start codon at position +1 is preceded by another ATG, 105 bases upstream of the start codon. Translation from this –105 ATG would result in a 3-amino-acid upstream open reading frame (uORF), MEA, whereas translation from the +1 ATG yields a protein of 2232 amino acid residues with high identity to other fungi and yeast acc gene products. Furthermore the +1 ATG has a purine (G) residue at the –3 and +4 positions, respectively which has been found to be important in translation initiation (Kozak, 1995). The promoter region of *accB1* is rich in C and T nucleotides, which is indicative of a strong fungal promoter (McNeil, 1988). A putative TATA box which provides TFIID binding site is present 267 bp upstream of the ATG.

3.2.4.5. The downstream region of *accB1*

The sequence of a 500 bp downstream flanking region of the *accB1* gene was determined. The consensus sequence, AATAAA, which is commonly used as a signal for polyadenylation, was not found in this region. However, at the 3'-end of the ACC*B1* flanking region, a tripartite consensus sequence, TAG…TATGT…TTT, was identified. Zaret and Sherman (1982) proposed that this motif is required for efficient polyadenylation/ termination in fungi.


A
BCP1
BCR2
Biotin
carboxylase




Trans-
carboxylase

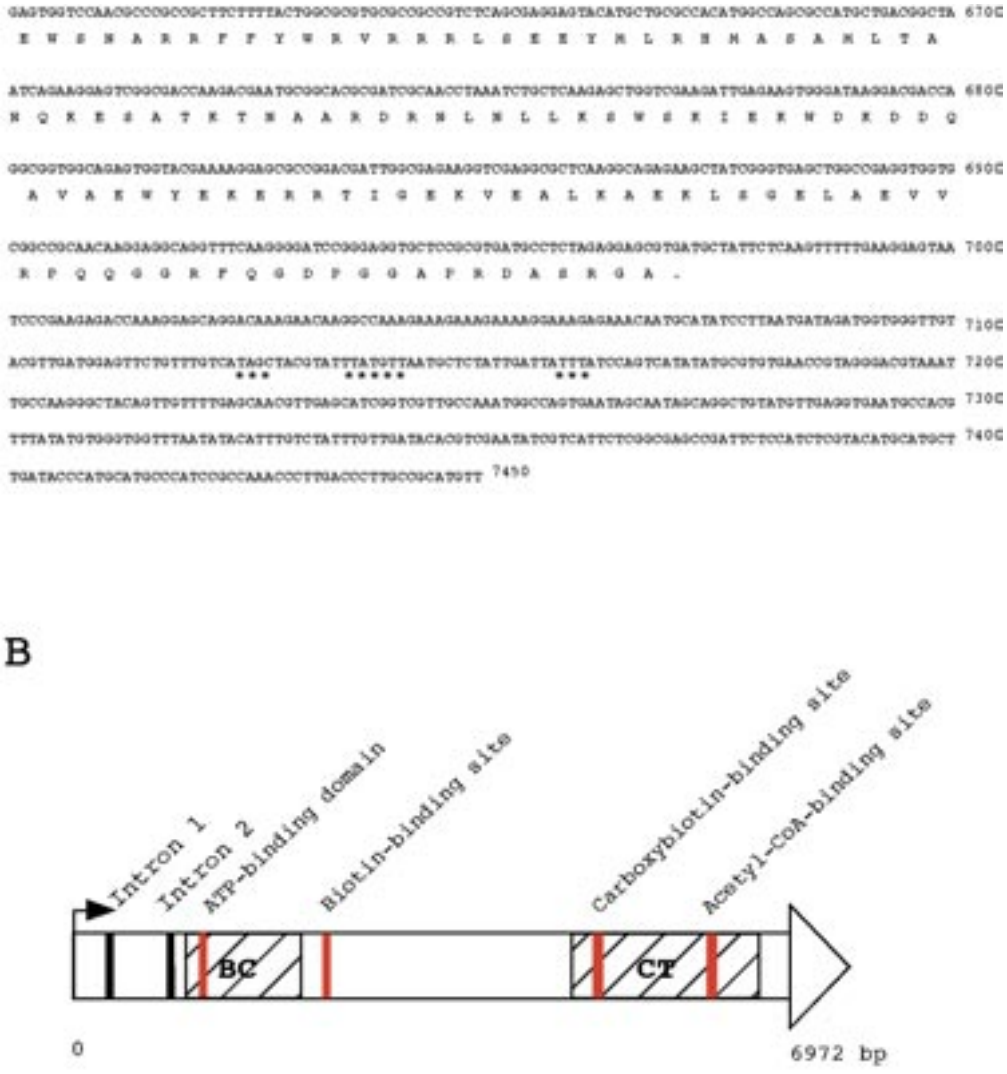


Fig. 3.6. A. Nucleotide and deduced amino acid sequence of the *B. bassiana 252 accB1* gene. The numbers are relative to the A of the start codon. The coding region of the gene, which is interrupted by two introns marked in shaded boxes, starts at position 1 and ends at position 6972. The position of degenarate primers BCF1 and BCR2 are indicated with arrow. The consensus sequences for the 5' and 3' splice site and for lariat formation are marked for each intron in bold. The positions of the conserved ATP-binding site (295-314 aa), biotin-binding motif (783-785 aa), carboxybiotin-binding domain (1685-1720 aa) and acetyl CoA-binding site (1961-1980 aa) are underlined. The tripartite consensus motif for polyadenylation was marked with asterisk below the sequence. **B. Schematic map of the *B. bassiana 252 accB1* gene.**

3.2.5. Construction of transformation vectors for identification of the core promoter sequence of the *B. bassiana accB1* gene

The vector construction was based on the pUC18 plasmid. An internal region of the *pyr* gene, encoding orotidylate decarboxylase, was used as the selectable marker for fungal transformation. It was chosen because simple positive (uridine prototrophy) and negative (5-FOA resistance) selection protocols have been developed. Upon transformation the truncated *pyrI* gene fragment is expected to disrupt the genomic *pyr* gene by a single-crossover recombination. Plasmid pRS31 (Suelmann *et al.,* 1997) containing the *sgfp* gene, in which the codon usage has been optimised for the expression in yeast *S. cerevisiae*, was obtained from Dr. Suelmann. This *sgfp* gene was used as a reporter gene.

The promoter region of *accB1* was amplified in various lengths from *B. bassiana* 252 genomic DNA and fused in-frame with the reporter *sgfp* gene. The resulting set of transformation vectors were designated pTGT-P*accB1* (Fig. 3.7).

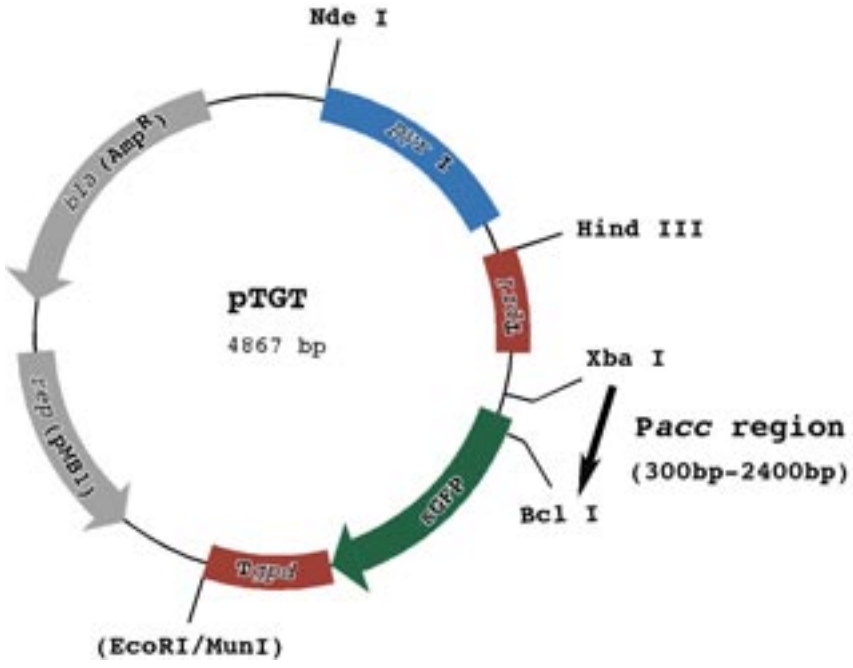


Fig. 3.7. Map of the plasmid pTGT-P*accB1*, a derivative of pUC18. *bla*,the ampicillin resistance gene. *rep*, the replication origin from plasmid pMB1. Tgpd, the terminator region of the *gpd* gene; T*pr1,* the terminator region of the *pr1* gene; *pyr* I, internal region of the *pyr* gene.

In order to provide a positive control plasmid for the promoter characterization experiments, a 700 bp promoter region of the gene encoding glyceraldehyde-3-phosphate dehydrogenase (*gpd)* was fused in-frame with the *sgfp* gene of pTGT , resulting in a plasmid designated pTGT-Pgpd. The *gpd* gene is constitutively expressed and is considered to contain a strong promoter. Selected gene segments were amplified by PCR to introduce new restriction sites and thereby simplifying the cloning process. All the cloned fragments except the *sgfp* gene were amplified from the genomic DNA of *B. bassiana 252*.

The promoter characterisation experiments and planed following experiments could not be completed at this point since the *B. bassiana* 252 transformation procedure is not yet available!

3.2.6. Expression of *E. coli* ACC in *Pichia pastoris* and effects on the resistance to soraphen A

The primary purpose of this thesis work was the development of the *sorR* dominant selectable marker for the transformation of *B. bassiana* 252 and to use it for site-specific gene disruption experiments. Unfortunately, at this time the transformation procedure for *B. bassiana* 252 was still being developed and optimised in our laboratory. Therefore, the applicability of *E. coli* ACC as an effective transformation marker was investigated in yeast *Pichia pastoris.*

The methylotrophic yeast *P. pastoris* is widely used as host for production of heterologous proteins and for other cell biological studies. Gene expression studies in *P. pastoris* frequently involves use of the promoter from the methanol-inducable alcohol oxidase I gene (*aox1*) which encoding the first enzyme in methanol metabolism (Cregg, 1989). In wild-type *Pichia*, this single-copy gene can be induced to give alcohol oxidase levels of up to 30% of total cell protein. The *aox1* promoter was chosen for expression of the *E. coli acc* marker genes.

In order to express the *E. coli acc* genes in the *P. pastoris* GS115 strain, the shuttle vectors pAO815 and pPIC3.5K were used for cloning. In both vectors, the cloning sites were flanked by the promoter and transcriptional terminator of the *aox1* gene. The vectors also carry the wild-type *his4* gene of *P. pastoris* for selection in a *his4 (-)*

strain. In addition, vector pPIC3.5K contains the bacterial kanamycin-resistance gene Tn903Kan^r, which confers resistance to geneticin (G418) in *P. pastoris*.

The *his4* negative *P. pastoris* strain GS115 (Invitrogen) was chosen in this experiment as a recipient strain for transformation. Genes of interest are integrated into the *P. pastoris* genome via homologous recombination either at the *aox1* locus or at the *his4* locus.

3.2.6.1. The sensitivity of *P. pastoris GS115* to soraphen A

Initial tests were carried out to determine the sensitivity of strain GS115 to soraphen A. The yeast cells grew rapidly on the control plate without soraphen A, but failed to grow in the presence of soraphen A even at the concentration as low as 0.02 µg/ml (Fig. 3.8). In accordance with these results, medium with concentrations of 0.02-0.1 µg/ml soraphen A was used for the transformation experiments.

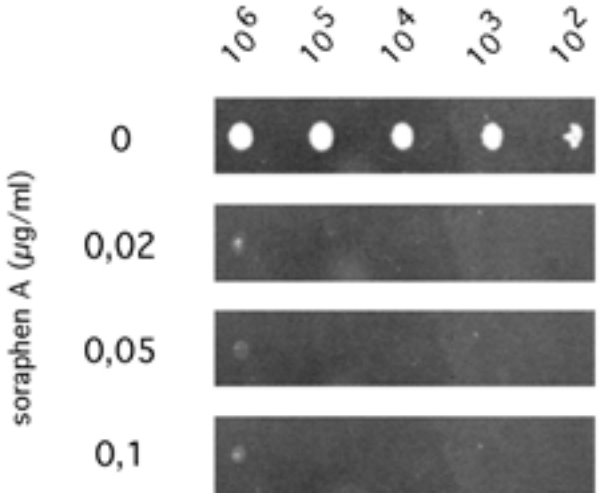


Fig. 3.8. *P. pastoris* strain GS115 is sensitive to soraphen A. Serial dilutions of the cells were grown on MM medium with different concentration of soraphen A for six days.

3.2.6.2. Construction of the *E. coli* ACC expression vector

Three plasmids carrying *E. coli* acc genes were kindly supplied by Prof. John.E. Cronan (Department of Microbiology, University of Illinois). Plasmid pLS151 (Shyr-Jiann Li *et al.,* 1992a) contained the *accA* gene, which is 960bp in length; plasmid pLS9 (Shyr-Jiann Li *et al.,*1992b) carries the *accD* gene which is 918bp in length; plasmid pLS1 (Shyr-Jiann Li *et al.,* 1992c) carries both the *accB* and the *accC* genes, 471bp and1350bp in length, respectively. These plasmids were used as templates for subsequent PCR amplifications.

The four genes coding for the subunits of *E. coli* ACC were amplified by PCR, using primers in which *Eco*R I restriction sites (for *accA, accB, accC* genes) or *Mfe* I sites (for *accD* gene) were introduced into the 5' and 3' end of the synthetic genes. In addition, all the 5'-primers contained substitutions that change the three nucleotides immediately preceding the start codon to ACC for optimal translational initiation in yeast (Kozak,1986).

The *Eco*R I or *Mfe* I digested PCR products containing the *E. coli acc* genes were fused to the *aox1* promoter by insertion into the *Eco*R I site of the plasmid pAO815 to create plasmid pAO815-A, pAO815-D, pAO815-BC and pAO815-BCCP, respectively. Each resulting plasmid carried one expression cassette containing one *E. coli acc* gene flanked by the *aox1* promoter and transcription terminator. This expression cassette was flanked by the *Bgl* II and *Bam*H I restriction sites.

In order to generate an *E. coli* ACC multipeptide expression vector,the *accB* expression cassette in pAO815-BCCP was excised by digestion with *Bgl* II and *Bam*H I and then inserted into the *Bam*H I site of plasmid pAO815-accA to give pAO815-ACC2. The same insertion process was repeated twice to include the *acc D* and *accC* expression cassettes, respectively, into pAO815-ACC2. The resulting plasmid was designated pAO815-ACC4 and contained all the four *E. coli acc* gene expression cassettes linked to a single *his4* gene (Fig.3.9)

In order to provide a negative control for the transformation experiment, plasmid pAO815-ACC3 was constructed with the same reinsertion process, with the exception of *accB* insertion. Thus the plasmid contained only three *E. coli acc* gene expression cassettes: *accA, accC, accD.*

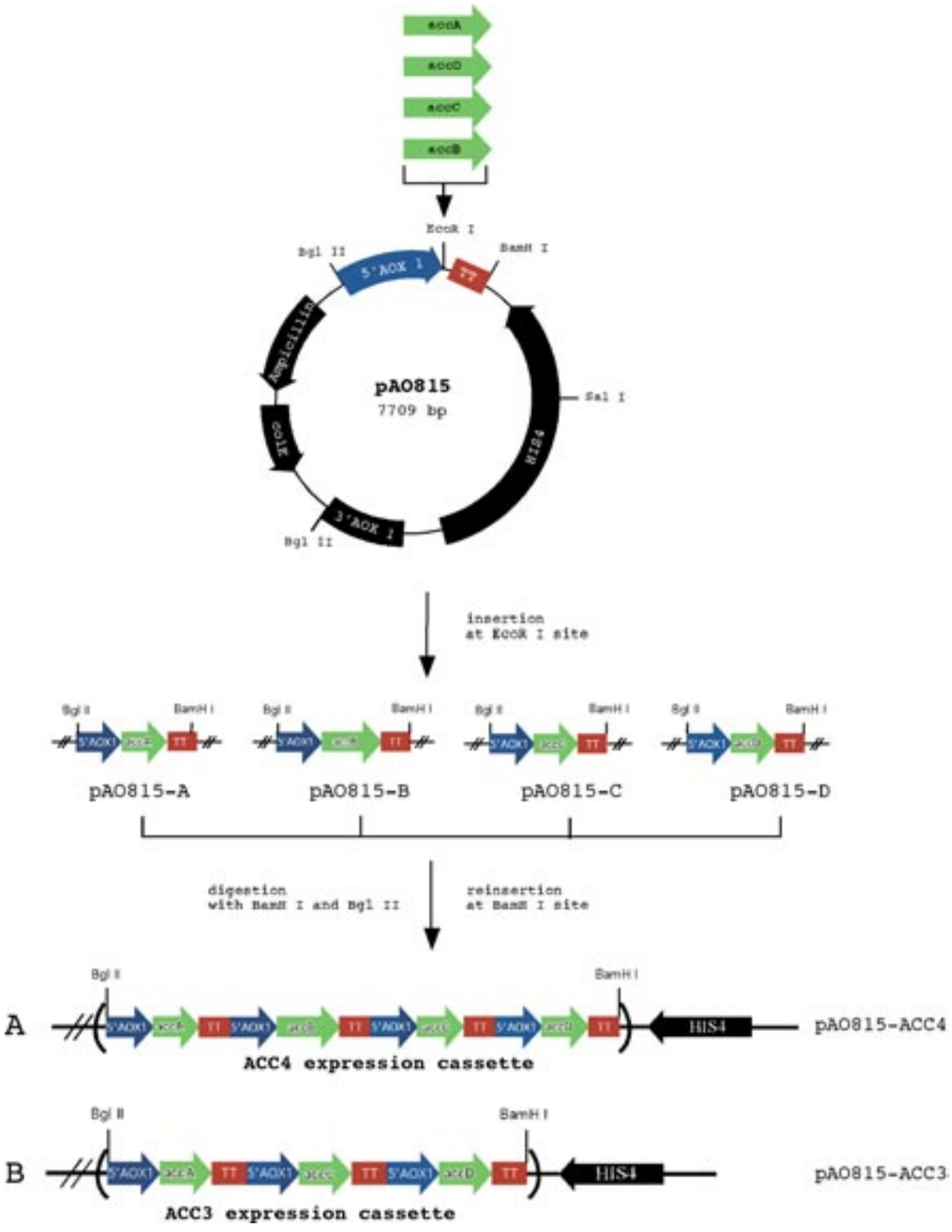


Fig. 3.9. Scheme for construction of *pichia* expression vectors.

A. pAO815-ACC4; B. pAO815-ACC3

The orientation of the cloned inserts was confirmed by sequencing. The expression vector was linearized with *Sal* I to allow homologous integration at the *his4* locus of GS115 cells.

3.2.6.3. Expression of *E. coli* ACC in *P. pastoris* GS115

10 µg of *Sal* I-linearized *E. coli* ACC expression vector was introduced into GS115 cells using the spheroplast transformation procedure following the recommendations of the manufacturer (Invitrogen). The transformants were selected initially on histidine-deficient plates (RDB) for histidine prototroph. The transformation yielded about 30 *his4* (+) clones /µg linearized DNA.

Transformation of the GS115 strain with the pAO815-ACC4 plasmid generated presumptive transformants designated HWA4. Transformation of the GS115 strain with pAO815-ACC3 plasmid resulted in presumptive transformants termed HWA3.

After the primary histidine selection, three *his* (+) clones from each of the HWA4 and HWA3 transformants were tested for soraphen A sensitivity by screen on MM plates containing increasing concentrations of soraphen A. Soraphen A did not inhibit the growth of the transformants expressing pAO815-ACC4 at a concentration of 0.02 to 0.1 µg/ml. One clone, termed HWA4-1, was completely resistant to soraphen (Fig. 3.10), whereas the growth of the two other two clones, HWA4-2 and HWA4-3, was slightly inhibited by soraphen A at the concentration of 0.1 µg/ml. In contrast, the transformants HWA3, expressing the incomplete *E. coli* ACC polypeptide, were as sensitive to soraphen A as the recipient GS115 strain. The growth of a representative clone, HWA3-2, on soraphen A plates is shown in Fig. 3.10.

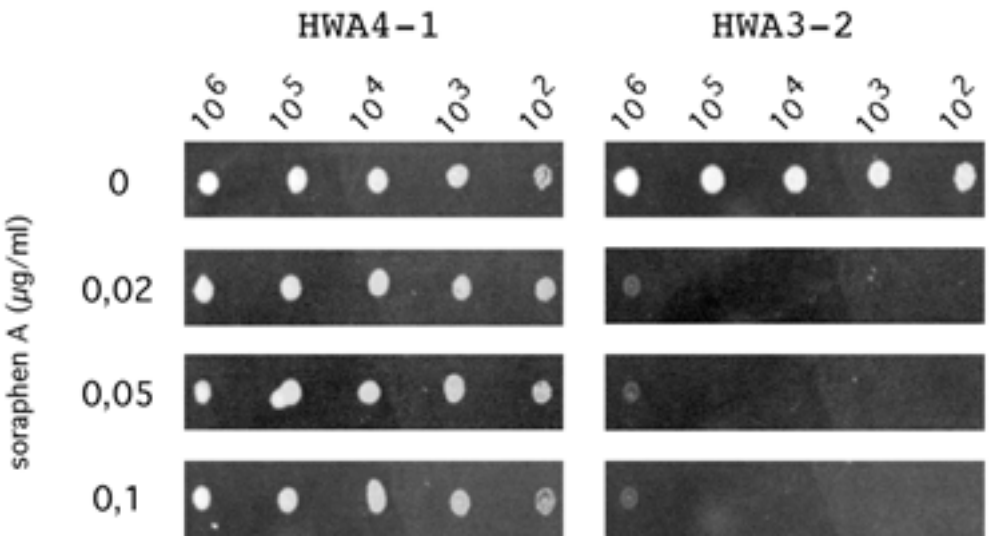


Fig. 3.10. Soraphen A sensitivity of the *P. pastoris* transformants. Serial dilutions of the cells were inoculated on MM plates containing different concentrations of soraphen A and incubated at 30°C for six days.

3.2.6.4. Chromosomal DNA analysis of putative Sor^R transformants

The presence of an integrated *E. coli* ACC expression cassette in the strains HWA4-1 and HWA3-2 was confirmed by Southern blot analysis using a *his4*-specific probe (Fig. 3.11). In the untransformed recipient strain GS115, this probe hybridised to a single band of about 2.7kb which indicates that the mutant chromosomal *his4* (-) gene of the recipient strain resides on this genomic DNA fragment (lane 2, Fig. 3.11A).

For the clone HWA4-1, the result of the southern blot indicated the expected single-copy homologous integration of pAO815-ACC4 at the *his4* locus. As shown in Fig. 3.11A, two bands were generated: a 3.6 kb band containing the mutant chromosomal *his4* (-) gene and a 11.8 kb band containing the wild type *his4* gene carried on the integrated plasmid pAO815-ACC4 DNA fragment which harbours the four *E. coli* ACC expression cassettes (lane3, Fig. 3.11A). These bands are of the same intensity indicating that both fragments contain a single copy of the *HIS4* gene. Different results were observed in two other investigated clones. With clone HWA4-2, the *his4*

probe recognized a 3.6 kb band and a band of about 18kb. With clone HWA4-3, four bands were recognized : 2.7 kb, 6.7 kb,15 kb and 22 kb. These results may arise from multi-copy integration at the *aox1* locus and/ or intramolecular rearrangement (data not shown).

In the clone HWA3-2, single-copy integration at the *his4* locus could be confirmed by Southern analysis (lane4, Fig. 3.11A). The expected bands were detected: a 3.6 kb band and, instead of a 11.8 kb band, a 10 kb band containing the wild type *his4* gene carried on the integrated plasmid pAO815-ACC3 which only contains three *E. coli* acc expression cassettes.

A

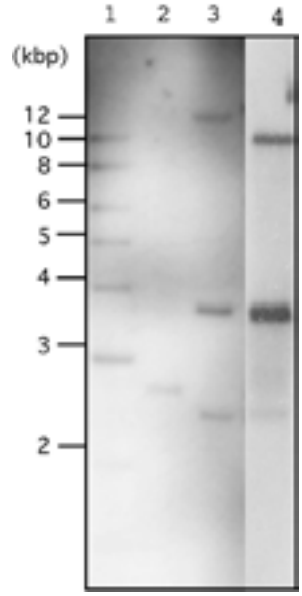
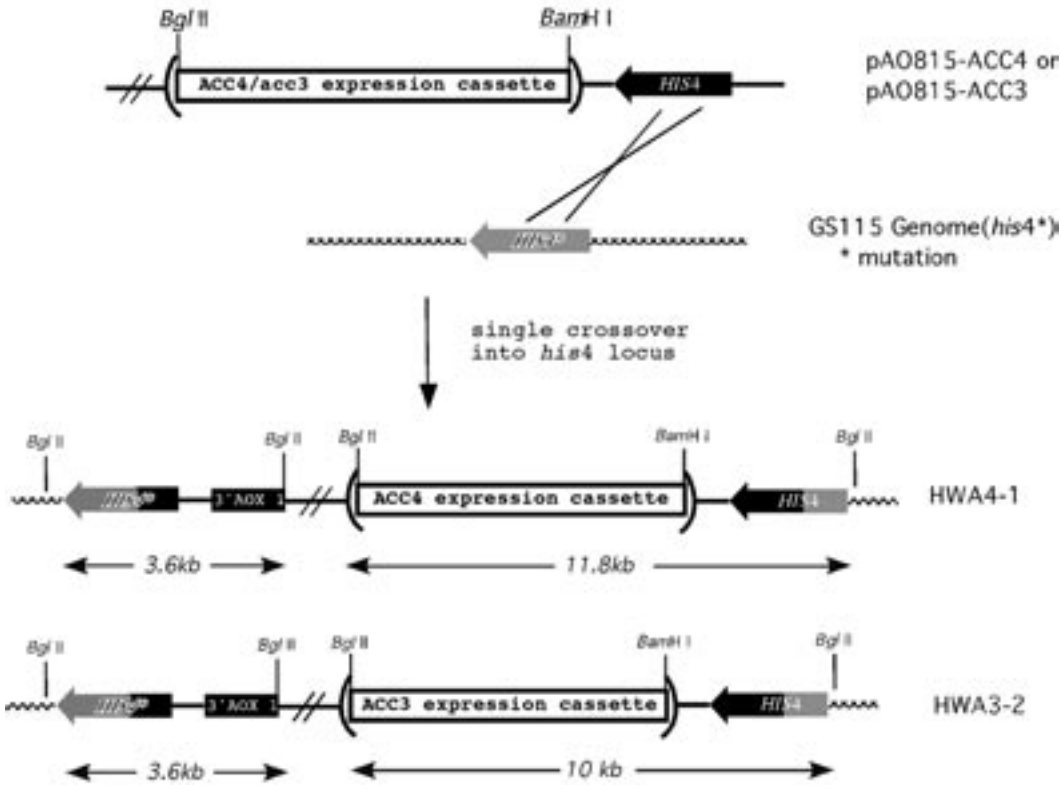


Fig. 3.11. (A) Southern blot analysis of chromosomal DNA from different Pichia strains. 5 µg chromosomal DNA was digested with *Bgl* II and hybridised to a biotinylated *his4* probe. Lane1, Molecular weight markers; lane2, DNA from GS115 strain; lane3, DNA from clone HWA4-1; lane4, DNA from clone HWA3-2. **(B) Predicted recombination events for transformants.** Clone HWA4-1 and HWA3-2 arised from single copy integration of pAO815-ACC4 and pAO815ACC3 at the *his4* locus. The size of the predicted restriction fragments is indicated below the map.

B



3.2.7. Development of a stepwise transformation procedure for *sorR* marker

The previous experiments confirmed that the complete expression of *E. coli* ACC polypeptides can offer *P. pastoris* resistance to soraphen A. The *sorR* marker consists of four *E. coli* ACC subunits. To facilitate handling and simplify the transformation method, a stepwise transformation procedure for the *sorR* selectable marker was developed. *P. pastoris* strain HWA3-2, that contains three *E. coli* ACC subunits was used as a recipient. The lacking *accC* gene encoding the BCCP subunit of *E. coli* ACC was transferred into HWA3-2 to determine whether the expression of all polypeptides will restore the resistance of the recipient strain to soraphen A.

3.2.7.1. Construction of the expression vector pPIC3.5K-BCCP.

A 471bp *Eco*R I fragment of the *accC* gene, which encodes the BCCP subunit of *E. coli* ACC, was subcloned from pAO815-BCCP into the vector pPIC3.5K into the *Eco*R I site to give a 9475 bp plasmid pPIC3.5K-BCCP. Gene expression could be driven by the *aox1* promoter (Fig. 3.12).

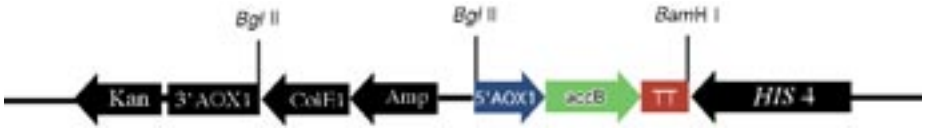


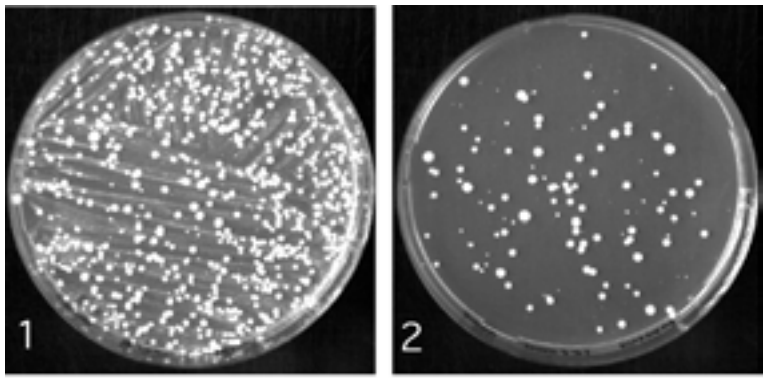
Fig. 3.12. The map of pPIC3.5K-BCCP. *Amp*, the ampicillin resistance gene; ColE1, the replication origin from *E. coli* plasmid ColE1. 3'AOX1, downstream region of *aox1* gene; *kan*, kanamycin resistance gene.

3.2.7.2. Expression of the *E. coli accC* gene in strain HWA3-2

pPIC3.5K-BCCP was linearized by *Sal* I for integration at the *his4* locus and introduced into HWA3-2 by electroporation. With the preparation of competent cells, three independent transformation experiments were done using different amounts of plasmid DNA (3 µg, 6 µg and 12 µg respectively). The electroporated cell suspensions were divided to select for either G418-resistance or soraphen A-resistance, in parallel. $G418^R$ transformants were selected on YPD plates with 0.25

mg/ml of G418, while sor^R transformants were selected on MM plates with 0.02 µg/ml of soraphen A. Resistant colonies appeared after two days (G418 selection) or seven days (soraphen A selection). The presumptive transformants were termed HWA3pG or HWA3pS, respectively. The transformation frequency is summarized in Fig. 3.13. The maximum transformation efficiency was observed when 3 µg DNA was used for electroporation. About 10 fold more transformants were obtained under G418 selection condition than under soraphen A selection condition (Fig. 3.13B). No colony formation was observed in the absence of plasmid DNA, both with G418 and soraphen A selection excepted that a lawn of background growht were observed under the selection of G418. The $G418^R$ transformants were transferred individually onto fresh G418 selective plates or further analysis.

A



B

	Transformants /µg DNA		
Selective medium	3 µg	6 µg	12 µl
YPD + G418 (0.25 mg/ ml)	4986	2839	4400
MM + Soraphen A (0.02 μg/ml)	246	167	150

Fig. 3.13. Transformation of *P. pastoris* strain HWA3-2 with pPIC3.5K-BCCP. (A) 100 µl electroporated cells were streaked on YPD-G418 plates for the selection of $G418^R$ clones (1) or on MM-soraphen A plates for the selection of Sor^R clones (2). 3 µg of plasmid was used for electroporation. (B) Transformation frequency (transformants /µg DNA) obtained with a different amount of plasmid DNA under the selection of G418 and soraphen A.

3.2.7.3.Soraphen A sensitivity assay of the transformants HWA3pG

In order to confirm that the G418-positive transformants were also soraphen A resistant, five $G418^R$ clones were picked up randomly from each transformation experiment, and tested for soraphen A sensitivity by a screen on MM plates containing 0.02 µg/ml soraphen A. After seven days incubation at 30°C, ten transformants grew in the presence of soraphen A (termed Sor^R) while five transformants failed to grow (Sor^S). Two representative transformants, designated HWA3pG31 and HWA3pG69, are shown in Fig. 3.14.

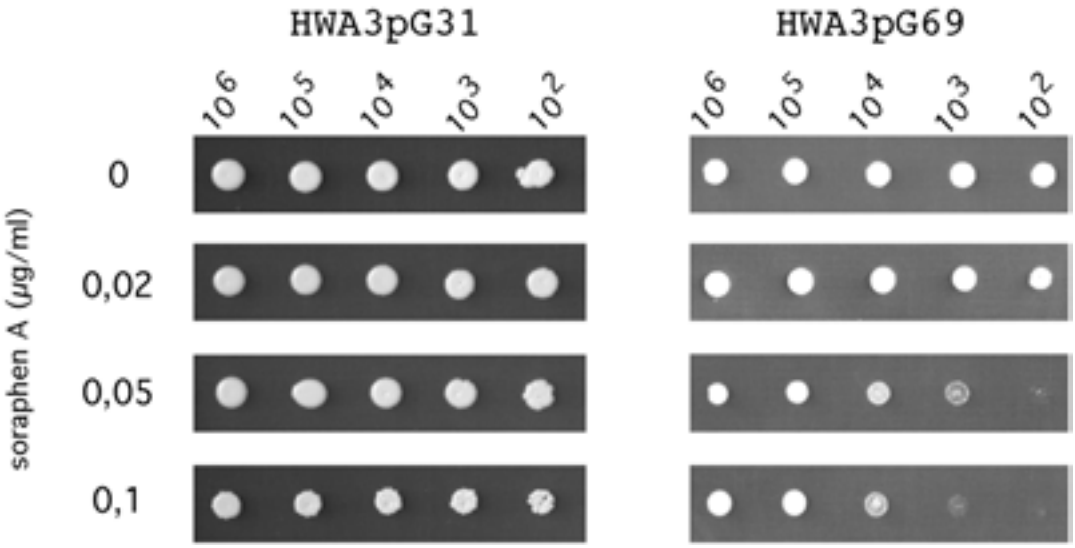


Fig. 3.14. Soraphen A sensitivity of the *P. pastoris* transformants. Serial dilutions of the cells were inoculated on MM plates containing different concentrations of soraphen A and incubated at 30°C for seven days.

3.2.7.4. Chromosomal DNA analysis of Sor^R transformants

In order to verify that the resistance against soraphen A was caused by the expression of the integrated *accC* gene, chromosomal DNA was isolated from the fifteen G418-resistant clones described in the previous paragraph and analysed by southern blot. Chromosomal DNA was digested with the restriction enzyme *Bgl* II and a biotin-labelled *his4* probe was used for detection.

Among the ten Sor^R clones, four different hybridisation patterns were identified:

(i) Single copy integration of pPIC3.5K-BCCP at the right *his4* locus of the recipient genomic DNA. As indicated in Fig. 3.16 (Rs), this kind of recombination would be expected to generate three bands: a 10 kb band containing the wild type *his4* gene carried on the integrated plasmid pAO815-ACC3 DNA fragment; a 5.8 kb band containing the *his4* gene carried on the integrated plasmid pPIC3.5K-BCCP DNA fragment; and a 4.9 kb band containing the chromosomal *his4* (-) gene. This predicted hybridisation pattern was observed in lanes 1 and 8 of Fig. 3.15.

(ii) Multiple, tandem insertion at the right *his4* locus of the recipient genomic DNA. As shown in Fig. 3.16 (Rs), multi-copy integration at the right *his4* locus will generate an additional plasmid sized band of 7.1 kb in addition to the 10 kb, 5.8 kb and 4.9 kb bands. This expected hybridisation pattern was observed in lane12 (Fig. 3.15).

(iii) Multiple tandem insertion at the left *his4* locus of the recipient genomic DNA. This kind of integration, as shown in Fig. 3.16 (Lm), will give four bands upon hybridisation : a 12.4 kb band containing the wild type *his4* gene carried on the integrated plasmid pAO815-ACC3 and the partial pPIC3.5K-BCCP DNA fragment; a 4.7 kb band containing the *his4* gene carried on the integrated plasmid pPIC3.5K-BCCP DNA fragment; a 7.1 kb band resulting from tandem repeated integration of the pPIC3,5K-BCCP plasmid; and a 3.6 kb band containing the mutated chromosomal *his4* (-) gene. The predicted hybridisation pattern was observed in lanes 6, 7, 9 and14 (Fig. 3.15).

(iv) Integration of plasmid pPIC3.5K-BCCP at the *his4* locus combined with an intramolecular rearrangements. As shown in lane 10, 13 and 15 (Fig. 3.15), unexpected bands of 7.8 kb, 13 kb, and 5.3 kb, respectively, were detected.

Among the five Sor^S clones, two hybridisation patterns were identified:

(i) There is no integration of the plasmid pPIC3.5K-BCCP. As shown in lane 2, 3, 4 and 5 of Fig. 3.15, only two bands were identified, 3.6 kb and 10 kb, which is identical with the hybridisation pattern of the recipient strain.

(ii) As found in lane 11 of Fig. 3.15, there were multiple integrations of the plasmid pPIC3.5K-BCCP at the left *his4* locus as indicated by the presence of a 3.6 kb band. However, the 12.4 kb band, representing the wild type *his4* gene carried on the integrated plasmid pAO815-ACC3 and partial pPIC3.5K-BCCP DNA fragment, was missing suggesting that intramolecular DNA rearrangement may have taken place.

The 2.4 kb band appearing in all the hybridisations could be a *Bgl* II fragment of the expression plasmid which houbours the *E. coli* ampicillin resistance gene and the ColE1 origin of replication.

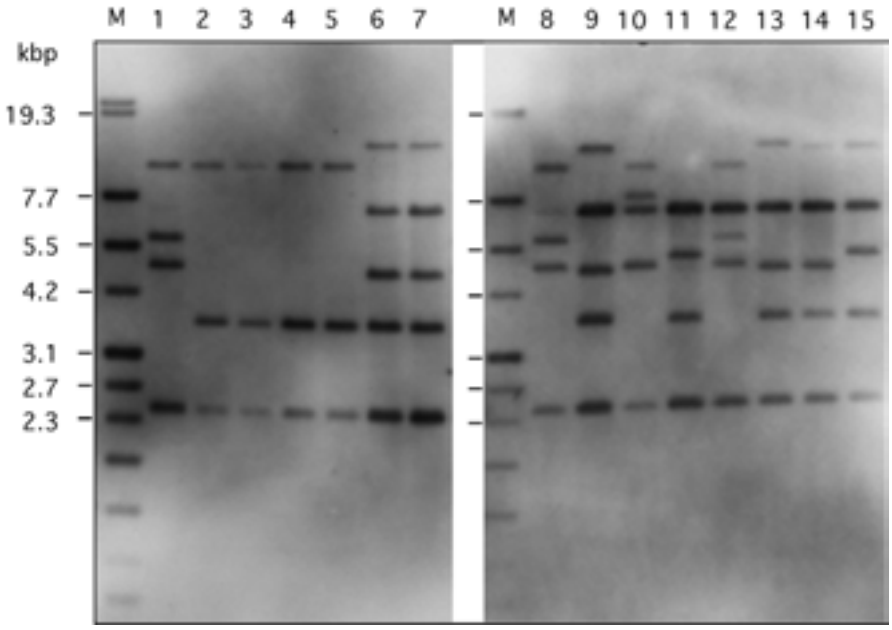


Fig. 3.15. Southern blot analysis of chromosomal DNA from transformants HWA3pG. 5 µg chromosomal DNA was digested with *Bgl* II and hybridised to a biotinylated *his4* probe. M, DNA molecular weight marker IV (Roche); lane 1 to 5, DNA samples from clones electroporated with 3 µg of plasmid DNA; lane 6-10, DNA samples from clones electroporated with 6 µg of plasmid DNA; lane 11-15, DNA samples from clones electroporated with 12 µg of plasmid DNA.

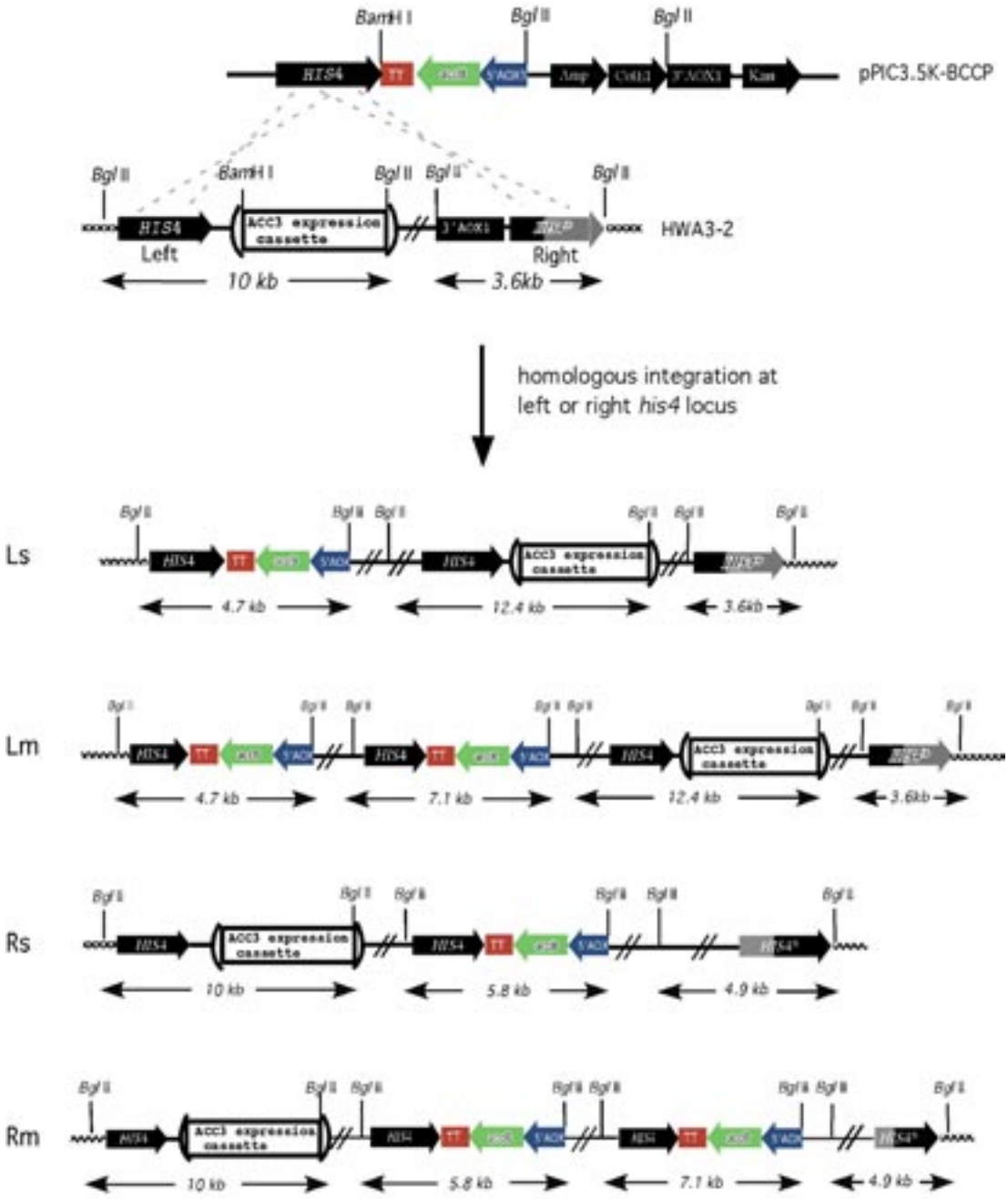


Fig. 3.16. Predicted molecular events for Sor^R clones arising from homologous integration of pPIC3.5K-BCCP at the *his4* locus of HWA3-2 recipient. Ls, single copy integration of pPIC3.5K-BCCP at the left *his4* site; Lm, multi-copy integration of pPIC3.5K-BCCP at the left *his4 site*; Rs, single copy integration of pPIC3.5K-BCCP at the right *his4* site; Rm, multi-copy integration of pPIC3.5K-BCCP at the right *his4* site. The size of predicted restriction fragments that should be detected by a biotin-labelled *his4* probe is indicated below the maps.

In lane 9, 11, 12,13 and 14 (Fig. 3.15), the 7.1 kb band is stronger than other bands, indicating that multi-copy integration may have occurred. To verify this, the chromosomal DNA represented in lane 9 and 14 was further digested with *Nae* I, which restricts pAO815-ACC3 once but is absent in pPIC3.5K-BCCP, and hybridised with a biotin-labelled *his4* PCR fragment. Integration of a single copy of this plasmid at the *his4* locus could be expected to generate two bands: either 13.5 + x kb and 12 + y kb or 23 + x kb and 2.5 + y kb (Fig. 3.17B) corresponding to integration into the right or left *his4* locus, respectively (x and y represent the border genomic DNA sequence that flanks the ends of the integrated vector). If more than one plasmid copy is integrated in tandem at the *HIS4* locus there should be a similar pattern of bands as above but with larger size corresponding to the plasmid pPIC3.5K-BCCP (9.5 kb). As predicted, two bands were detected from both clones, a 5 kb band and a band which is larger than 50 kb, indicating the multi-copy integration of the plasmid (Fig. 3.17A).

In addition, four independent clones HWA3pS, selected directly from the soraphen A plates, were analysed by southern blot. The hybridisation pattern indicated that the plasmid was integrated into the genome at both *his4* sites. The presence of the *E. coli acc* genes in the Sor^R clones transformed with pPIC3.5K-BCCP was also confirmed by PCR amplification using the respective cloning primers (data not shown).

A

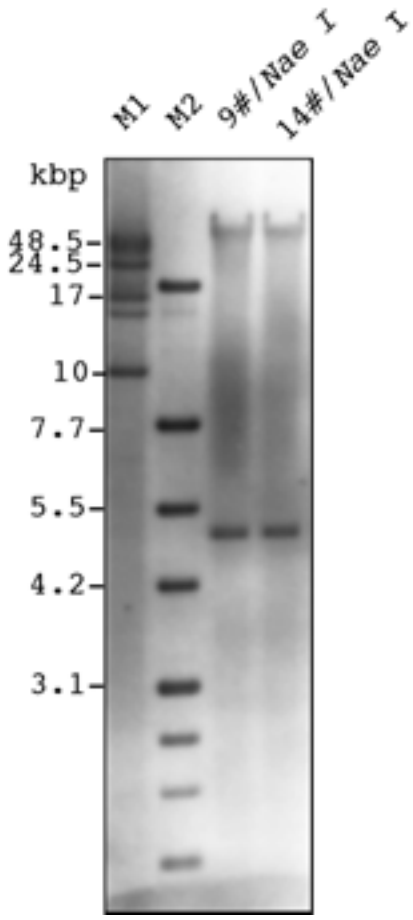
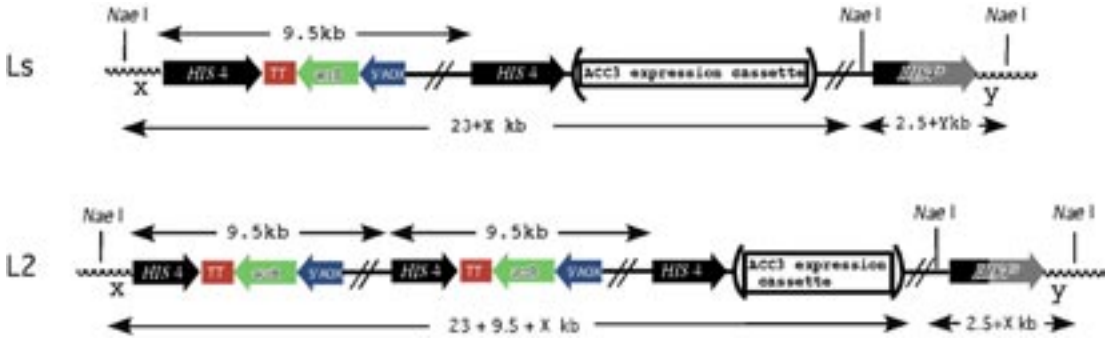


Fig. 3.17. (A) Southern blot analysis of the multi-copy integration of pPIC3.5K.BCCP into the genome of the recipient HWA3-2. 5 µg chromosomal DNA of clone 9# and 14# as shown in lane 9 and lane 14 of Fig.3.15. was digested with *Nae* I and hybridised to a biotinylated *his4* probe, respectively. M1, DNA molecular weight marker (lambda cut mix, NEB); M2, DNA molecular weight marker IV (Roche); **(B) Predicted recombination events for the transformants.** Ls, single-copy integration of pPIC3.5K-BCCP at the left his4 site; L2, double-copy integretion of pPIC3.5K-BCCP at the left his4 site. The size of the predicted restriction fragments that should be detected by a biotin-labelled his4 probe is indicated below the maps.

B



3.3. Discussion

3.3.1. Sequence of the gene encoding acetyl-CoA carboxylase in *B. bassiana* 252

The gene encoding acetyl-CoA carboxylase was isolated from *B. bassiana* 252. This gene, designated *accB1*, has an ORF of 6972 bp, coding for a protein of 2232 amino acids. Two small introns of 76 and 95 nucleotides have been mapped near the 5' end of the gene. This is in good agreement with the size of ACCs from other eukaryotic organisms. The *B. bassiana accB1* gene has 69% identity at the amino-acid level to the *A. nidulans acc1* gene. Based on sequence homology to the *A. nidulans* and *S. cerevisiae acc1* genes, biotin carboxylase, transcarboxylase domains and a biotin binding-site have been assigned.

Sequence analysis suggested the presence of two AUG start codons, 105 bases apart, at the 5'-end of the *accB1* mRNA. Translation from the first AUG would give a 3-amino-acid peptide before termination, whereas translation from the second AUG yields a protein of 2232 amino acids which has high homology to other acetyl-CoA carboxylases. The sequence context around these two AUG codons suggests they could allow leaky scanning, as described by Kozak (1995) and Arst and Sheerins (1996). The presence of uORF was also observed in the *acc1* gene from *A. nidulans*. A study from Wanke *et al.* (1997) showed that the presence of uORF in *Aspergillus spp*. is not uncommon, particularly in genes involved in cell growth and differentiation. Although the exact function of uORF is unknown, it is believed to affect the efficiency of mRNA translation and as such may be important in the regulation of expression of some genes. They are often found in mRNAs encoding transcription factors, growth factors, and receptors, all important regulatory proteins (Kozak, 1991).

The mRNA of *accB1* has an unusually long leader sequence. Primer extension and 5'RACE experiments determined the location of the transcriptional initiation site to be 380 bp upstream of the main ATG. The typical CAAT-like elements were not found in the upstream region of the *accB1* gene. The main feature of the upstream region are several CT-rich stretches of nucleotides in proximity to the transcriptional start site and the translational start site. Dobson (1982) and McNeil (1988) found that CT regions near transcriptional start sites are indicative of strong promoters in *S. cerevisiae* and appear to increase fidelity of transcription initiation in highly expressed genes.

The synthesis of long-chain fatty acids from acetyl-CoA also requires the heteromultimeric fatty acid synthase (FAS) complex. FAS consists of two multifunctional proteins, FAS1 (α subunit) and FAS2 (β subunit), that are organized in an $\alpha_6\beta_6$ complex. In yeast *S. cerevisiae*, these three genes are co-ordinately repressed by fatty acids and the activities of FAS and ACC are reduced in the presence of inositol and choline. This regulation is mediated through transcriptional regulation at the UAS_{INO} (upstream activation sequence) with the consensus sequence TTCACATG which functions in either orientation. The same sequence motif is found in the upstream regions of all so far characterized yeast genes encoding enzymes of phospholipid biosynthesis, suggesting that this sequence modulates the expression of these genes so that an appropriate membrane lipid composition is maintained (Chirala,1992). The sequence of the yeast *acc1* regulatory region has one UAS_{INO} and the function of this UAS has been verified by deletion and mutational analysis (Chirala,1994). However, the conserved sequence of UAS_{INO} could not be found in the regulatory region of *B. bassiana accB1*.

The conserved biotin-binding site Met-Lys-Met is located at residues 783-785 in *B. bassiana accB1*, inside the second subdomain BCCP. Comparison of amino acid sequences near the biotin-binding sites of all known biotin-containing enzymes showed that (i) this site is preceded by an Val instead of a Ala residue, as in other carboxylase enzymes. This is also true in the rat, chicken, and yeast *acc* sequences, but not in *U. maydis acc1* where it is preceded by Ile (Bailey,1995), and (ii) this domain is located closer to the NH2 terminus of the polypeptide, whereas in other biotin-containing enzymes, e.g., yeast pyruvate carboxylase, this domain occurs near the COOH termini. It has been suggested that this positioning of the biotin-binding site may increase the efficiency of the biotinylation of the proteins (Stucka, 1991 and Bai, 1989). In all the biotin-containing enzymes, the biotinylated residues are located 25-29 amino acids downstream of a short amino acid sequence flanked by two Pro residues [-Pro-(Xaa)n-Pro-], which might act as a hinge to permit the biotin-containing arm to move between the carboxyl donor and acceptor sites (Al-Feel, 1992). *B. bassiana accB1* and the *acc* gene from yeast, rat and chicken also contain similar proline-flanked sequences.

Despite the conservation of biotin-binding sites among all biotin-containing proteins, the amino acid sequences surrounding these sites are divergent. Indeed, the sequence of *B. bassiana accB1* between residues 650 and 1350 is the least homologous domain as compared with that of *acc* genes from other fungal species. The lack of

conservation of the sequences in these regions of the enzymes may pertain to the assembly of the enzyme subunits into polymer forms that make up the enzymatically active carboxylases. Also, this lack of conservation suggests that the amino acid sequences within the BCCP domains of the carboxylases may be involved only in providing a scaffold for the critical functional regions. Indeed, the biotin protein ligase (also called holocarboxylase synthetase), a highly conserved enzyme required to attach biotin to the lysine residue of BCCP, recognizes only properly folded domains (Chapman-Smith , 1999). In this regard, the biotin in the BCCP domain may be akin to the 4'-phosphopantetheine prosthetic group of the acyl carrier protein domain of the fatty acid synthase, where the quaternary structure of the protein and the need for the pantetheine-bound fatty acyl intermediates to interact with the various catalytic domains of the synthase play a crucial role in the overall activity of the enzymes.

3.3.2. Expression of *sorR* conferred soraphen A resistance in *P. pastoris*

In these experiments, a yeast expression system was utilized for the development of a new dominant selectable marker, *sorR*, for fungal transformation. The *sorR* marker is based on the expression of *E. coli* ACC polypeptides which confer soraphen A resistance. For yeast transformation, the marker gene was made up of the structural genes of *E. coli* ACC fused to promoter and terminator sequences of the *P. pastoris aox1* gene. This chimeric gene containing the homologous *aox1* promoter was expected to be efficiently expressed in *P. pastoris*.

The primary growth assay in the presence of soraphen A demonstrated that both *B. bassiana 252* and *P. pastoris* GS115 were sensitive to this fungicide compound. A concentration of 0.5 to 1 µg/ml inhibits growth of *B. bassiana 252,* whereas 0.02 µg/ml is enough to inhibit growth of *P. pastoris.* This data indicate that the optimal inhibitory concentration of soraphen A to be used for transformation is likely to vary from strain to strain and will thus require empirical determination.

Using plasmid pAO815-ACC4, which contains the complete genes encoding *E. coli* ACC, *P. pastoris* cells could be transformed to soraphen A resistance. The transformant HWA4-1 can grow on a soraphen A plate with a concentration five times higher than that which inhibits the growth of the untransformed recipient strain completely. Strain HWA3-2, which contained an incomplete *E. coli* ACC resulting from the transformation with plasmid pAO815-ACC3 that harbours three *E. coli acc*

genes, showed the same sensitivity to soraphen A as the recipient strain GS115. To demonstrate that the expression of the four *E. coli* ACC polypeptides is a prerequisite for its function as a dominant selectable marker and to develop a transformation procedure for simplified use of *sorR*, a further expression vector pPIC3.5K-BCCP that contains the lacking *accC* gene, was constructed and introduced into the strain HWA3-2. The soraphen A resistance in transformant HWA3p-8, containing all four *E. coli* ACC polypeptides, as confirmed by southern blot analysis, was restored. This indicates that the yeast were made soraphen A-resistant by the introduction of all four *E. coli acc* marker genes. The fungicide resistance was shown to be dominant and the *sorR* genes, fused with an appropriate promoter, also have the potential to be exploited as a selectable marker for transformation of other fungal species under the following conditions: (i) that cells can be transformed with a linear DNA fragment; (ii) availability of a functional promoters which can drive the transcription of the *sorR* gene in the fungal cell; (iii) the fungal species of interest must be sensitive to soraphen A.

These experiments demonstrate that a bacterial ACC can be assembled and functionally expressed in yeast, indicating that the subunits are expressed in full-length form and with appropriate biotinylation. It has previously been shown that the *E. coli* ACC subunits are able to catalyse distinct partial reactions as purified subunits, moreover, the purified subunits retain the ability to perform the overall ACC reaction when they are recombined. In addition, several plant ACC have been expressed in *E. coli* and shown to encode proteins having the expected activity and the plant BCCPs are readily biotinylated by *E. coli* (Sasaki, 2001).

The importance of providing the proper stoichiometry of interacting subunits for macromolecular structures has been demonstrated in several systems, including phage morphogenesis, bacterial chemotaxis, and yeast histone genes. The results obtained in this work suggest that this principle may apply to the *E. coli* ACC. Multi-copy integration of the plasmid pPIC3.5K-BCCP into the yeast genome occurred in several transformants as shown by Southern blot experiments. The transformants, as represented by clone HWA3p-12, can grow on the control plate and survive when soraphen A was supplied at low concentration (0.02 µg/ml). However, growth was partially inhibited when the concentration of soraphen A was increased to 0.05 and 0.1 µg/ml. These results suggest a correlation between the level of soraphen A resistance and the stoichiometry of polypeptides in the *E. coli* ACC. Although over-expression of the BCCP subunit seems to have no effects on cell

growth under nonselective condition, as evidenced by the lack of change in growth rate and cell morphology in comparison with that of recipient GS115 cells, the function of *E. coli* ACC as a selectable marker may be sensitive to changes in subunit stoichiometry. Clare (1991) observed that multiple insertion of plasmid DNA into the chromosomal DNA was a common event in *P. pastoris* transformations, and suggested that the religation of transforming DNA fragments in vivo allowed the repeatedly insertion of circularised DNA forms into the chromosome by single crossovers. In addition, circularised fragments could insert wherever there is homology to chromosomal DNA, e.g., at the *aox1* locus. Such events could explain why the clone HWA4-3 generated four bands, 2.7 kb, 6.7 kb, 15 kb, and 22 kb, which hybridised with the *his4* probe in Southern blot experiments. The presence of the 2.7 kb band indicated that the original chromosomal *his4* (-) gene was not affected by the recombination.

In order to introduce exogenous plasmid DNA into recipient cells, two transformation methods were applied: spheroplasting was used to obtain histidine prototroph and electroporation was used to obtain G418 or soraphen A resistant transformants. In general, both methods provide a high transformation efficiency. Electroporation is a more convenient method because it does not require the generation and maintenance of spheroplasts. In this work, the electroporation procedure gave a higher transformation frequency than the spheroplasting method. In one experiment, three different transformations by electroporation were done in parallel by adding 3 µg, 6 µg and 12 µg plasmid DNA/ 60 µl competent cells, respectively. The electroporated cells were incubated at room temperature under nonselective condition for three hours and then selected for G418 or soraphen A resistance. The highest transformation frequency was obtained when 3 µg plasmid DNA was applied. This observation was consistent with the conclusion from Becker and Guarente (1991) who also noted a decreased transformation efficiencie when increased amount of DNA were applied for electroporation. Furthermore, in each transformation experiment, the transformation frequency under G418 selection conditions was at least ten-fold higher than under soraphen A selection conditions. This great difference may be an artefact of the selection method that was used. Transformed cells require a growth period in the absence of selection to allow the primary regeneration of transformed cells. Also, integration and full expression of the resistance gene require additional time. In this work, the rich medium YPD was used for G418 selection and a minimal methanol medium (MM) was used for soraphen A selection. In general, the doubling time of log phase *Pichia* in YPD

medium is about two hours, in comparison to that in MM medium which is about four to six hours, although the growth characteristics may vary depending on the recombinant strain. In this work, the addition of either G418 or soraphen A for selection of the transformed cells was delayed for three hours. In such a situation, the timing of selection becomes critical; relatively early addition of the selection drug may result in inhibition of many putative transformants while relatively delayed addition may result in large amount of replicates of original transformed clones. In addition, the density of cells plated onto G418 plates is also critical. It has been noticed that a large number of false G418 positive colonies can occur even when the cells are plated at low density. Spontaneous G418-resistant colonies were found in these experiments, especially when low amounts of DNA (3 µg) was applied for electroporation: four from five randomly chosen clones were false positive colonies.

The *sorR* marker gene can be used to directly select for soraphen A-resistance following the electroporation of *Pichia* strain HWA3-2 with the plasmid pPIC3.5K-BCCP. But the frequency of transformation was lower when compared with that after G418 selection. Further improvements of the selection protocol may be possible. For example by optimising the time point for addition of soraphen A. In addition, the stepwise transformation procedure developed for the *sorR* selection marker facilitates handling of DNA and simplify the practical use of the transformation method. Thus, the development of *sorR* selection marker not only adds an additional drug resistance gene to the list of selectable markers for fungal transformation but also enhances the versatility of fungal genetic manipulation studies, especially when successive transformations are performed.

IV. Materials and Methods

4.1. Materials

4.1.1. Chemicals and consumables

5-Brom-4-Chlor-3-Indolyl-β-D-Galactopyranosid (X-Gal)	Biomol
Ammonium peroxidisulfate (APS)	Serva
Bovine serum albumin (BSA)	Serva
Disodium 3-(4-methoxyspiro{1,2-dioxetane-3,2'-(5'-chloro) tricyclo[3.3.1.1 ^{3,7}] decan}-4-yl)phenyl phosphate (CSPD)	Roche diagnostics
Diethylpyrocarbonate (DEPC)	Sigma
Dithiothreitol (DTT)	Serva
Isopropyl-β-D-thiogalacto pyranoside (IPTG)	Sigma
Maleic acid	Merck
Ni-NTA-agarose	Qiagen
Phenylmethylsulfonylfluoride (PMSF)	Sigma
Sobitol	Applichem
Trichloroacetic acid (TCA)	Sigma
Biodyne A, B Blotting Membrane	Pall
Glass wool, silanized	Serva
Glass beads (0.5mm)	Serva
Mirocloth	Calbiochem
X-ray film	Kodak

Soraphen A was obtained from Prof. Dr. Hans Reichenbach, Gesellschaft für Biotechnologische Forschung m.b.H., Braunschweig, Germany

4.1.2. Radioisotope

γ^{32}P-dATP, 3000 Ci/mmol, 10 mCi/ml	Amersham

4.1.3. Antibodies

Anti Dehydrofolate reductase	Prof. R. Herrman, ZMBH
Anti rabbit IgG, alkaline phosphatase-conjugated	Dianova
Anti rabbit IgG, peroxidase-conjugated	Dianova

4.1.4. Enzymes and kits

Enzymes

T4 DNA Ligase	Roche Diagnostics
Ampli Taq DNA polymerase	Perkin Elmer
AMV Reverse Transcriptase	Promega
Biotin High Prime	Roche Diagnostics
Caylase C3	Sigma
Deep Vent DNA polymerase	Biolabs
Expand Long Template PCR System	Roche Diagnostics
Glucanex	Novo Nordisk Ferment
Lyticase	Fluka
Lysing Enzyme	Sigma
Proteinase K	Roche Diagnostics
Restriction endonucleases	Roche Diagnostics, BioLabs, Promega
Shrimp alkaline phosphatase	Roche Diagnostics
SuperScript RNaseH$^-$ Reverse Transcriptase	GibcoBRL
SUPERaseIn RNase Inhibitor	Ambion
T4 Polynucleotide Kinase	BioLabs
Terminal Transferase	Diagnostics
Zymolyase	Invitrogen

Kits

Access RT-PCR system	Promega
Bio Rad Protein Assay	Bio Rad
Biotin Luminescent Detection Kit	Roche Diagnostics
DNA-free^{TM} Kit	Ambion
Lambda DASHII/BamH I Vector kits	Stratagene
Minelute PCR Purification Kit	Qiagen
Multi-Copy pichia Expression Kit	Invitrogen
Nucleospin Plasmid Purification Kit	Macherey & Nagel
QIAquick PCR Purification Kit	Qiagen
QIAquick Oligonucleotide Purification Kit	Qiagen
QIAquick Nucleotide Removal Kit	Qiagen
Rneasy Plant Mini Kit	Qiagen
S.N.A.P. Gel Purification Kit	Invitrogen

Wizard Lambda Preps DNA Purification System	Promega
Zero Blunt TOPO PCR Cloning Kit	Invitrogen

4.1.5. Plasmids

pRS31	(Suelmann *et al.,* 1997)
pLS151	(Shyr-Jiann Li *et al*., 1992a)
pLS9	(Shyr-Jiann Li *et al*.,1992b)
pLS1	(Shyr-Jiann Li *et al*., 1992c)
pAO815	Invitrogen
pPIC3.5K	Invitrogen
pBSSK II+	Stratagene
pQE40	Qiagen
pUC18	(Yanish-Perron *et al.,* 1985)

4.1.6. *E. coli* strains

ABLE C	*E. coli C lac*(*LacZω*$^{-}$) [Kan^{r} *McrA*$^{-}$ *McrCB*$^{-}$ *McrF*$^{-}$ *Mrr*$^{-}$ *HsdR* ($r_K^{-}m_K^{-}$)] [F'*proAB lacIqZΔM*15 *Tn10* (Tet^{R})]
ABLE K	*E. coli C lac*(*LacZω*$^{-}$) [*Kan^{r} McrA*$^{-}$ *McrCB*$^{-}$ *McrF*$^{-}$ *Mrr*$^{-}$ *HsdR* ($r_K^{-}m_K^{-}$)] [F'*proAB lacIqZΔM*15 *Tn10* (Tet^{R})]
SG13009[pREP4]	Nal^{S}, Str^{S}, Rif^{S}, *Thi*$^{-}$, *Lac*$^{-}$, *Ara*$^{+}$, *Gal*$^{+}$, *Mtl*$^{-}$,*F*$^{-}$, *RecA*$^{+}$, *Uvr*$^{+}$, *Lon*$^{+}$
SCS110	*rpsL* (*Str^{R}*) *thr leu endA thi-1 lacY galK galT ara tonA tsx dam dcm supE44Δ (lac-proAB)* [F' *traD36 proAB lacIqZΔM*15]
XL1-Blue MRA (P2)	Δ(*mcrA*)183 Δ(*mcrCB-hsdSMR-mrr*)173 *endA1 supE*44 *thi*-1 *gyrA*96 *relA*1 *lac*(P2 lysogen)
XL-1-Blue MRF’	Δ(*mcrA*)183 Δ(*mcrCB-hsdSMR-mrr*)173 *endA1 supE*44 *thi*-1 *recA*1 *gyrA*96 *relA*1 *lac* [F'*proAB lacIqZΔM*15 *Tn*10 (Tet^{R})]

4.1.7. Fungus and yeast strains

Beauveria bassiana (ARSEF 252) was obtained from the USDA-ARS Collection of Entomopathogenic Fungi Cultures (ARSEF), U.S. Plant, Soil, and Nutrition Laboratory, Tower Rd., Ithaca, NY, USA. The strain was originally isolated from

Leptinotarsa decemlineata. The strain was grown on YPG medium at 25 ºC and stored at 4 ºC.

Pichia pastoris host strain GS115 was obtained from Invitrogen. GS115 has a mutation in the histidinol dehydrogenase gene (*his4*$^-$) that prevents histidine synthesis. GS115 cells were grown on the complex medium YPD or on minimal media supplemented with histidine at 30ºC and stored at 4ºC.

4.1.8. Antibiotic stock solutions

Antibiotic	Stock Solution	Working Concentration
Ampicillin	100 mg/ml in H_2O	60 µg/ml
Chloramphenicol	34 mg/ml in Ethanol	34 µg/ml
Kanamycin	30 mg/ml in H_2O	50 µg/ml
Tetracyclin	12.5 mg/ml in Ethanol	25 µg/ml
Geneticin (G418)	10 mg/ml in H_2O	0.25-1.0 mg/ml
Soraphen A	1 mg/ml in Methanol	0.02-0.2 µg/ml

4.2. Methods

4.2.1. Microbiological techniques

4.2.1.1. Growth of *E. coli*

E. coli was either inoculated in liquid LB medium or streaked on LB agar (2% agar) plates and allowed to grow overnight at 37°C. Appropriate antibiotics were added using the concentrations described above.

LB medium:

Bacto Trypton	1% (w/v)
Bacto Yeast Extract	0.5% (w/v)
NaCl	1% (w/v)
pH 7.2	

LB agar:

LB medium plus 2% (w/v) agar

4.2.1.2. Electroporation of *E. coli*

Preparation of cells: 1 liter of LB medium was inoculated with 10 ml of a fresh E. coli liquid overnight culture and incubated at 37 °C with agitation until the OD_{600} of the culture reached 0.5 to 1.0. After chilling the culture on ice for 30 minutes, the cells were harvested by centrifugation at 4000g for 15 minutes and washed twice with ice-cold sterile water. Thereafter, the cells were washed twice with ice-cold 10% glycerol and finally resuspended in 2 ml ice-cold 10% glycerol. The cell suspension contained 1-3 x 10^{10}cells/ml and stored at -80°C in aliquots.

Electroporation: about 100 ng of plasmid DNA was mixed with 45µl of competent cells and incubated on ice for 1 minute. Electroporation was performed with the Biorad GenePulser under the following conditions: Field strength 1.5kV/cm, Electric capacity 25 µF, Resistance 200Ω. Thereafter, 1 ml prewarmed LB medium was added immediately, the cell suspension was incubatede at 37°C with agitation for 1 hour and spreaded onto LB agar plates containing the appropriate antibiotics.

4.2.1.3. Growth of *B. bassiana*

Preparation of *B. bassiana* conidia

B. bassiana was inoculated onto YPG agar plates and allowed to grow for 10 days at 25 °C. 10 ml sterile water was added to the plate. The conidia were detached from the mycelium by gentle stirring with a sterile glass funnel stuffed by glass wool at the end. The suspension was filtered through glass wool in order to separate mycelium and transfered to a sterile tube. The concentration of conidia was determined by counting with a hemocytometer.

Liquid culture of *B. bassiana*

Conidia of B. bassiana were inoculated in YPG medium to a final concentration of 1 x 10^6/ ml, and incubated at 25 °C with agitation (150 rpm in a rotary shaker). The culture was incubated for 20 hours to obtain mycelium or for four days to obtain spores.

YPG medium:

Bacto Yeast Extract	0.2% (w/v)
Bacto Peptone	1% (w/v)
Glucose	2% (w/v)

YPG agar plate:

YPG medium plus 2% (w/v) agar

4.2.1.4. Media transfer of *B. bassiana* culture in order to achieve C/N derepression

100 ml of complete medium (CM) in a 250 ml Erlenmyer flask was inoculated with 5 x 10^6 conidia and incubated in an orbital shaker (150 rpm at 25 °C) for four days in order to allow extensive fungal growth. The spores were harvested ba filtration through three layers of sterile mirocloth, washed once with steril BSM1 and incubated in 100 ml BSM1 for 24 hours under the same conditions as above. The culture was then harvested by centrifugation at 1500 g for 10 minutes, washed once with steril BSM2 and inoculated in 100 ml BSM2 supplemented with 0.8% (w/v) beetle cuticle pieces. The culture was incubated for a further 4-12 hours under the same conditions. The mycelium and supernatant were seperated by centrifugation and stored at -80°C for further analysis.

Complete medium (CM):

Sucrose	1 % (w/v)
$NaNO_3$	0,2 % (w/v)
KH_2PO_4	0,1 % (w/v)
$MgSO_4.7H_2O$	0,05% (w/v)
$FeSO_4.7H_2O$	0,2 µg/ml
$ZnSO_4.7H_2O$	1,0 µg/ml
$NaMoO_4.2H_2O$	0,02 µg/ml
$CuSO_4.5H_2O$	0,02 µg/ml
$MnCl_2.4H_2O$	0,02 µg/ml

The pH was adjusted to 5,5 with NaOH

Basal salts medium 1 (BSM1):

The composition of BSM1 is the same as that of CM except for the addition of:

Sucrose	0.2 % (w/v)
$NaNO_3$	0.05 % (w/v)

Basal salts medium 2 (BSM2):

The composition of BSM2 is the same as that of CM except for the addition of:

Sucrose	0.1 % (w/v)
$NaNO_3$	0.02 % (w/v)

Cuticle Medium (Cu):

0.8% beetle cuticle pieces was added to previously sterilized BSM2 (121 °C, 15 min) and autoclaved for 5 min at 115 °C.

4.2.1.5. Sensitivity assay of *B. bassiana 252* to soraphen A

1000 spores of *B. bassiana* 252 were plated on the YPG plates containing increasing amounts of soraphen A and duplicate cultures were incubated at 25°C for six weeks. The growth of *B. bassiana* 252 was assessed by counting the number of resistant colonies that appeared on the plates.

4.2.1.6. Growth of *P. pastoris*

Pichia strain GS115 was streaked onto a YPD plate and incubated for 2 days at 30°C in order to obtain single colonies. A single colony of GS115 from the plate was picked and inoculated in YPD medium and allowed to grow at 30°C with vigorous shaking (200rpm). The obtained culture is ready for further analysis or may be stored at 4°C for several days.

YPD medium:

Bacto Yeast Extract	1% (w/v)
Peptone	2% (w/v)
Dextrose	2% (w/v)

The medium was supplemented with 2% (w/v) of agar for the preparation of YPD plates.

4.2.1.7. Sensitivity assay of *P. pastoris* to soraphen A

For point inoculation, 10-fold serial dilutions of 10^6 cells were spotted on MM plates containing 0 to 0.1 µg/ml soraphen A. For whole surface inoculation, 5000 cells were streaked on MM plates containing soraphen A. Duplicate inoculations were incubated at 30°C for four days. The sensitivity of *P. pastoris* to soraphen A was assessed by the ability of their growth on different concentrations of soraphen A.

4.2.1.8.Transformation of *P. pastoris*

Transformation by spheroplasting

For the preparation of spheroplasts, a single colony of GS115 cells was picked and inoculated in 10 ml YPD medium and grown overnight at 30°C with vigorous shaking. 1 ml of the culture was inoculated in 200 ml of fresh YPD medium and allowed to grow at 30°C with vigorous shaking until the OD_{600} of the culture reached 0.2-0.3. The cells were harvested by centrifugation at room temperature for 5 min at 1500xg. After washing with 20 ml of sterile H_2O, 20 ml of freshly made SED and 20 ml of 1M Sorbitol, the cells were resuspended in 20 ml of SCE buffer and one aliquot of the cell suspension was taken to monitor the optimal time of digestion with Zymolyase that resulted in approximately 70% spheroplasting. This step was done following the manufacturers instructions and approximately 30-40 minutes of Zymolyase treatment was found to achieve optimal spheroplasting. Therefore, 7.5 µl Zymolyase was added to another aliquot of the cell suspension and the cell wall was digested at 30°C for 30-40 minutes. The resulting spheroplasts were harvested by centrifugation at 750 xg for 10 min, washed with 10 ml of 1M sorbitol and with 10 ml of CaS. After centrifugation at 750xg for 10 minutes, the spheroplasts pellet was gently resuspended in 0.6 ml of CaS for transformation.

For spheroplast transformation, 10 µg of linearized DNA was mixed with 100 µl of the spheroplast preparation and incubated at room temperature for 10 minutes. Thereafter, 1 ml of a freshly made PEG/CaT solution was added to the spheroplast-DNA solution. After incubation for another 10 minutes at room temperature, the cells together with DNA were harvested by centrifugation at room temperature for 10 minutes at 750xg. The pellet was dried from PEG/CaT solution briefly and suspended in 150 µl of SOS medium, after incubation at room temperature for 20 minutes, 850 µl of 1M sorbitol was added to the spheroplast- DNA suspension. 250 µl aliquots of the spheroplast- DNA suspension was mixed with 10 ml of melted RD top agarose and poured on a RDB plate. After the top agarose was solidified, the plates were inverted and incubated at 30°C for 4-6 days until the transformants appeared.

SE:	1 M Sorbitol, 25 mM EDTA, pH 8.0
DTT:	1 M DTT in water
SED :	19 ml of SE and 1 ml of 1 M DTT per 20 ml solution

SCE:	1 M Sorbitol, 1 mM EDTA and 10 mM Sodium citrate, pH 5.8
CaS:	1 M Sorbitol, 10 mM Tris-HCl, pH 7.5, 10 mM $CaCl_2$
CaT:	20 mM Tris, pH 7.5 and 20 mM $CaCl_2$
PEG/CaT:	1:1 mixture of 40% (w/v) PEG 3350 and CaT
SOS:	1 M sorbitol, 0.3 x YPD, 10 mM $CaCl_2$
RD top agar:	1 M sorbitol
	2 % (w/v) dextrose
	1.34 % (w/v) Yeast Nitrogen Base (YNB)
	0.00004 % (w/v) biotin
	0.005 % (w/v) amino acids
	1 % (w/v) agar
	For growth of *his 4(-)* strains, 0.004% histidine was supplemented.
RDB plate:	1 M sorbitol
	2 % (w/v) dextrose
	1.34 % (w/v) YNB
	0.00004 % (w/v) biotin
	0.005 % (w/v) amino acids
	2 % (w/v) agar
	For growth of *his 4(-)* strains, 0.004% histidine was supplemented.

Transformation by electroporation

For the preparation of competent cells, a single colony of GS115 cells was picked and inoculated in 10 ml YPD medium and incubated overnight with shaking at 30°C. 200 µl of the overnight culture was inoculated to 500 ml of fresh YPD medium and allowed to grow overnight with shaking at 30°C until the OD600 of the culture reached 1.3-1.5. The cells were harvested by centrifugation at 1500xg for 5 minutes at 4°C and then washed three times with 500 ml of ice-old, sterile H_2O, followed with 250 ml of ice-cold, sterile H_2O and finally with 20 ml of ice-cold 1 M sorbitol. The cell pellet was resuspended in 1 ml of ice-cold 1 M sorbitol.

For electroporation, 3-16 µg of Sal I-linearized plasmid DNA was mixed with 80 µl of the cell suspension and then transferred to an ice-cold 0.1 cm electroporation cuvette. After incubation for 5 minutes on ice, the cells were pulsed with a Bio-Rad Genepulser under the following conditions: charging voltage 1.5 kv, capacitance 25 µF, resistance 200 Ω. Immediately thereafter, 1 ml of 1 M sorbitol was added to the cuvette and the cells were allowed to regenerate for 1 hour at room temperature. Then the cell suspension was aliquoted into two sterile Eppendorf tubes. 0.5 ml of

YPD medium or MM medium was added, respectively, to each aliquot and the cells were allowed to further regenerate for 3 hours at room temperature with shaking. 50-200 µl of the cell aliquots in YPD medium were spread on YPD-G418 plates (0.25 mg G418/ ml) in order to select G418 resistant transformants. Cell aliquots in MM medium were spread on MM-soraphen A plates (0.02 µg soraphen A/ ml) for direct selection of soraphen A resistant transformants.

YPD-G418 plates:

YPD agar was autoclaved and the temperature of the medium was cooled down to around 55°C. The approprate amount of G418 stock solution was added.

The agar solution was mixed well by swirling and poured into the petri dishes.

MM medium

YNB	1.34%
Biotin	0.00004%
Methanol	2%

The medium was supplemented with 2% (w/v) of agar for the preparation of MM plates.

4.2.1.9. Preparation of comminuted cuticles from adult colorado potato beetle

Beetles were frozen at -20°C and thereafter homogenized in 1% (w/v) potassium tetraborate on ice. The cuticle pieces were washed extensively in distilled water, stirred overnight in 1% (w/v) potassium tetraborate and dried at room temperature. The cuticle pieces were milled to a fine powder (0.5 mm) with a mortar and pestle. The powder was washed in 1% potassium tetraborate and rinsed in distilled water, allowed to settle and any floating material was removed. The cuticle powder was finally dried at room temperature and stored at -20 °C before use.

4.2.2. DNA techniques

4.2.2.1. Isolation of chromosomal DNA from *B. bassiana*

Spores of *B. bassiana* 252 were harvested from 10 days old YPG plates using sterile water and inoculated into YPG medium to a final concentration of 2 x 10^6 spores/ml. The culture was incubated at 25°C for 5 days on a rotary shaker at 150 rpm. Blastospores were filtered through three layers Miracloth and inoculated into 60 ml of YPG medium to the final concentration of 1x10^7 spores/ml medium. Nikkomycin

and ampicillin were supplemented to a final concentration of 2µg/ml and 60µg/ml, respectively. The culture was incubated for 12-13 hours at 25°C on a rotary shaker at 150 rpm allowing the blastospores start to germinate. The mycelium was harvested by centrifugation at 2500 rpm for 10 min, the pellet was washed once with 20 ml of sterile distilled water and resuspended in 30 ml of the SCE buffer plus 60 mg of Glucanex, 30 mg of Caylase C3 and 30 mg of Lysing Enzyme. The mixture was incubated at 37°C for 20 hours to ensure that >80% of the cells have been protoplasted and the cells were collected by centrifugation at 4000 rpm for 15 min at room temperature. The pellet was washed once with SCE buffer and resuspended in 15 ml lysis buffer containing 5 mg of proteinase K, mixed well and incubated at 55°C overnight until the solution cleared. The lysates was extracted with phenol/chloroform/isoamyl alcohol (25:24:1, v/v) and the DNA was precipitated from the aqueous phase by addition of 0.1 volumes of 3 M sodium acetate (pH 7.0) and 0.54 volumes of isopropanol. The DNA pellet was dissolved in TE containing 20 µg/ ml of DNAse free RNAse A.

SCE buffer:

Sorbitol	1 M
Sodium citrate, pH 7	0.1 M
EDTA, pH 8	50 mM

Lysis buffer:

Tris-Cl, pH 8	20 mM
EDTA, pH 8	25 mM
NaCl	0.25 M
SDS	1%

4.2.2.2. DNA fragmentation by partial cleavage with *Mbo* I

A number of reactions were set up where 10 µg of genomic DNA was mixed with 0.25 units of *Mbo* I and incubated at 37°C. The reactions were terminated after 5, 10, 20. 30, 45, 60 minutes by adding EDTA to 20 mM. A reaction incubated for 60 min without MboI was used as negative control. The partial digests were analyzed by electrophoresis on a 0.8% agarose gel to determine the optimum digestion time under which the size distribution of fragments will peak in the range between 9 and 23 kbp. The optimal time point was estimated to be between 45 and 60 min. Therefore, a digestion time of 50 minutes was chosen for phage library construction.

4.2.2.3. Construction of phage genomic library of *B. bassiana* 252

Library construction was performed following the instruction manual of the Lambda DASH® II /*Bam*H I vector kit (Stratagene). 0,4 µg of fragmented genomic DNA was dephosphorylated with 1 unit of shrimp alkaline phosphatase at 37°C for 1 hour in order to prevent ligation of the genomic fragments to each other while permitting ligation to the vector arms. Thereafter, the DNA was ligated to 1 µg of *Bam*H I predigested λ DASH II vector in a total volume of 5 µl and the ligation was incubated at 4°C overnight. 1 µl of DNA ligates (0,3 µg/µl) was mixed with 25 µl Gigapack® III gold packaging extract (Stratagene) and the recombinant phage DNA was packaged by incubation of the mixture at room temperature for 100 minutes. After the addition of 500µl of SM buffer, the phage library was ready for further analysis.

For titering the package reaction, a single colony of XL1-Blue MRA (P2) host cells was picked and inoculated in 5 ml LB medium supplemented with 10 mM $MgSO_4$ / 0.2% (w/v) maltose. The cells were allowed to grow at 37°C with agitation for 3 to 4 hours until the OD_{600} reached 0.7 and then were harvested by centrifugation. The cell pellet was diluted to an OD_{600} of 0.5 with 10 mM $MgSO_4$. Consecutive dilution of packaged phages were mixed with 200µl of the diluted host cells and the attachment of the phages to the host cells was achieved by incubation of the mixture at 37°C for 15 minutes. Then 3 ml of prewarmed LB top agar was added and the mixture was plated on LB agar plates. The plates were incubated at 37°C until the cleared plaques appeared. The titer of the phage library was assessed by counting the number of plaques on the plates.

LB Top agar:

LB medium plus 0.7% (w/v) agar

SM buffer

NaCl	0.58% (w/v)
$MgSO_4.7H_2O$	0.2% (w/v)
Tris-HCl (pH 7.5)	50 mM
Gelatin	0.01% (w/v)
add deionized H_2O to 1 liter	

4.2.2.4. Phage library screening

For amplification of the library, a single colony of XL-1 Blue MRA(P2) host cells was picked and inoculated in 20 ml LB supplemented with 0.2% maltose and 10 mM $MgSO_4$ and grown overnight at 37°C. 1 ml of the culture was inoculated in 50 ml of fresh, prewarmed medium and allowed to grow for 3-4 hours under vigorous agitation until the OD_{600} reached to around 0.7. The cells were harvested and resuspended to an OD_{600} of 0.5 in 10 mM $MgSO_4$. In order to amplify a phage library with 1.5 x 10^6 plaques, 20 aliquots were prepared. In each aliquot about $7x10^4$ pfu of the lambda phages were incubated with 600 µl of the diluted host cells for 15 minutes at 37°C and then seeded into 7 ml of LB top agar containing 0.2% maltose and 10 mM $MgSO_4$. The phage mixture was poured onto agar plates (145mm) and the plaques were allowed to grow overnight to a diameter of 0.5-1.0 mm. Thereafter 10 ml of SM buffer was added to the plates, the phages were allowed to diffuse into the buffer by incubation for overnight at 4°C and the phage suspensions were harvested from the plates and stored in aliquots at -80°C in 7% (v/v) DMSO.

For plaque blotting, Biodyne A nylon membranes (PALL) of appropriate size were placed on the surface of the prechilled agarose plates containing plaques, removed after 2 min and dried briefly by placing, plaques side up, on sterile filter paper. Thereafter the membrane was treated on a piece of filter paper soaked in denaturing solution for 2 min and subsequently transferred for 5 min, plaques side up, to filter paper soaked in neutralizing solution. The filters were rinsed for 30 seconds on filter paper soaked in washing solution and then dried briefly on Whatman 3MM paper. To fix the nucleic acids to the filters, the blots were UV crosslinked with a Stratalinker (Stratagene). Filters were prehybridised in hybridization buffer for 2 hours at 42°C in a rotary oven. Biotin-labelled DNA probes were generated by random priming using a Biotin High Prime enzyme system following the manufacturer's instructions. After denaturation the labelled probe was added to the hybridization buffer. Hybridization was carried out overnight at 42°C. The subsequent washing and detection steps were performed under "Southern blot analysis", section 5.2.2.7. Positive plaques were excised from the agarose and transferred to Eppendorf tubes containing 500 µl SM buffer supplemented with 20 µl chloroform and stored overnight at 4°C. The phage solution was titered as described above and 5000 pfu of phages were inoculated with 200 µl of host cells diluted in 10 mM $MgSO_4$ to an OD_{600} of 0.5. Phage and host cells were incubated for 15 min at 37°C in Eppendorf tubes. After the addition of 3

ml LB top agar, the phage solutions were poured onto LB agar plates and allowed to grow at 37°C until plaques formed in the top agarose. The second and tertiary screenings were performed as described above until pure phage was obtained.

20 x SSC

NaCl	3 M
Sodium citrate	0.3 M
pH 7	

Denaturing solution:

NaOH	0.5 M
NaCl	1.5 M

Neutralizing solution:

NaCl	1.5 M
Tris-HCl (pH8)	0.5 M

Washing solution:

Tris-HCl (pH7.5)	0.2 M
2xSSC	

4.2.2.5. Subcloning of *try1, try2, plb1* and *plb2* genes from the phage genomic library

Phage DNA was isolated using a promega lambda preps DNA purification kit, following the manufacturer's instructions. The isolated phage DNA was digested with *Bam*H I, *Eco*R I, *Sal* I, *Xba* I, *Xho* I, and *Not* I restriction endonucleases and submitted to electrophoresis on 0.8% agarose gels. Separated fragments were transferred to Biodyne A nylon membranes by vacuum blotting and probed with biotin-labelled probes specific for the respective genes. DNA fragments containing the genes of interest were excised from the gel, purified and cloned into a pUC18 vector and sequenced.

4.2.2.6. Isolation of genomic DNA from *P. pastoris*

20 ml cell suspension with an OD_{600} of 5-10 was harvested by centrifugation at 1500 x g for 10 min at RT. The cell pellet was washed once with distilled water and resuspended in 2 ml freshly prepared SCED buffer, the suspension was incubated at 37°C for 2 hours after the addition of 15 µl 10U/µl Lyticase. The cells were lysed by addition of 2 ml 1% SDS followed by incubation for 5 minutes on ice. The lysates

were neutralized by addition of 1.5 ml 5 M potassium acetate (pH 8.9), transferred to centrifugation tubes and extracted with one volume of phenol/chloroform twice. The DNA in the aqueous phase was precipitated in 100% ethanol and resuspend in 750 µl TE buffer. The DNA in TE buffer was purified further by extraction with phenol/chloroform (1:1) followed with chloroform/isoamyl alcohol (24:1) and by precipitation with 100% ethanol after addition of 0.1 volumes of 3 M sodium acetate. Finally, the DNA pellet was dissolved in 100 µl nuclease-free TE buffer.

SCED buffer

Sorbitol	1 M
Na.Citrate	0.01 M
EDTA	0.01 M
DTT	0.01 M
pH 7.5	

4.2.2.7. Southern hybridisation

5-10 µg of genomic DNA restriction fragments were separated by agarose gel electrophoresis. 20 ng DNA marker was electrophoresed in parallel to determine the size of the detected bands. The gel was depurinated by soaking in 0.25 M HCl solution for 15 minutes, denatured by soaking in 0.5 M NaOH/1.5 M NaCl for 2x15 minutes and neutralized by soaking in 1 M Tris-HCl, pH7.5/ 1.5M NaCl for 30 minutes. All soaking steps were performed at room temperature with gentle shaking. In a 10x SSC transfer buffer, the DNA fragments were transferred onto a Biodyne B nylon membrane using a vacuum Blotter (Appligene). After UV-cross linking, the DNA fragments bound on the membrane were prehybridized for 1 hour at 42°C in hybridization solution and hybridized at 42°C overnight with a denatured Biotin-labelled probe diluted in hybridization solution. Thereafter, the membrane was washed with washing solution I for 30 minutes followed with washing solution II for another 30 minutes at 42°C. The membrane was subjected to detection according to the manufacturer's recommendations and autoradiographed.

Hybridization solution

Formamide	50% (v/v)
Blocking solution (Biotin Lum. kit)	2% (v/v)
N-Lauroylsarkosin	0.1% (w/v)
SDS	0.02% (v/v)
5x SSC	

Washing solution I

2 x SSC	
SDS	0.1% (w/v)

Washing solution II

0.2 x SSC	
SDS	0.1% (w/v)

4.2.2.8. Construction of the transformation vector pTGT-PaccB1

A 681 bp DNA fragment corresponding to an internal region of the *pyr* gene (*pyrI*) was amplified with the primers pyrIF and pyrIR with *Nde* I overhangs. After digestion, the PCR product was cloned into a *Nde I* site of the pUC18 vector with an opposite orientation to *bla*. A 452 bp DNA fragment harbouring the terminator sequence of the serine protease gene (T*pr1*) was amplified with the primers pr1TF and pr1TR containing a *Hind* III restriction site and was cloned downstream of *pyrI,* at a *Hind* III site, to completely block the possible transcription from the *pyr* gene. This vector was termed pUCpyrI.

A 331 bp DNA fragment harbouring the terminator region of the glyceraldehyde-3-phosphate dehydrogenase gene (T*gpd*) was amplified with the primers gpdTF and gpdTR containing *Eco*R I and *Mun* I restriction sites, respectively. The digested PCR amplicon was inserted at an *Eco*R I site of pUCpyrI to give the vector pTT. Restriction sites for *Mun* I and *Eco*R I, which generate compatible DNA ends, were designed at the 3' and 5' end of the T*gpd* fragment respectively. After ligation, the *Eco*R I site at the 5' end of the T*gpd* fragment was still cleavable for the next cloning procedure.

The 717-bp full length *sgfp* gene was amplified from the plasmid pRS31 by PCR with the forward primer gfpF, containing the ATG codon, and the reverse primer gfpR which contains the stop codon of the *sgfp* gene. In the forward primer, two additional restriction sites, *Bcl* I and *Eco*R I, were introduced immediately preceding

the start codon of the *sgfp* gene. In the reverse primer, one additional stop codon (TAA) was introduced to completely block the translation, followed by another restriction site for *Mun* I. The *Eco*R I-*Mun* I-digested fragment was inserted in the vector pTT at the *Eco*R I site upstream to Tgpd, generating the 4867-bp vector pTGT.

Using primers containing *Xba* I and *Bcl* I sites in forward and reverse orientation, respectively, the promoter region of *accB1* was amplified in various lengths from *B. bassiana* 252 genomic DNA. The *Xba* I/*Bcl* I restricted PCR products were fused in-frame with the reporter gene, *sgfp* by insertion into the *Bcl* I and *Xba* I sites of the pTGT vector. The resulting set of transformation vectors were designated pTGT-P*accB1*. The PCR primers used for vector construction are listed below (restriction sites are underlined).

Pyr-IF: 5’-GGCAGCATATGACATGGTCTCGGGTTGGGA
Pyr-IR: 5’-GTGACCATATGTGCACAAAAGCATCGTCGT
pr1-TF: 5’-CCACCTAAGCTTGACTAGCAGAGTGT
pr1-TR: 5’-GACGCCCAAGCTTCGGCTACCGGAA
gpd-TF: 5’-GTAGGAATTCGACTCCTCAGCTAAGCCAAG
gpd-TR: 5’-CGCGCAATTGGGACCGATTGCACCCGCCGT
gpd-PF: 5’-TATTGACAGGTGAATACAGATGGATCCATTGGG
gpd-PR: 5’-GCGCGTCGACTGATCAATTGAAAGGGTGAGGTG
gfp-F: 5’-GCGGAATTCTGATCACAATGGTGAGCAAGGGCGAG
gfp-R: 5’-AGCCGTACCTGCTCGACATGTTCATTGTTAACGCG

4.2.2.9. primers used for construction of pAO815-ACC4

accA forward 5’-GACTAATACGAATTCACCATGAGTCTGAATTTCCTTG
reverse 5’-CAGAACTTTGAATTCTTACGCGTAACCGTAGCTC
accB forward 5’-AGAGTACGGGAATTCACCATGGATATTCGTAAGATT
reverse 5’-AGCATGTTCGAATTCTTACTCGATGACGACCAG
accC forward 5’-TCGAGTAACGAATTCACCATGCTGGATAAAATTGTT
reverse 5’-GACGCTTTAGAATTCTTATTTTTCCTGAAGACC
accD forward 5’-CAGACAGAACAATTGACCATGAGCTGGATTGAACG
reverse 5’-CCCTGCCCTCAATTGTTATCAGGCCTCAGGTTC

4.2.2.10. Recovery of DNA fragments from crystal violet agarose gel

In order to increase the cloning efficiency when using long PCR products, gel-purification was performed. The PCR products were visualized using crystal violet DNA stain which is less mutagenic than visualization under UV-light using ethidium bromide. 0.8% (w/v) agarose gels containing 1.6µg/ml of crystal violet were prepared. PCR products were mixed with crystal violet loading buffer and loaded onto the gels and the gels were runned at 80 volts. The PCR products are visible while the gel is running and were excised as soon as they were sufficiently resolved. Isolation of the PCR product from the crystal violet gel was done by purification on a spin column as recommended by the the TOPO® XL PCR cloning kit (Invitrogen) instruction manual.

6 x crystal violet loading buffer:

Glycerol	30%
EDTA	20 mM
Crystal violet DNA stain	100 µg/ml

4.2.2.11. DNA sequencing

DNA was sequenced by the internal ZMBH sequencing facility.

4.2.3. RNA techniques

4.2.3.1. Isolation of RNA from *B. bassiana*

Total RNA was isolated from *B. bassiana* using a RNeasy plant mini kit. 1 g of mycelium harvested from *B. bassiana* culture was grinded under liquid nitrogen to a fine powder using a prechilled mortar and pestle. The tissue powder together with liquid nitrogen was transferred to a 50 ml tube and the liquid nitrogen was allowed to evaporate. Immediately 2 ml of RLC buffer containing 1% (v/v) β-Mercaptoethanol was added to the tissue powder followed by vigorous vortexing. The lysate was aliquoted and loaded onto four QIAshredder spin columns and homogenized by centrifugation. The cleared lysate was transferred to a new tube and 0.5 volumes of 100% ethanol was added and mixed well by pipetting to creat conditions which promote selective binding of RNA to the RNeasy membrane. The

sample was then applied to the RNeasy spin column. Total RNA binds to the membrane, contaminants are washed away, and RNA is eluted in RNase-free water. The integrity and size distribution of total RNA isolates was investigated by formaldehyde agarose gel electrophoresis and ethidium bromide staining.

A DNA-free Kit (Ambion) was used to remove contaminating DNA from RNA preparations, and to subsequently remove the DNase and divalent cations from the RNA sample. Less than 50 µg of RNA was treated in a single DNA-free reaction. For each reaction it was added 0.1 volume of 10 x DNase I buffer, 3 µl of DNase I (2 U/µl) and 2 µl of 40U/µl RNase inhibitor to the RNA followed by gentle mixing and incubation at 37°C for 1 hour. Thereafter 0.2 volumes of the slurry of the DNase inactivation reagent was added to the sample, the sample was mixed and incubated for 2 minutes at room temperature. After centrifugation the tube for about 1 minute to pellet the DNase inactivation reagent together with DNase and divalent cations, the purified RNA solution was removed and stored at -80°C for RT-PCR experiments. A control RT-PCR reaction using intron-spanning primers and without reverse transcriptase was performed to monitor the purity of the RNA.

4.2.3.2. RT-PCR

The Access RT-PCR system (Promega), which offers reverse transcription and PCR amplication in a single reaction, was used to detect mRNA expression. For each 50 µl reaction, a reaction mix containing 0.2 volumes of 5 x AMV/ Tfl reaction buffer, 0.2 mM dNTP Mix, 1 µM specific forward and reverse primers, and 1 mM $MgSO_4$ was prepared in a thin-walled 0.2 ml PCR reaction tube (Biozyme) on ice. Thereafter 1 µl of AMV reverse transcriptase (5U/µl) and 1 µl of Tfl DNA polymerase (5U/µl) were added and the reactions were mixed by brief vortexing. The reaction was initiated by adding 1 µg of the RNA template. First strand cDNA synthesis was performed by incubation at 48°C for 45 minutes, thereafter the reaction was incubated at 94°C for 2 minutes to inactivate the AMV reverse transcriptase and to denature the RNA/cDNA/primer mixture. Second strand cDNA synthesis and the amplification followed directly through 40 thermal cycles (30 sec at 94°C, 1min at 50/55°C, and 1 min at 68°C). This was followed by a final extension for 7 minutes at 68°C. After electrophoresis on an agarose gel, the cDNA was stained with the ethidium bromide for 30 minutes and analysed by UV illumination with a fluorimager (Fuji). The primers used for RT-PCRs are listed below.

Gene	Forward primer	Reverse primer	Tm(°C)	bp
acc	5'-CATATGCCGAGAAGC ATAGCATTGC	5'-CTGGACTTCCAAATGTCT GGC	63/60	929
try1	5'-CGACGACAATACTATC TAGTCTTGTC	5'-CTGATCACGCACTTCTTT ATCAGCCC	62/65	498
try2	5'-CCTGTACTCGTACCAT ACATACAGC	5'-ATTCCGGGGTCAATTAAC TTCTGCCG	63/65	861
plb1	5'-ATTGGCTGGTCCGTCT TTTGCTGGAG	5'-CAGCTGAAATGACAACA TACGG	66/58	720
plb2	5'-TCCAGCCTTTTGCAAT AACACGGCCC	5'-GTCGCTGGCTGAGACGT TAAAG	66/62	781
pr1	5'-CGACCACGTTTGACTA CGACTCG	5'-CAGGTAGACAGAATGCC AGTGCC	64/64	576

4.2.3.3. Primer extension

Radioactive labelling of the primer

5'-end labelling of the oligonucleotide primer was carried out using T4 polynucleotide kinase (PNK) and γ^{32}P-ATP according to standard protocols (Sambrook *et al.,* 1989). 10 pmol oligonucleotide was mixed with 1µl 10 x PNK buffer, 30Ci γ^{32}P-ATP and 10 unit T4 PNK in a 10 µl reaction system. The labelling reaction was incubated for 1 hour at 37°C and stopped by adding 2µl 0.5 M EDTA followed by an additional incubation at 65°C for 20 minutes. The labelled primer was purified using the QIAquick oligonucleotide purification Kit (Qiagen).

Radioactive labelling of a 25bp DNA ladder

2µl 10-fold diluted 25bp DNA ladder (Biolabs) was mixed with 2µl 10xPNK buffer, 60µCi γ^{32}P-ATP and 20 unit T4 PNK in a 20 µl reaction system. The labelling reaction was incubated for 30 minutes at 37°C, stopped and purified as described above. 1µl of labelled DNA ladder was loaded as control for gel electrophoresis analysis of primer extension.

Primer extension

Approximately 10 µg of *B. bassiana* total RNA in 50 mM dNTP was mixed with 1.2 pM of γ^{32}P-labelled primer 5'-GAGAAGAGCAGACAATGTCGGTTTCGAGGTG. After incubation for 10 minutes at 70°C the primer was allowed to anneal by cooling down the reaction on ice. Subsequently an extension solution was added [12µl 5 x 1st strand buffer(Gibco), 3 µl of 10 mM dNTP, 6 µl 0,1M DTT, 1 µl 40U/µl RNase inhibitor, 2 µl Superscript Reverse transcriptase(Gibco, 25 U/µl)]. The extension reaction was allowed to proceed for 90 minutes at 43°C and was stopped with 20 µl 0.5 M EDTA. After 15 minutes incubation with DNase-free RNase, the reaction was purified using the minelute PCR purification kit and the synthesised cDNA was eluted in 9 µl of elution buffer. The cDNA samples were mixed with 4.5 µl loading buffer, denatured at 65°C for 5 minutes followed by cooling on ice and separation on a 8% polyacrylamide gel containing 7 M urea. After electrophoresis the gel was dried and submitted to autoradiography with a phosphoimager.

Loading buffer:

Bromphenol blue	0.05% (w/v)
Xylene cyanol FF	0.05% (w/v)
EDTA	20 mM

4.2.3.4. 5'RACE

First strand cDNA synthesis was primed using the gene-specific primer PE3. In a 120 µl reverse transcription reaction, 10 pmol PE3 and 60 mM dNTP were mixed with 40 µg total RNA and DEPC treated water was added to a final volume of 76 µl. The RNA mixture was denatured for 10 minutes at 65°C and quenched on ice. cDNA-synthesis reaction mix [24 µl 5 x 1st strand buffer (Gibco), 12 µl 0,1M DTT, 6 µl 40U/µl RNase inhibitor] was added and the primer was allowed to anneal the RNA template by incubating at 42°C for 2 minutes. Thereafter, the cDNA synthesis was initiated by adding 2 µl Superscript reverse transcriptase(Gibco, 25 U/µ) and incubated at 42°C for 90 minutes. The reverse transcriptase was inactivated by heating the reaction at 70°C for 15 minutes and the RNA was degraded by adding a RNase mix and incubating at 37°C for 20 minutes. cDNA strands were purified by extracting with phenol/chloroform/isopropanol and precipitating twice in ice-cold ethanol. After centrifugation, the cDNA pellet was resuspended in 10 ml of 5 mM Tris.

In order to add homopolymer tails to the cDNA fragments, 9 µl of cDNA was mixed with 6 µl of 5 x reaction buffer, 4.5 µl of 5mM $CoCl_2$, 7.5µl of 0.02 mM dCTP, 2 µl of destilled water, and 1 µl of 25U/µl terminal transferase in a 30 µl tailing reaction. The reaction will add a homonucleotide tail of around 20 dC at the 3' end of the cDNA after the reaction is incubated at 37°C for 10 minutes. The terminal transferase was inactivated by heating the reaction for 5 minutes at 70°C.

For the 5'RACE reaction, a nested-PCR procedure was performed using Ampli Taq polymerase (Perkin Elmer). The specific primer PE3 (5'-GCTATTGCCTCCAATAAAGTGAGG) was used in combination with a polyG20 oligonucleotide for the first round of PCR amplification (5 min at 94°C; 5 min at 45°C; 40 min at 70°C; 40 cycles of 40 sec at 94°C, 2 min at 50°C, and 2 min at 72°C; and a final extension for 20 min at 72°C). Thereafter, the gene-specific internal primer PE1 (5'-CCATTTGTGGTCTCATAAGCCATG) in combination with polyG20 were used for a second round of PCR amplification with 2µl of the first round PCR amplicons as the template (5 min at 94°C; 5 cycles of 40 sec at 94°C, 1 min at 50°C, and 1 min at 72°C; 30 cycles of 40 sec at 94°C, 1 min at 55°C, and 1 min at 72°C; and a final extension for 30 min at 72°C). The PCR products were purified using Qiaquick columns, subcloned into a TOPO-TA cloning vector and submitted for sequencing.

RNase mix:

Denatured salmon sperm DNA	100 µg/ml
DNAase-free RNase in TEN 100 buffer	20 µg/ml

TEN 100 buffer:

100 mM NaCl in TE buffer, pH 7.5

4.2.4. Protein methods

4.2.4.1. Protein preparation from *B. bassiana* mycelium

The mycelium from a 2 ml culture of *B. bassiana 252* was harvested by centrifugation at 10,000 x g for 2 minutes. The pellet was resuspended in 200 µl of breaking buffer and an equal volume of acid-washed glass beads (0.5mm) was added. The cell wall was disrupted by sonication for several cycles (7 x 20 seconds) and the sample was cooled on ice between treatment. After centrifugation for 10

minutes at 13,000 rpm, the clear supernatant containing proteins was collected for further analysis. All steps were be performed at 4°C

Breaking buffer

Sodium phosphate, pH 7.4	50 mM
PMSF	1 mM
EDTA	1 mM
Glycerol	5% (v/v)

4.2.4.2. Protein preparation from supernatant of *B. bassiana* culture

The supernatants of *B. bassiana* cultures could be loaded dierectly on SDS-PAGE gels and the proteins were analysed by Coomassie staining or Western blot. To increase the concentration of proteins in the supernatant, a trichloroacetic acid (TCA) protein precipitation was performed. A 50% TCA solution was added to the supernatant to a final concentration of 10% and the proteins were allowed to precipitate for 30 minutes on ice. The precipitated proteins were harvested by centrifugation at 13,000 rpm for 20 minutes at 4 °C. After washed once with 5 volumes of ice-cold acetone, the protein pellet was resuspended in protein buffer and used for further analysis.

Protein buffer:

Urea	6 M
CHAPs	2% (w/v)
β-Mercaptoethanol	5% (v/v)
Tris-HCl, pH 6.8	60 mM

4.2.4.3. Construction of pQE-PR1 and pQE-PLB2

A cDNA fragment corresponding to a region of high antigenicity of PR1, from amino acid residue 181 to 297, was amplified from *B. bassiana252* genomic DNA with primers containing a *Bgl* II (pr-BF) and a *Sal* I overhang (pr-SR). A cDNA fragment corresponding to a high antigenic region of PLB2, from amino acid residue 218 to 588, was amplified with primers containing a *Bgl* II (plb2-BF) and a *Pst* I overhang (plb2-PR). The resulting PCR products were digested, gel purified and cloned directionally in a pQE-40 expression vector (Qiagen) generating the expression vectors pQE-PR1 and pQE-PLB2, respectively. The constructs encoding

N-terminal Dehydrofolate reductase (DHFR) and hexahistidine tagged PR1 or PLB2 peptide fusions were sequenced to ensure the absence of point mutations and frame shifts. Both expression constructs were transformed into SG13009 competent *E. coli* cells for protein expression. The primers used for vector construction are listed below (restriction sites are underlined).

pr1-BF: 5‘-ATTGGCTCCAGATCTTACGGCGTAGCTAAGAAC
pr1-SR: 5‘-AACAACTCGTCGACAGTTGGAAAAGCTGGAGCG
plb2-BF: 5‘-TGGAGCTCCAGATCTAATGATGCAGACTTTG
plb2-PR: 5‘-ACGCGGCCGACTGCAGCGTTATCGTTGAC

4.2.4.4. Expression of fusion proteins

E. coli GS13009 cells carrying a pQE-expression plasmid were grown in LB medium containing 100 µg/ml ampicillin and 25 µg/ml kanamycin at 37 °C overnight. Fresh medium was inoculated with the overnight cultures at a ratio of 20:1 and incubated at 37°C with vigorous shaking until the OD_{600} reached 0.5 to 0.7. The fusion protein expression was induced by adding IPTG to a final concentration of 1 mM and maximum expression was obtained after 4-5 hours additional growth. After centrifugation, total bacterial protein extracts were obtained by boiling 10 µl of the bacterial pellet in 1 x protein loading buffer for 5 minutes. The sample were resolved on 12.5% SDS-PAGE gels and stained with R-250 Coomassie blue (Sigma).

Stacking gel:

Acrylamide: bis-acrylamide (29:1)	4.5% (v/v)
Tris-HCl, pH 6.8	125 mM
SDS	0.1% (w/v)
TEMED	0.4% (v/v)
APS	0.4% (w/v)

Separating gel:

Acrylamide: bis-acrylamide (29:1)	12.5% (v/v)
Tris-HCl, pH 8.8	375 mM
SDS	0.1 (w/v)
TEMED	0.25% (v/v)
APS	0.5% (w/v)

10 x SDS-Tris-Glycin buffer:

Tris-HCl, pH 8.6	250 mM
glycine	1.92 M
SDS	1% (w/v)

5xSample buffer

Tris-HCl,pH 6.8	0.5 M
Glycerol	10% (v/v)
SDS	10% (w/v)
ß-mercaptoethanol	5% (v/v)
Bromophenol blue	0.05% (w/v)

4.2.4.5. Large scale protein preparation and affinity purification from *E. coli*

1 litre of the induced bacterial cultures was centrifuged and the pellet was resuspended in 4 ml lysis buffer (300 mM NaCl, 50 mM NaH_2PO_4, 10 mM imidazole, pH 8.0) and subjected to sonication to lysis the cells. The supernatants containing the soluble protein fraction were recovered after centrifugation at 20,000 rpm for 20 minutes in a refrigerated beckman ultracentrifuge. After the addition of 1 ml of Ni-NTA agarose slurry (Qiagen) the suspension was mixed for 2 hours at 4 °C. Bound proteins were washed twice with washing buffer and eluted in the same buffer but containing 250 mM imidazole. Eluted fractions were separated on 10 or 12.5% SDS-polyacrylamide gels and visualised by Coomassie staining.

Lysis buffer:

Urea	8 M
NaH_2PO_4	0.1 M
Tris-HCl, pH 8	0.01 M

Washing buffer:

Urea	8 M
NaH_2PO_4	0.1 M
Tris-HCl, pH 6.3	0.01 M

Elution buffer:

Urea	8 M
NaH_2PO_4	0.1 M
Tris-HCl, pH 6.3	0.01 M
Imidazole	0.2-0.3 M

4.2.4.6. Immunoblot analysis

After electrophoresis, the proteins on the SDS-polyacrylamide gel were transferred by electroblotting to a Hybond PVDF membrane (Amersham Pharmacia) in the presence of transfer buffer. The membrane was blocked with 5% dry milk in TBST at room trmperature for one hour and incubated with the primary antiserum (PR1 1:3000, PLB2 1:1000, DHFR 1:4000) diluted in TBST solution. After washing, bound antibodies were detected by incubation with a secondary antibody conugated to horseradish peroxidase (anti-rat IgG 1:20,000). The immune complexes that formed were detected by enhanced chemiluminescence (ECL) according to the manufacturer's instructions (Amersham Pharmacia Biotech)

Transfer Buffer

Tris-HCl	48 mM
Glycine	39 mM
Methanol	20% (v/v)
SDS	0,0375% (w/v)

TBST Buffer

Tris-HCl, pH 8	10 mM
NaCl	150 mM
Tween-20	0.05 (v/v)

4.2.5. Raising of antisera

Preparation of PR1 and PLB2 antigens

Fusion-protein expression and purification under denaturing conditions was done as described above. The purified fusion protein fraction was separated by SDS-PAGE. After electrophoresis, the gel was washed briefly in water and stained for 10 minutes with 0.05% Coomassie blue G-250 prepared in water. The gel was washed repeatedly in water, and the stained protein band corresponding to the expressed fusion protein was excised with a scalpel. The gel slices were lyophilized, grounded into a fine powder, and resuspended in Freund's complete adjuvant for immunization.

Generation of antisera

Rabbit anti-Pr1 and rabbit anti-PlbA polyclonal antisera were produced commercially (Peptide Specialty Laboratories GmbH). Two rabbits were immunized for every antisera and a standard protocol was followed for antibody production. Briefly, on day 0 a preimmune bleeding was performed. On the same day, 50 µg of purified antigen was injected intramuscularly. On day 14, 28, and 56, the antigen was injected again under the same conditions. A test bleeding was performed on day 38 post-immunization. A large bleeding and a final bleeding was performed on day 66 and 80, respectively, post-immunization. Antisera were stored frozen (-80 °C) in aliquots.

4.2.6. Soft ware and computational analysis

Platform	Software	Task
Apple Macintosh	DNA Strider	Sequence editor
	Laser gene	Sequence editor
		Mapping of restriction enzyme sites
		Multiple alignments of sequences
		Protein analysis
	Adobe Photoshop	Image processing
PC	Viewfinder Lite	Image processing
WWW	BLAST	Protein and nucleotide similarity searches
		Protein second structure analysis

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VI. Summary

The entomopathogenic fungus, *B. bassiana,* is attracting increased attention as potential biological control agent against insect pests. Understanding mechanisms of fungal pathogenesis in insects will provide a rational basis for strain selection and improvement. The research of this thesis focused on a *B. bassiana* strain, ARSEF 252, that is highly pathogenic for the Colorado potato beetle, *Leptinotarsa decemlineata*. Because of both the strong evidence supporting the role of cuticle-degrading proteases (PR1 and PR2) as well as phospholipase B (PLB) in fungal pathogens and their correlations to virulence observed in either *M. anisopliae* or *C. albicans*, the genes encoding PR2 and PLB were cloned from *B. bassiana 252* (the gene encoding PR1 has been cloned in our lab).

Two PLB-encoding genes (*plb1* and *plb2*, 57% identity) and PR2-encoding genes (*try1* and *try2*, 22.4% identity) are detected in *B. bassiana 252.* The predicted peptide encoded by *try1* is homologous to the trypsin-like proteases from fungi, whereas the putative TRY2 peptide is homologous to the trypsin-like proteases from insects. The structure similarity of TRY2 protease to insect enzymes might allow the fungal cells to evade host "non-self" recognition and thus might represents one important virulence determinant.

To study the significance of cuticle-degrading enzymes and their formation in the course of infectious process, the antisera against PR1 and PLB2 were raised. PR1 is a serine protease that degrade rapidly cuticular proteins. Production of PR1 is transcriptionally modulated by carbon catabolite and nitrogen metabolite repression. Further induction is obtained in poor media by the addition of insect cuticles. High expression of the *pr1* gene and the secretion of the enzyme by *B. bassiana 252* grown on the insect cuticle-induction media was proved by RT-PCR and western blot analysis, respectively. Whereas, PR1 was not produced in rich media containing carbon/ nitrogen sources. The formation of PLB2 was not influenced by carbon or nitrogen sources. In poor media containing insect cuticles, the synthesis of PLB2 was prevalent. These results are consistent with the idea that the infectious process that involves enzyme production and penetration of host cuticle occurs only when a nutritional relationship with the host is necessary.

The detailed analysis of the role of putative pathogenic factors depends on the transformation-mediated site-specific disruption of the specific genes. Because *B.*

bassiana252 is naturally resistant to many selection antibiotics, a new selectable marker, sorR, was developed for transformation. It is based on the sensitivity of *B. bassiana252* to fungicide soraphen A. Soraphen A is a macrocyclic polyketide, which is produced by the myxobacterium *Sorangium cellulosum*. Soraphen A specifically inhibits the eukaryotic Acetyl-CoA Carboxylase (ACC) but not prokaryotic ACC. The sorR marker is constructed based on the expression of *E. coli* ACC polypeptides in fungi which confer soraphen A resistance.

In an alternative approach, the *acc* gene from the *B. bassiana252* was cloned. The aim was to construct a mutated form of the *acc* gene that is resistant to soraphen A. This gene may be used as selection marker and transformation vectors were constructed. However, the experiments could not be completed since the transformation procedure for *B. bassiana252* was not yet available. Therefore, *P. pastoris* was chosen for marker development. The expression of *sorR* successfully confers the recipient strain resistance to soraphen A. The *sorR* marker consists of four *E. coli* ACC subunits. To facilitate handling and simplify the transformation method, a stepwise transformation procedure for the *sorR* selectable marker was developed. A *P. pastoris* strain that contains three *E. coli* ACC subunits was constructed as a recipient. This strain is sensitive to soraphen A. The integration of the fourth ACC subunit restored soraphen A resistance. Thus, soraphen A resistance is dominant. For high expression of *E. coli* ACC subunits in fungi, each of the four cistrons was fused to an efficient promoter. The prerequisites for fungal transformation are: (i) cells should be accessible to transformation with plasmid or linearized DNA; (ii) availability of a promoter which can drive the transcription of the *sorR* gene in the fungal cell; (iii) the fungal species should be sensitive to soraphen A. This novel marker system was submitted to be considered a patent.

Cloning, virulence evaluation and modification of putative virulent genes have more thoroughly pursued only for *M. anisopliae*. The molecular biologic investigation of the pathogenicity of *B. bassiana* is still at an early stage. The data presented in this thesis may contribute to improve our understanding of the molecular mechanisms that control pathogenicity of *B. bassiana.*

VII. Appendixes

Appendix1. Papers in preparation and patent application arising from this work

Hong Wan, Andreas Leclerque, Hans Ulrich Schairer. Development of a new dominant selectable marker, sorR, for fungal transformation. (Biotechniques, In preparation)

Andreas Leclerque, Hong Wan, Anette Abschütz, Esther Duperchy, Galina V. Mitina, Gisbert Zimmermann, Hans Ulrich Schairer. Agrobacterium-mediated insertional mutagenesis of the entomopathogenic deuteromycete *Beauveria bassiana.* (Current Genetics, submitted)

The results from the thesis work "sorR, a new dominant selection marker for fungal transformation" was submitted to be considered a patent to:

Technology-Licens-Office (TLB)
Of the Universities of Baden-Württemberg state
76137 Kalsruhe
Germany

Name of the invention: Soraphen-resistant marker
Inventors: Hong Wan and Andreas Leclerque

Appendix 2. Abbreviations

A. asgypti	*Aedes aegypti*
A. darlingi	*Anopheles darlingi*
A. fumigatus	*Aspergillus fumigatus*
A. gambiae	*Anopheles gambiae*
A. nidulans	*Aspergillus nidulans*
A. niger	*Aspergillus niger*
B. bassiana	*Beauveria bassiana*
B. moritai	*Bacillus moritai*
B. popilliae	*Bacillus popilliae*
B. thuringiensis	*Bacillus thuringiensis*
C. albicans	*Candida albicans*
C. neoformans	*Cryptococcus neoformans*
D. melanogaster	*Drosophila melanogaster*
E. coli	*Escherichia coli*
F. oxysporum	*Fusarium oxysporum*
M. anisopliae	*Metarhizium anisopliae*
N. crassa	*Neurospora crassa*
N. rileyi	*Nomuraea rileyi*
P. chrysogenum	*Penicillium chrysogenum*
P. nodorum	*Phaeosphaeria nodorum*
P. pastoris	*Pichia pastoris*
S. calcitrans	*Stomoxys calcitrans*
S. cerevisiae	*Sacchromyces cerevisiae*
S. hygroscopicus	*Streptomyces hygroscopicus*
S. hindustanus	*Streptoalloteichus hindustanus*
S. pombe	*Schizosaccharomyces pombe*
T. harzianum	*Trichoderma harzianum*
U. maydis	*Ustilago maydis*

5’-RACE	rapid amplification of cDNA 5’-ends
ACC	acetyl CoA carboxylase
amp^r	ampicillin resistance
ATP	adenosine 5’-triphosphate
bp	base pair
BSA	bovine serum albumin
cpm	counts per minute
Da	Dalton
dATP	deoxyadenosine 5’-phosphate
DEPC	diethyl pyrocarbonate
DNA	deoxyribonucleic acid
DNAase	deoxyribonuclease
dNTP	deoxynucleoside triphosphate
DTT	dithiothreitol
DTX	destruxins
EDTA	ethylenediaminetetraacetic acid
g	gram/gravity
IgG	immunoglobulin G
kb	kilobasepair
kDa	kilodalton
l	liter
LB	Luria-Bertani bacterial medium
M	molar
m	milli
nmol	nanomol
µ	micro
mRNA	messenger ribonucleic acid
O.D.	optical density
ORF	open reading frame
PAGE	poly-acrylamide gel electrophoresis
PCR	polymerase chain reaction
PLA	phospholipase A

PLB	phospholipase B
PLC	phospholipase C
PLD	phospholipase D
pfu	plaque forming unit
RNA	ribonucleic acid
RNAase	ribonuclease
rpm	revolutions per minute
RT	room temperature
RT-PCR	reverse transcriptase polymerase chain reaction
Tris	tris-(hydroxymethyl)-aminomethane
Tyr	trypsin
U	unit
UV	ultraviolet
v/v	volume to volume
w/v	weight to volume
℃	degree Celsius

Appendix 3. Sequences

1. Sequence of *try* 1 gene

ATGGTAGGCAAGGCTTCTCATCTATTCGTCACAGCACTCTGTGCTGCTTTGGCAGCAGCC 60
GGAACTATTGACAGGAGAATCATTGGCGCAGAGAACGCCACGGAAAACGAATTTCCATCC 120
ATTGTCAGCGTCCAGAATGCGGGTTCTCACATTTGCGGAGGCACCTTGCTGGACAACACC 180
ACGGTTCTCACTGCTGCTCACTGCGCCTACTCGCTCAAGCCTCGAGGCACTCGAAGGTAC 240
TCAGTAAGAGCAGGAACTCTG*gtaagcctgctctgcattcctcatttgcctattgaacta* 300
ctccttgtctaccagatttccagcatctgaatctcgcaacggaccccagcatttggaagc 360
*agcacaaggactaataacatacag*GACCCGCAGACTGGCGGCGTAGTTGCAGCAGTAGCA 420
TCGATCACAAAACACCCGGCTTACACTACGCGCAGAAGTAAAGCAGAGCGCTATCCTGCC 480
AATGATGTTGCTATCATCAAACTATTGACTCCGATCGGAGAGAGCGAAAAAATTCGCTTT 540
GCGACGCTACCGCCGGATGGTTGGACTGCTACAGAAAACTCTACTGCGATTGCCGCGGGC 600
TG*gtaagtgcctctgccattgagcgcagcatcgccgggcaagaaccactcactaactgat* 660
*tcggctcag*GGGCATACAGAACTTCAACGAGAACGTCGCTACCACCACGCTCGCCAAAGT 720
GCTGGTCCCGATTCGCCCCAGTCAAGACTGCGCTGCGTTTTCCGTAACCGAGGACACAAT 780
ATGCGCCGGTGCAATAGGCAAGGATACAGCCAAAGGAGACAGTGGCGGCCCTCTTATCGA 840
CCCGAACACGGGACATTTGATTGGCGTCACAACAACTGGCCCTGCGAATCAAGTAAACAA 900
AGGAGTGTATGCG*aagacctcgagctacatccccttcatcaacgaaaatctcggctggac* 960
ggggcggtctacccagtcgcccaagcctgtggatcaacaagtcgagaattactgcggatg 1020
ctccggatacgacaaggctgcctgtttagaggccgccgctcgctgcaaagcggaagtgaa 1080
*gccagacgcagttgtgcccctacacctcgaatgtattgatagg*CTCCAAGCCTGCTCCGG 1140
TTCAAAATTGAGACTTGCATGGGATTCGCAAAGGAGTGCCAATATGATCACGAGTTCCCT 1200
TTTGGGGACCTAG 1213

2. Sequence of *try* 2 gene

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ATGGTCTCTCTGCGACCCCACCGACTTGTCTTTTTGGCATGTCTCATGGCTTTCCTGACT 60
GCTCTGCCAGTAACCGCGATTGCTGGTGGCCAAGACGCTAAGTTGGCTTTCTTTCCATAT 120
ACCGTTAGCCAGCGAACCCTTGGTCGCCATAACTGCGGAGGAATCATCATTGATCACTCT 180
TCGATTCTGACCGCCGCCCACTGCATTGTCGGTCTAAGCGCAGACAAGCTTAGTATCGTC 240
GCTGGTGCGGTAAAACTTGACTCTGGAGGAACTACGTACCAGGTGTCGGCGGTTCATCCG 300
CATCCCGGCTATAATTCCTCGACGTTGACTAACGACATTGCCGTTCTGAAACTTGCAAAA 360
CCCCTATCATATAACCCCTATATCCGACCCATTGCTCTTGCAACACAAGATCCGCCTGCT 420
GGGTCGGACACTCTCGTCTCTGGGTGGGGAGTCAGTCGCAGCTCCGGGCCAATATCCAAC 480
ACTCTTCAATGGATCTCTCTACGGAGCTTGGATCACAAAGTTTGCCAGGAACAAATGCGT 540
GTTACTGTTGGTCAGATTTGTACTACGAGCGCAAACAAGGGGGGATCGTGTCGTGGAGAT 600
TCTGGTGGGCCACTTACTGACGAGCGCATCACGTTGGTGGGAGTGAATTCACGGGGTAGG 660
GGCTGTGGAGATGGATACCCTGATGTTTACGCCAGTGTAGCTTACCATCGTCATTGGATA 720
GGGAGCTATATGGGTTAG 738
```

3. Sequence of *plb* 1 gene

ATGCGCCAACCACAATTCTTGGTGGCAGCTACGGCTCTGGCATTGGCTGGTCCGTCTTTT 60

GCTGGAGTTGCCGACGCTAACGCTCTAG*gtacgatgactttgatcccatataaatattct* 120

*cacatttgctaatacaacgccag*CTCTTCGAACTGCAGATCTGACTCCAGAGGATGTTGA 180

ATTCTTGGTTCGCCGAGCTACAGCGCAAGCGCCAGACGGATATGCGCCTTCTGAAGTTAC 240

TTGTCCCGGCACGAGGCCGTTCATTCGGGATAGCTCCAGTTCGGTCCTGAGCCCAGAAGA 300

AAAGGCATGGCTTCCTATTCGCCGCCAAGAAACAATCACACACATCAAGGATTTCCTCAA 360

GCGTGCAGCCATCCCCAACTTCGACAGCGCCAAGTATTTGGATAATGTCGGCTCCAACTC 420

AACGGCCCTGCCCAACATCGGCATTGCCGTCTCTGGCGGCGGCTACCGTGCAATGCTTAA 480

TGGCGCTGGTGCAATCAAGGCTTTTGACAGTCGTTCAACCGGCAGTACGGATAAAGGCAA 540

CCTCGGCGGTTTGCTGCAAAGTGCCACCTACCTTTCCGGCCTGTCCGGAGGCGGCTGGCT 600

TGTAGGGAGTATTTTTACCAACAACTTTACCACTGTCCAGGATGCCGTCGCCTCCAAAGA 660

CATCTGGCAGTTTGGTGAATCAATTCTTGAGGG*ttagtctactcaagtaaccatgcgaaa* 720

*aaatcagctatatgctaacgcaggattggtcaaagg*TCCCGAGAATATTGGCATAGTAGA 780

CTACTACTCTACCATCATCGACGAACTGGAAAAGAAGCGTGACGCAGGCTTCAATCGTTC 840

CATTACCGACATTTGGGGCCGTATGTTGTCATTTCAGCTGGTGAAAGCAAAGCATGGCGG 900

TCCCCGTTATACTTTCTCGTCCATCGCCAATGATACCGAGTTTGCTGCGGGAAGGACGCC 960

ACTCCCTATACTGGTTGCCGACAGCAGAGCCCCGGGCGAAAAGAACACCACGATTGAGTC 1020

CGTCCTGTTCGAATTCAACCCCTGGGAGCTCGGATCCACGGATCCTGGCATGACTGGCTT 1080

CGCGCCGCTCAAGTATACCGGCTCCAAGTTTGACGGTGGAAGGCTATCGAGTGATGCCAA 1140

ATGCATCAATGGCTTTGACAATGTCGGTTACGTTATGGGAACATCTAGCAGTCTGTTCAA 1200

CCAAATTATTCTTCGCATGAAGAGCGATCCCGCCAAGTATCAGCCCAAAAATATCCCCAA 1260

GACTGTCGTGGCTTTCATCACCGATTTTCTTACCACATTGGGCGACCGCAGCGACGACGT 1320

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CGCTGACTGGACTCCAAACCCCTTCAAGAACTGGAACTCGGGCAAGAATTATGTTGCCAG 1380

TTCCGATAGGCTGACTCTGGTCGACGGAGGAGAGGACGGACAGAACGTTCCCTTCCACCC 1440

TCACACTGTCCACGAGCGAAAAGTCGACGTTGTCTTTGCCGTGGACTCGAGCGCCGACAC 1500

CAGCAATTGGCCCGACGGCGCTTCTGCGCTTGCAACATATCAGCGAACCATGAGTAATTC 1560

GTCGGCTGGCACGTCTTTCCCTGTCGTCCCCGATAAGAACACATTTATAAACCTTGGATT 1620

GAATACGCACCCAACTTTCTTTGGTTGTGATACCAGCAAATCGAGCGGCTCAGTACCCCC 1680

GCTGGTCGTTTACCTGCCCAACTATCCATACGTCTTCCACTCGAACCTATCCACCTTCAC 1740

GATGAGCATCAATGATACCGAAAGAGACGCCATCATATCAAACGGCTGGGCCGTTGTTAC 1800

TCAAATGAACAGCACGCGCGACACCAACTGGCCGACTTGCGTCAGCTGCGCCATGTTGTC 1860

ACGCAGCTTCGATCGCACCAAGACTGAGGTTCCGGCTGCCTGCAAGGATTGTTTTACCTC 1920

ATACTGTTGGAATGGCACTGTCGACAGCAGCCAACCTGCTACGGCTTACGCACCCAAGAT 1980

GTGGAGTAACACTACAATCGATGTTGCTTCCAAGAAGGACAGTGCTGGCGCTCGAGCCAC 2040

GAGTGGCTCTGCCACGTTGATTGTCTTGGCCATCGCCTCGGCTATGTTGCTGCTCTAA   2098
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4. Sequence of *plb* 2 gene

ATGAAGCACCTGGCGTTCCTTGTTGCTGCCGCGTCAGCTTGCGTATCCCAAGCACACGCC 60

GACTCTCCCCATATCG*gtacgtccattcaactcgacaatctcggcccgcgcaacttcctt* 120

*aaccccagccttgacaatgtttcccacgacaagagctaacacatgaaactcag*CTCGAGT 180

AGAGTACGCAGAGCCGCTTGACCTGCCAGAAGCCTCCGCCCAAGTCGACACACAAGATGC 240

TGCAGTCATAGTCGACCGCGCGATAGACGCTGCACCTTCTGGATACATTCCATCCTCGTC 300

AGCATGCCCCGATCCACCTCCCACGATCCGCTCAGGTAGCACCATCGGCCCGGACGAAAA 360

GACATGGCTCCCCAAGCGTCGCAAAGAGACGATACCTTATATGCGCCGTCTTATCAAGCG 420

CCTGGCCATTACCGATTTCGACAGCGAAAACTACTTTACCAATGTCACACACAATTCCAC 480

GAAGCTGCCCAACATTGCCATCGCCATATCTGGAGGCGGCTACCGTGCCATGGTTGGTGG 540

CGCAGGCGCAATTGCCTCTTGGGATGCTCGCTCCGCTGGCAGCGAGGAGAAGGGCAACCT 600

CGGTGGCCTGCTGCAAAGCGCAACCTATATATCCGGACTTTCGGGCGGCGACTGGCTCGT 660

CGGCAGCTTATATGTTAATAATTTCACCTCGGTTCAAAGCGCTGTGGACGCTCCTCTCAT 720

CTGGCAGTTGGAAAACAGCATCTTCAAA*Ggcatgctctccatggccaactaccaagtgct* 780

*ctcaattcgatactaacttgccactcaatag*GTCCCGACCAGTATAGTGTCCGAGGATAC 840

TACACTGACATCTTCAACCAAGTAGAGGCCAAGTCCAACGCCGGCTTTAACGTCTCAGCC 900

AGCGACTACTGGGGTCGCATGCTGGCATATCAGATGGTCAACGCCAGCAATGGTGGGCCA 960

GGATATACTTGGAGCTCCATCGCCAATGATGCAGACTTTGCAGCCGGCAAGACGCCCATG 1020

CCATTTCTGCTGGCAAACGGCCGTAGCCTTACAAAGACTACCATTGCTTCCACCAACTCG 1080

ACAGTTTACGAGTTCACGCCCTGGGAGCTCGGCTCTTCAGACCCGACCCTGTCTGGATGG 1140

GTGCCGCTCCGGTACGTGGGCACAGTCTTCAAAAATGGCCAAGTGGCGGACCAGGATAAA 1200

TGCGTCACGGGCTTCGACAATGCCGGTTTCGTCATGGGCACCACGTCGTCTATTTACACA 1260

CAATCCATCTCGTATCTCAAAGACAATAACAAGAAATACGTCCCCGGCGACGTCCCTGAT 1320

TTCGCCATCAAGACGGCCGCGAAGCTCATTTCCGCGCTGAGTGACTCCAACTCGTACGAT 1380

ATTGCCGAATGGTCACCGAACCCTTTTCGCGGCTTCAACACGGCCACCAACCGCAACGCC 1440

AACAACTCGCGCCTCAACCTCGTCGACGGCGGTGAAGACGCCCAAAATATCCCCTTTCAC 1500

CCGCACTTGCTCCCCGACCGCGATGTAGACGTCATCTTCTCCTACGACTCGTCTTCGGAC 1560

ACGGAGTATGGCTGGCCCGACGGCTCCGCCCTCGTTGCGACCTACGAACGCTCGCAGCAG 1620

TCGGCATTCAAGGATACGCCCTTTCCGTCGGTCCCGGACAAGAACACCATGCGCAACCTG 1680

GGCTTTAACACACGGCCGACTTTTTTCGGTTGCAACGCCACCAACTTTACCGCGCACGGC 1740

AGCGACGTTGCGCCTCTGGTCGTCTGGCTGCCTAACCACCCCTACGTCTACGACGGCAAT 1800

TTGACCACATTTACCTGGACCGTCAACGACGGCGAGCGCGCCGCCATCATCGACAATGGC 1860

TGGGCCGTCGCCACCCAGCTCAATGCCACCCGCGATACCGAGTGGCCCGCCTGCGTCGCG 1920

TGCGCCATCCTTGCCCGCAGCTTCTACCGCGCCAATGCCACCGTCCCCGTGCAGTGCACC 1980

AAGTGCTACGACCGCTACTGCTGGAAGGGAAACACGAATACCACCACCCCCAAGGGGCCC 2040

TACGACCCCACTTACTATGGCAAACCCATCATTGTCAACGATAACGCTGCTGTCGGCCGC 2100

GTGCCAGTCGCCACGTCCCAGCTGCTCACGGCGATGCTGTTTGTGACAATGATGCTTGTG 2160

CTATGA 2166

Hereby I certify that this thesis work was done independently and that I only used the stated resources.

Heidelberg, in January 2003

Hong Wan