BACILLUS BASED BIOCONTROL ON BRASSICA

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Abstract

Many bacterial strains have been shown to mediate protection to biotic stress and promote growth of plants. Different bacteria can mediate protection in different ways e.g. by inhibition, competition or increasing plant resistance. Examples of bacteria that mediate protection to plants include different *Pseudomonas, Serratia* and *Bacillus* strains. Bacillus strains have one major advantage toward other biocontrol strains and that is the ability to form spores that are resilient against chemicals and mechanical damage. I have studied the effect of four closely related Bacillus strains on plants in two different projects, one concerned with oilseed rape (*Brassica napus*) and the other using *Arabidopsis thaliana* to allow mechanistic studies of the interaction. The bacterial strains are all classified as *Bacillus amyloliquefaciens*. These bacterial strains have been tested for phenological effects on plants and for plant protection towards pathogens like *Alternaria brassicae, Botrytis cinerea, Leptosphaeria maculans*, and *Verticillium longisporum*. Production of antifungal compounds by the strains and the effects on the different pathogens were investigated.

Two potential candidates for biocontrol were identified. Both Bacillus strains were found to provide significant protection of oilseed rape against the four pathogens. The effects of Bacillus treatment on the *B. napus* transcriptome were studied using the cDNA-AFLP technique. Bacillus priming had strong systemic effects on leaf transcripts but small effects on roots. This far 65 differentially expressed plant genes have been identified due to Bacillus treatment, of which many seem related to metabolism.

The effect of Bacillus seed treatment has also been studied on Arabidopsis. Significant protection was achieved also here using the same two strains toward *Alternaria* and *Leptosphaeria* as well as *Pseudomonas syringae* as pathogens. Arabidopsis signalling mutant studies showed that functional jasmonic acid (JA) and ethylene (Et) signalling as well as *Npr1* were needed for Bacillus biocontrol. Expression levels of marker genes depending on these signalling pathways showed no increase upon Bacillus treatment, while an increase of the JA dependent marker occurred after Bacillus treated plants were infected by *P. syringae*. Altogether, Bacillus primed biocontrol seems to be based on induced systemic resistance (ISR).

Keywords: Bacillus, Brassica, Biocontrol, Arabidopsis

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Contents

List of Publications

This thesis is based on the work contained in the following papers, referred to by Roman numerals in the text:

I Danielsson J, Reva O and Meijer J (2007) Protection of oilseed rape (*Brassica napus*) toward fungal pathogens by strains of plant-associated *Bacillus amyloliquefaciens*. Microbial Ecolology 54, 134-140.

II Sarosh BR, Danielsson J and Meijer J (Submitted Manuscript) Transcript profiling of oilseed rape (*Brassica napus*) primed for biocontrol to differentiate genes involved in microbial interactions with beneficial *Bacillus amyloliquefaciens* from pathogenic *Botrytis cinerea*.

III Danielsson J, Reva O and Meijer J (Submitted manuscript) Protective ability of *Bacillus amyloliquefaciens* strains to *Arabidopsis thaliana* pathogens.

IV Danielsson J and Meijer J (Submitted manuscript) Specificity and mechanism of plant protection to pathogens by *Bacillus amyloliquefaciens* UCMB-5113 on different *Arabidopsis thaliana* genotypes.

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Abbreviations

AFLP	Amplified Fragment Length Polymorphism
cDNA	Complementary DNA
Et	Ethylene
HR	Hypersensitive Response
ISR	Induced Systemic Resistance
JA	Jasmonic Acid
SA	Salicylic Acid
SAR	Systemic Acquired Resistance
PGPR	Plant Growth Promoting Rhizobacteria
UCM	Ukranian Collection of Microorganisms (strain code)

1 Introduction

1.1 General Introduction

Plants exist in a changing environment with many challenges to handle. Abiotic stresses as drought and frost as well as biotic stresses like insect infestations and pathogen mediated diseases all need to be overcome. To accomplish this plants have different options. Some of them are defences that are activated upon need (inducible) and some are always present (constitutive). Some are accomplished with the help of other organisms.

Each year more than ten percent of the total crop yield is lost due to disease (Strange and Scott, 2005). Many different approaches to decrease this loss are continuously being developed to be one step ahead of pathogen evolution. Development of new agricultural practises, breeding of resistant cultivars and genetic engineering are examples of important measures to decrease yield loss. Chemical pesticides and fungicides are important tools to maximize yield in modern agriculture. Every year pesticides and fungicides corresponding to 768,000 tonnes of active ingredient are used world-wide, which of course leads to an additional strain on the environment. In the US 80,000 tonnes are used yearly to a total value of over four and a half billion dollars (U.S. Environmental Protection Agency, 2008). Biocontrol, the use of organisms to combat disease and pests, has the potential to become a complement or alternative to more traditional chemical treatment. This is a more environmentally friendly option than chemicals. Another advantage is that biocontrol might be effective against pathogens that are difficult to control by conventional means. A pathogen that infects plant roots might be hard to control using chemical treatment while a biocontrol bacterium introduced in the soil is in the appropriate place to combat the pathogen.

Biocontrol may be mediated in many different ways. By use of natural enemies that parasitize harmful insects, by introduction of a new insect species or bacteria into an ecological niche or as in this case by spreading bacteria in soil by seed treatment, hence giving these bacteria an advantage in colonisation. All these methods have the same goal, to keep the levels of one or several pests or pathogens at a lower level than it would be without the biocontrol agent.

The main focus of the thesis is the study of a *Bacillus amyloliquefaciens* potential biocontrol strain, UCMB-5113, and elucidate the effectiveness and function of this bacterial strain.

1.2 *Brassica napus* and Brassica

Brassica crops have been cultivated at least since 1500 BC (Doweny and Röbbelen 1989) . The genus Brassica consists of three species, *B. oleracea*, *B. rapa* and *B. napus*. *B. oleracea* include many important vegetables like cauliflower and broccoli. *B. rapa* and *B napus* are important oil crops grown all over the world. *B. napus* consists of two subspecies, Swede (subspecies Brassica), and oilseed rape (subspecies oleifera). Oilseed rape is the most important oilcrop in Sweden (Svensk Raps AB, 2008) .The oil can be used in various applications, most importantly as cooking oil and biofuel while the seed press cake can be used as a protein rich animal feed. Oilseed rape seed oil contains both omega-6 and omega-3 fatty acids, which makes it nutritionally valuable (McKevith, 2005). According to the United States Department of Agriculture, rapeseed was the third leading source of vegetable oil in the world in 2000, after soybean and oil palm, as well as the world's second largest source of protein meal. World production is growing rapidly, with the UN Food and Agriculture organisation reporting a production of 36 million tonnes of rapeseed in the 2003-04 season increasing to 46 million tonnes in 2004-05. Considering that in 1965 the production was only 5.2 million tonnes a dramatic increase of the importance of this crop has taken place. China is the largest producer followed by India and Canada. The country with the largest production in Europe is Germany (Food and Agriculture Organisation of the United Nations, 2008) . This makes *Brassica* an important crop world-wide. Unfortunately there are many serious pests and pathogens that attack Brassica, some of the more serious ones being fungal diseases like *Alternaria*

brassicae and *Botrytis cinerea* as well as insect pests like Diamond back moth (*Plutella xyllostella*) and flea beetles (*Phyllotreta spp*.).

1.3 Arabidopsis thaliana

The Brassicaceae plant *Arabidopsis thaliana* is a dicotydeloneous weed that can be found in most parts of the world (Alonso-Blanco and Koornneef, 2000). It is a close relative to *Brassica* species such as *B. napus*. It is firmly established as a plant model organism since the 1980s with the advantages of having a small size and short lifecycle (6 weeks or longer). Specimens of this plant have been collected from various places all over the world, which has provided a huge collection of ecotypes. An ecotype is a distinct entity of an organism that is closely linked (in its characteristics) to the ecological surroundings it inhabits. This gives a great possibility to study natural variation and adaptation and its genetic background (Koornneef et al, 2004).

Arabidopsis is also easy to transform, which has led to a multitude of genetic tools being available. There are well defined mutants, T-DNA mutants and extensive marker information available. The Arabidopsis genome sequence was completed in the year 2000 and it was the first plant to be completely sequenced. A genome with the size of 119 Mb with approximately 27,000 genes was thus described (AGI, 2000). The high sequence similarity between Arabidopsis and Brassica species is a great advantage when using Arabidopsis in Brassica research. All this and the large community working on Arabidopsis as well as the tools developed by this community make Arabidopsis a very advantageous plant to work with.

1.4 Pathogens and pests

In this study several different pathogens and pests have been used to study the effectiveness of the selected bacteria in biocontrol. Four important fungal pathogens on *B. napus* - *Alternaria brassicae, Botrytis cinerea, Leptosphaeria. maculans* and *V. longisporum*, one Brassica specialist insect *P. xylostella* and the bacteria *P. syringae* have all been used to study the biocontrol effect. The Ascomycete *Alternaria* is a necrotrophic fungus, i.e. it kills plant cells with the help of toxins and then feed on the dead plant tissue. It is the pathogen responsible for black spot disease (Glazebrook, 2005). The Ascomycete *Botrytis* is also a necrotrophic fungus and the cause

of the grey mould disease (Glazebrook, 2005). The Deutoromycete *Verticillium longisporium* has a biotrophic lifestyle, utilizing a living host. It is the causal agent of wilting disease (Granér et al, 2003). The Ascomycete *Leptosphaeria* causes blackleg disease and is a hemibiotroph. Hemibiotrophs usually start as biotrophs but turn necrotrophic later in its life cycle (Howlett et al, 2001). All these fungi can be found on crops in Sweden (Svensk Raps AB, 2008).

Pseudomonas syringae is not a major pathogen on *B. napus* but this bacterium is commonly used in Arabidopsis signalling studies and plant pathology (Nobuta and Meyers, 2005).

Plutella is an insect pest specialised on Brassica crops and its feeding can mediate serious damage.

1.5 Bacillus

Bacillus is a genus of gram positive, rod shaped, endospore forming bacteria (Reva et al, 2004). Members of the genus are very diverse, they can be found as pathogens as well as beneficial bacteria. Bacillus produce many antibiotic compounds such as Iturin and Zwittermycin (Romero et al, 2007; Raaijmakers et al, 2002). Some members of the Bacillus genus are *B. amyloliquefaciens, B. anthracis, B. cereus* and *B. subtilis*. *B. subtilis* is an established model organism for research on gram positive bacteria and the genome is sequenced. Several Bacillus strains can protect plants from pathogens. Strains able to protect plants are most commonly *B. subtilis*, *B. cereus* and *B. amyloliquefaciens*. *B. amyloliquefaciens* was first isolated in 1943 and named after its ability to produce amylase (Fukumoto, 1943; Priest et al, 1987). It is known to produce several antibiotics and is often found in soil and associated with plants (Yu et al, 2002).

1.6 Bacterial lifestyles

Bacteria can exist in very diverse niches. Different habitats such as soil, animal intestines and even boiling water house different microorganisms. Certain bacteria live in close relationships with other organisms as plants. This relationship can be harmful, neutral or beneficial. Bacteria can be found on plants living as endophytes, colonising the plants internally, or epiphytes, colonising plant surfaces, and colonisation occur on the aerial

parts, the phyllosphere, as well as on below ground tissues, the rhizosphere. Bacteria can sustain themselves by different lifestyles contrasted by symbionts and pathogens. Symbionts help the plants to obtain nutrients, for instance nitrogen fixators in root nodules helping the plants to harvest nitrogen in exchange for nutrients and protection inside root nodules (Denison and Kiers, 2004). Pathogens on the other hand utilize plant tissues and nutrients as resources compromising plant growth and reproduction.

Bacteria that colonise the plant rhizosphere get access to nutrients exudated from the plants. At the same time the bacteria protects the plants from potentially harmful organisms trying to establish in the rhizosphere. Certain bacteria can promote growth of plants. This effect can be due to increasing nutrient availability (Idriss et al, 2002). Bacteria may also produce plant hormones that stimulate plant growth (Timmusk et al, 1999; Martens and Frankenberger, 1993). Some bacteria produce ACC deaminase that degrades the precursor of the hormone ethylene. Ethylene promotes plant growth at a low concentration but is inhibitory at higher levels. By degrading the ethylene precursor ACC, the bacteria can manipulate the plant to increase root mass and at the same time use breakdown products as nutrients (Abeles et al, 1992).

1.7 Life in the Biosphere

The biosphere is composed of all living organisms that depend on transformation of matter for their survival. Autotrophic organisms, including plants, convert compounds such as CO_2 to glucose and nitrate, ammonium and phosphate into amino acids and nucleotides. These compounds are then utilised by fungi and bacteria as exudates and or as living or dead plant tissue. On the other hand several bacteria in soil have a big influence on plant growth by increasing amounts of necessary plant nutrients. A condensed picture of the interactions taking place in the biosphere can be seen in Fig. 1.

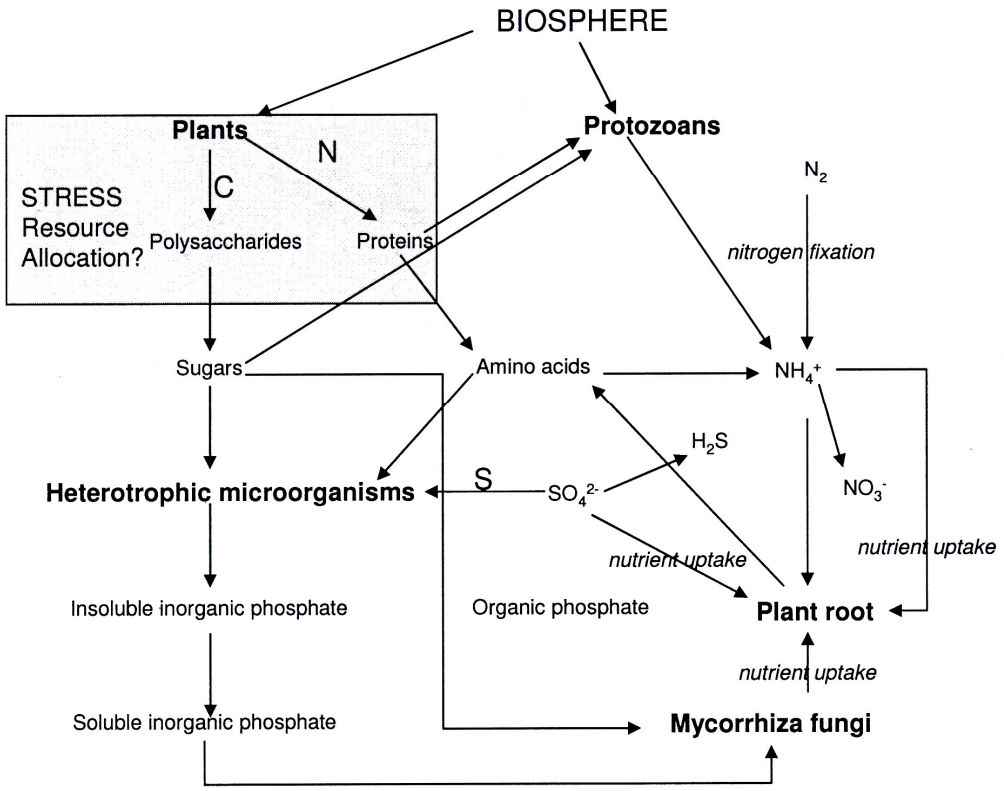

Figure 1. Nutrient cycling in the biosphere (Varma et al, 2004).

1.8 Life in the rhizosphere

The rhizosphere is the region of soil surrounding plant roots and that is influenced by the roots. Bacteria that are able to colonise this region are called rhizobacteria and take advantage of the root exudates released by the plant. This interaction is beneficial both for the plant as well as the bacteria. The presence of non-pathogenic bacteria at the roots confers protection to the plants as it makes it more difficult for pathogenic bacteria to become established. This protection by bacteria can be mediated in different ways. It can be due to production of antibiotics harmful to other microorganisms (Wulff et al, 2002a and 2002b; Bais et al, 2004; Whipps, 2001). Another factor is competition for available nutrients and growth space e.g. production of siderophores that helps the bacteria to out compete other bacteria for iron (Handelsman and Stabb, 1996; Whipps, 2001). The fact

that these bacteria also colonise highly exposed plant parts available for pathogens makes appropriate rhizosphere bacteria very important for plant fitness.

Successful root colonisation is influenced by many factors such as genetic factors, abundance of growth substrates, indigenous bacteria as well as abiotic factors such as soil humidity, pH and temperature (Garbeva et al, 2004; Smith et al, 1999; Varma et al, 2004). Root exudates mainly consist of carbohydrates, organic acids and amino acids (Lugtenberg et al, 2001; Nelson, 2004). The amounts and composition of these different metabolites vary between different plants. The requirement of the bacteria for different nutrients might explain why bacteria most often colonise plants in a species specific manner (Dunn et al, 2003). Close to 20% of the net photosynthesis products are exudated in wheat seedlings (Lugtenberg et al, 2001). Bacteria that colonise the plant can alter the composition of the exudates as well as the amount (Lugtenberg et al, 2001).

Bacteria colonise certain areas of the roots more densely than others. These areas are mainly junctions between epidermal root cells and side roots. Root tips are usually less colonised than other parts of the root (Lugtenberg et al, 2001).

1.9 Biocontrol

Biocontrol is the use of an organism to limit number and negative effects of unwanted organisms. The biocontrol organisms can be insects, bacteria or fungi. Insect based biocontrol is perhaps the most well known example. The release of predatory insects like wasps that feed on the pest, spread of fungi or bacteria that can infect pests or produce antibiotics that kills pathogens are all methods used in biocontrol. A classic example of successful biocontrol is the release of a small wasp, *Trichogramma ostriniae*, that helped to control the European corn borer (Wang et al, 1999). Spraying with fungi or bacteria that cause disease in insect is also used. When the pest feed upon the plant these biocontrol organism are also eaten and can hence infect the insect. Another example of this is the use of *Bacillus thuringiensis*, which produces a toxin in the insect gut that kills the insect (Roh et al, 2007). Biofertilisation, using bacteria to increase available nutrients is a common practise (Bloemberg and Lugtenberg , 2001). Several different commercial variants of Bacillus based biocontrol products already exist (Table 1). Kodiak

is for instance used on almost all cotton planted in the US and mediates good protection against fungal disease (Jacobsen et al, 2004).

Table 1. Commercial Bacillus based biocontrol products (Schisler et al, 2004).

Bacterial strain	Primary target	Product name
B. subtilis QST 713	Fungi and bacteria on vegetables and fruit	Serenade
B. licheniformis	Fungi on turf	Ecoguard
B subtilis GB03	Fungi on cotton and soybeans	Kodiak
B. pumilis GB34	Fungi on soybeans	Yield Shield
B. amyloliquefasciens and *B. subtilis* GB122	Fungi on bedding plants	BioYield
B. subtilis MBI600	Fungi on cotton and soybeans	Subtilex
B. subtilis MBI600 and *Rhizobium*	Fungi on soybeans	Hi Stick

1.10 Bacterial biocontrol

Many different bacterial strains can mediate biocontrol. Most important are Pseudomonas and Bacillus strains but several other bacteria are also known to mediate plant protection (Table 2). Pseudomonads are probably the most studied rhizobacteria in biocontrol but Bacillus has one big advantage over Pseudomonads. Bacillus produce spores that are resistant to stress. It can survive high temperatures, extreme pH, drought, chemical and mechanical stress. Accordingly, Bacillus bacteria are more covenient to use in the fields as it is easier to handle and apply providing commercial benefits (Schisler et al, 2004).

The protection mechanism differs among strains. Probably several different methods can be used at the same time to combat the pathogen. Alteration of the plant cell wall that causes an increased protection to pathogens has been found to occur with both *B. subtilis* and *Pseudomonas aeruginosa* (Benhamou et al, 1996). Formation of biofilm on plant roots by the bacteria makes the plant less sensitive to infection (Bais et al, 2004; Rudrappa et al, 2008). Competition for growth space and nutrients is another important factor (Handelsman and Stabb, 1996). Production of antibiotics and other harmful compounds by the bacteria is also important (Raaijmakers et al, 2002; Whipps, 2001). Synthesis of salicylic acid by bacteria can make the plant more tolerant to pests and pathogens by

stimulating systemic acquired resistance (SAR), a common defense program induced in plants to combat pathogens (Bostock, 2005). Induction of induced systemic resistance (ISR) in the plant is another way that bacteria can protect plants (van Loon et al, 1998).

Table 2. Selection of bacteria known to mediate biocontrol. (Dunn et al, 2003; Schisler et al, 2004; Rudrappa et al 2008; van Loon et al, 1998).

Organism
Bacillus amyloliquefaciens
Bacillus subtilis
Bacillus polymoxa
Bacillus licheniformis
Bacillus cereus
Bacillus pumilis
Pseudomonas fluorescens
Pseudomonas putida
Pseudomonas chlororaphis
Enterobacter agglomerans
Enterobacter cloacae
Serratia marcescens

1.10.1 Antibiotics

Bacteria are known to produce a wide array of antibiotics. Many bacteria are able to produce several different antibiotics that have a broad range and sometimes overlap in their function (Raaijmakers et al, 2002; Yu et al, 2002; Risøen et al, 2004; Leifert et al, 1995). These antibiotics play a significant role in biocontrol. Bacteria are also able to synthesize enzymes like chitinases, proteases, lipases and beta-1,3-glucanases that are all harmful for microorganisms and further improves the biocontrol efficiency (Whipps, 2001; Varma et al, 2004). Some bacteria are genetically improved to produce more or new antibiotics to provide better protection (Bainton et al, 2004).

1.10.2 Competition

One way that beneficial bacteria can protect plants from pathogens is through competition. Established rhizobacteria at the best spots in the

rhizosphere, like junctions between epidermal cells where there are plenty of exudates, do not want any intruding microorganism to use "their" nutrients and growth site. The fact that some pathogens use these places as sites of infection makes the presence of the beneficial bacteria even more important. Bacteria also compete with the pathogens for essential nutrients, this is made more efficient with the help of siderophores (Whipps, 2001). Siderophores are low molecular weight Fe(III) specific ligands that are used for bacteria to scavenge iron from the environment. Siderophores solubilises iron which then is transported into the bacterial cells using specific receptors. This gives the bacteria the possibility to deplete the available iron source from other potentially harmful bacterial strains. Siderophores have earlier been shown to be essential to some bacteria that protect plants (Whipps, 2001).

1.10.3 PGPR

Plant growth promoting rhizobacteria (PGPR) can increase plant growth and vitality through production of phytohormones like auxins, gibberellins, abscisic acid, ethylene and cytokinins (Varma et al, 2004). These hormones can be produced by various microorganisms such as algae, bacteria and fungi. These hormones are involved in many aspects of plant life such as root elongation, cell elongation and proliferation (Varma et al, 2004). Another way is to increase the amounts of available nutrients like fixed nitrogen, phosphorous and iron solubilised from soil (Varma et al, 2004).

1.10.4 Plant innate immunity

A major plant defence against pathogens has evolved as the innate immunity system. Using various pattern recognition receptor proteins plants can identify pathogen-associated molecular patterns (PAMPs) of potential pathogens and elicit a basal defence response (He et al, 2007). Flagellins are examples of a structure that such receptors can recognize and react to. The innate immunity system is widespread in nature and seems to have evolved early explaining extensive similarities found between animals, insects and plants (Iriti and Faoro, 2007). Plant pathogens have through co-evolution developed effectors to suppress or circumvent this recognition and resulting plant defence response and thus become virulent and cause disease. Plants have also specific disease resistance (R) proteins to counteract microbial

virulence effectors but different plant genotypes vary in the defence repertoire (deWit, 2007). The elicited defence is manifested as a local and rapid hypersensitive response (HR) that includes formation of reactive oxygen species and programmed cell death to restrict pathogen growth and disease development. The HR can then through systemic signalling mediated by salicylic acid (SA) or other hormones result in systemic acquired resistance (SAR) where distal tissues are activated and contains e.g. a plethora of pathogenesis related (PR) proteins that target different pathogens (Glazebrook, 2005). A successful pathogen recognition will lead to systemic responses, which make plants more resistant to subsequent pathogen attacks during a long time period.

1.10.5 ISR

Some bacteria mediate a more direct protection. This protection is referred to as (ISR). This is a type of protection induced in the plant by certain bacteria, commonly *Pseudomonas* and *Bacillus* (van Loon et al, 1998; Iavicoli et al, 2003; Kloepper et al, 2004). It is a latent defence, not activated until the plant is under pathogen or pest attack (Conrath et al, 2006). This defence system differs from the better known plant defence SAR by means of not being dependent on SA. Instead most reports show a need of functional jasmonic acid (JA) and ethylene (Et) dependent signalling as well as Npr1 (Fig. 2) (Pieterse and van Loon LC, 2004). Bacteria can induce ISR in different ways too, some depending on PR proteins and

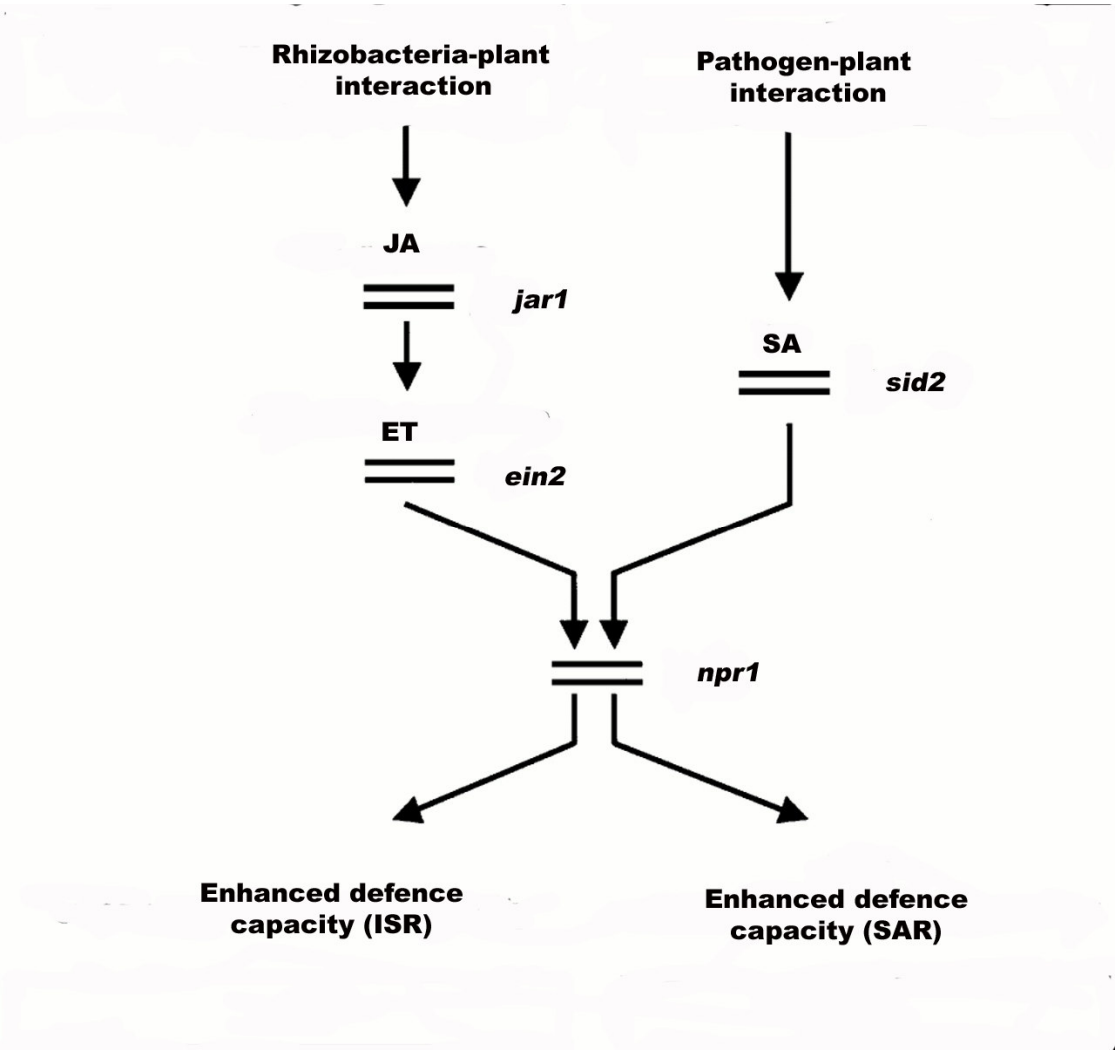

Figure 2. Signalling pathways leading to SAR and ISR (van Loon et al, 1998).

some on only Et (Ryu et al, 2004). Here, I will mainly focus on ISR as described for Pseudomonads WCS417r which is also similar to many Bacillus strains and also seem to be true for UCMB-5113 (Danielsson and Meijer, submitted manuscript IV). ISR has earlier been shown to be effective against several different pathogens on many different plants (van Loon et al, 1998). This induced plant protection is not associated with any increase of defence related marker genes, there is no increase in either JA, Et or SA dependent genes after bacterial treatment (van Wees et al, 1999). But still a need for a functional JA and ethylene signalling exists. When infected by *P. syringae*, an increase of *Vsp1* (a JA dependent marker) has been observed (van Wees et al, 1999). Priming of the plant defence is mediated by the bacteria allowing the plant to react faster and/or stronger to the presence of a pathogen. This means that ISR is an inducible defence, only truly activated after infection by the pathogen. An inducible system has the advantage of not being as expensive to maintain as a constitutively

active defence (van Hulten et al, 2006). The presence of the bacteria once in a sufficient amount, over 10^6 cfu, gives a priming of the defence system that is active for months (van Loon et al, 1998). In order to colonise the plant the beneficial bacteria must avoid to trigger the plant innate immunity system but still allow the plant to recognize other microbes as potential pathogens. How this delicate balance between plants and microorganisms can develop is intriguing and deserves further study.

Transcriptome studies have shown subsets of genes being up-regulated upon colonisation by bacteria (Danielsson et al, 2007; Ongena et al, 2005). Most commonly these genes are involved in signalling or plant metabolism. Direct effects that have been found during ISR include increased phytoalexin levels as well as increase of callose apposition and phenolics at the site of infection compared to untreated plants (Conrath et al, 2006; Ongena et al, 2000; Benhamou et al 1996).

2 Aims

The aims of this study were to investigate the effects that treatment with closely related *Bacillus amyloliquefaciens* strains have on plants. To isolate a potential biocontrol candidate. To investigate the protective range of the candidate as well as elucidate the function and mechanisms involved in the biocontrol interaction.

3 Results and methods

3.1 Experimental setup

The experimental setup is rather straightforward in this Bacillus-plant-pathogen system we have utilised. We have used seeds treated with Bacillus spores that were planted into autoclaved soil to give the bacteria an advantage in colonisation. Spore solutions were prepared by heat treating three days old Bacillus cultures to select for spores.

3.2 Screening

This project started with three different *Bacillus amyloliquefaciens* strains that had mediated some protection in an earlier study (Reva et al. 2004). To find out which strain that was most effective to mediate disease suppression we screened these three closely related strains against different pathogens. On Brassica we tested four fungal pathogens, *Alternaria brassicae, Botrytis cinerea, Leptosphaeria maculans* and *Verticillium longisporum* (I). We also tested if any protection could be observed on Arabidopsis and here we challenged the plants with *P. syringae, Lepthosphaeria and Alternaria* (III). Two Bacillus strains, UCMB-5036 and UCMB-5113, showed protective ability on both plant species towards all pathogens tested. UCMB-5113 was also screened against *Plutella xylostella* but no difference in feeding compared to untreated plants could be observed (III).

3.3 Plant fitness

For a potential biocontrol strain it is of course important to study if the plant is affected by the treatment. Therefore, a screen was performed where Arabidopsis and Brassica plants treated with the different Bacillus strains were compared with control treated plants. Here we measured important characteristics as seed yield, flowering and number of true leaves. We also carefully analysed the plants for any signs of disease. No disease symptoms or significant increase or decrease of growth rate was found (I and III). We choose UCMB-5113 for more mechanistic studies to address the mechanism of this bacteria plant interaction.

3.4 Antibiotic production

As Bacillus bacteria are known to produce several effective antifungal compounds we have studied this in vitro by growing the pathogen together with the Bacillus strain on agar plates. A clear zone of inhibition could be observed around the bacterial colonies. We also collected growth medium from Bacillus cultures, sterile filtered the liquid and added fungal spores to study if we could observe any antifungal compounds in the media. The result showed that most strains produced some kind of antifungal compound, but only UCMB-5036 produced antifungal compounds that was effective against all fungi studied (I).

3.5 Specificity

To study how specific the interaction between UCMB-5113 and plants are we studied colonisation and protection by Bacillus after seed treatment of different Arabidopsis ecotypes. All ten ecotypes were colonised to a high level with insignificant differences among them. Four out of ten ecotypes showed a significant decrease of disease symptoms after Bacillus treatment (IV).

3.6 Signalling

We investigated if SAR or ISR were induced after UCMB-5113 treatment with the use of Arabidopsis signalling mutants. Signalling mutants impaired in SA, JA and Et signalling as well as the *Npr1* mutant, which is impaired in both SAR and ISR expression, were used. This study showed a need for functional JA and Et as well as functional *Npr1* to protect against *P. syringae*. SA impaired mutants are as protected as wildtype (IV). To further confirm this, a PCR was run using primers for genes regulated by these defense signalling pathways. We compared the gene expression of untreated plants, Bacillus treated plants, *P. syringae* infected plants and Bacillus treated infected plants. This showed no increase of any of the markers with the exception of a slight increase of the JA dependent marker when the Bacillus treated plants were infected compared to plants only inoculated with Pseudomonas (IV).

To investigate what is essential for a protective effect we have tried to induce protection not only by a spore solution. We have used sterile filtrated growth medium as well as killed bacteria and spores and compared this effect with a viable spore solution when applied to plant. No protective effects could be found using any of the different treatment but the spore solution (IV).

3.7 Transcription

We have also performed a cDNA-AFLP study to investigate the transcriptome of UCMB-5113 treated *B. napus* (II). cDNA-AFLP is a highly reproducible method, which can be used with out any prior sequence knowledge (Sarosh and Meijer, 2007). Transcripts differentially expressed are visualised on a gel, where they can be cut out and sequenced. Sequences can then be used to identify homologues in other species. Since Arabidopsis is sequenced and we are working on the close relative *B. napus*, sequence homology is high. We used *Botrytis* to infect UCMB-5113 treated and untreated plants and compared these with untreated plants. Leaves as well as roots were collected and studied. All results were confirmed by northern blots. This far we have identified 76 differentially expressed transcripts but there are still many left to investigate. Ten differentially expressed transcripts were found in Bacillus treated roots, 29 in Bacillus treated leaves, 11 in Botrytis infected leaves and 26 in Botrytis infected

Bacillus treated leaves. Intriguingly, fewer transcripts were observed in roots compared to leaves of Bacillus treated plants suggesting a strong systemic effect in priming. Most of the transcripts identified are involved in metabolism and signal transduction. Some examples of genes that are induced are a beta-1,4-glucanase and protein kinases. Several genes with unknown function were also found that may provide new information on how priming is operating. Analysis showed several genes to be induced also by brassinosteroids and other hormones triggering plant growth. Accordingly Bacillus colonisation seems to affect formation of hormones that promote growth especially in the root tissue.

4 Conclusions and Discussion

Relatively big differences have been identified among these closely related Bacillus bacterial strains. This shows us that interactions and recognition between beneficial bacteria and plants may be as specific as plant pathogen interactions. The result from the ecotype screen gives further credence to this. This is not so surprising since the interaction between biocontrol agents and plants have earlier been shown to differ on cultivar level (Dunn et al, 2003). The ecotypes were isolated from different parts of the world but no correlation between protected ecotypes and location of ecotypes could be discerned.

UCMB-5113 gives a broad protection against several different pathogens with different lifestyles and infection strategies. No protection could be observed towards Plutella but to be sure that UCMB-5113 can not protect plants from insects other insects need to be tested.

One reason we choose to continue with UCMB-5113 and not UCMB-5036, which had a stronger inhibitory effect, is that UCMB-5113 does not produce any effective antifungal compounds against some pathogens in vitro while it could confer protection on plants. This means that protection can not be entirely dependent on production of antifungal compounds.

That no PGPR effect could be found was of course disappointing. Unfortunately these strains do not seem to promote growth but on the other hand they do not seem to retard growth. This experiment occurred in controlled environment and maybe an increase in plant fitness can be found if the plants are exposed to a more natural environment and subject to the stresses and challenges inherent in natural plant life.

The signalling mutant study gave us results similar to the observations made using Pseudomonas WCS417r. It is similar to the most studied type of ISR. The results of the marker genes are also similar to results obtained with Pseudomonas and show that priming of the plant defence takes place.

The cDNA-AFLP study identified several genes involved in the plant bacteria interaction. This showed that Bacillus colonisation of oilseed rape roots cause a genetic reprogramming of plant cells both in local (root) and distal (leaf) tissues. Majority of the genes affected seem to be involved in metabolism, energy generation and regulation (II). There are still many bands left to sequence so further interesting genes can be found. The fact that many genes are unknown, i.e. lack homology to genes with known function, make these genes very interesting for further study. That signal transduction genes showed up was not a surprise since we have already shown the need for functional signalling in Bacillus based priming of plant defence. The increase in transcription of genes involved in metabolism is not unexpected, since it could be due to an increase in exudates caused by the presence of the bacteria in the rhizosphere. Maybe this is the prize the plants have to pay for the service of increased protection mediated by bacteria.

5 Future studies

This study has identified certain bacterial strains effective in protecting plants from several pathogens under laboratory conditions. But as conditions in a laboratory are not the same as in nature, field trials are essential to truly see the potential of this strain. Plants in nature are subject to several different stresses, and to really study the efficiency of the Bacillus strains, plants have to be monitored during a lifetime of fungal infection, pest attacks, drought, and all other facets of plant life. Seed yield, which may be considered to be the foremost indicator of plant fitness, is an important factor to study. Bacteria tagged with GFP that is stably maintained during many generations would be of great assistance. Not only would this enable tests to study how long the bacteria are maintained in soil but it would also greatly facilitate studies of horizontal and vertical spread. To know how these bacteria spread in soil is essential for consequence analysis, to study how long the bacteria is able to withstand the competition from other naturally occurring bacteria is also of interest. Further, it would be interesting to study how the bacteria colonise the plant, is it only present in the rhizosphere or can it be found on other parts of the plant?

Another important study is to evaluate these bacteria for toxicity on humans, several different Bacillus strains are human pathogens and this would of course be a drawback.

It would also be interesting to study the bacteria more closely. The use of Bacillus microarrays could potentially identify genes that are essential for the plant-bacteria interaction. Since we have a closely related bacterial strain, UCMB-5033 that does not give protection, a comparative study with UCMB-5113 would be very interesting. Genes involved in plant bacteria signalling and colonisation could possibly be identified. As I have

discovered that these bacteria produce antibiotics it would also be interesting to identify these compounds.

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