Biological control of apple scab and fire blight by the application of the non-pathogenic bacterium *Pseudomonas fluorescens* Bk3 to the leaf surface

S. Schmoock[1], S. Kürkcüoglu[1] and A. E. Gau[1]

Abstract
The biological control of plant diseases by application of antagonistic microorganisms to the plant phyllosphere is an alternative strategy to prevent the frequent treatment of plants by pesticides. Microbiological antagonists can firstly interact directly against the pathogen by releasing antimicrobial compounds and/or secondly induce the plant resistance of the host plant by expression of pathogenesis-related proteins (PR proteins). The focus of our study is on the interaction of the non-pathogenic bacterium Pseudomonas fluorescens *Bk3 to the plant phyllosphere of* Malus domestica *cv. Holsteiner Cox. After application of* P. fluorescens *Bk3 to the phyllosphere of* M. domestica *cv. Holsteiner Cox we observed dramatic changes in the protein composition of the apoplast of the host plant. Sequencing of the induced proteins by ESI-Q-ToF mass spectrometry and homology search identified these additional proteins as pathogenesis related proteins (PR) like ß-1,3- glucanase, thaumatin-like protein, chitinase and hevein-like protein. To confirm these findings, a suppressive subtractive hybridization with total RNA from leaves before and after inoculation of* P. fluorescens *Bk3 to the leaves of the host plant was performed. It revealed an increased expression level of many PR and stress related genes.*
The induction of PR proteins and plant defence genes in host plants after application of non-pathogenic bacterial antagonists to the plant phylloshere can presumably prevent or reduce successful infections by plant pathogens.

Keywords: biological control, bioluminescence, *Erwinia amylovora*, pathogenesis related proteins, *Venturia inaequalis*

Introduction

Apple scab, caused by the fungus *Venturia inaequalis* and fire blight, caused by the bacterium *Erwinia amylovora*, are the most important diseases of apples worldwide, and they very likely occur in every country where apples (*Malus* × *domestica* Borkh.) are grown. Without any control, the pathogens can cause extensive production losses in regions with humid, cool weather during the spring months.
In some circumstances, the losses from apple scab can be 70% or more of the total fruit value (Agrios, 2005). Despite the tremendous amount of research on scab management (MacHardy, 1996), its control still requires up to 20 fungicide applications annually. Independent of the cost of chemical control, seasonal plasticity of fungicide sensitivities (Köller et al., 1995) is known and the long-term efficacy of fungicides is questionable. The only promising treatment against fire blight is the application of antibiotics which causes the risk of the establishment of antibiotic resistances.
Therefore the application of natural microbial antagonists to control microbial plant diseases could be a promising alternative to the application of synthetic pesticides. Spurr and Knudsen (1985) concluded that biological control of leaf diseases by management of bacteria on the phylloplane is a strategy with great potential and is needed to relieve the pressures and failings of other control measures.

[1]Institute of Botany, Leibniz University Hannover, Herrenhäuserstr. 2, D-30419 Hannnover, e-mail: achim.gau@botanik.uni-hannover.de

Biological pest control with epiphytic living antagonists (*e.g.*, bacteria, yeast and other fungi) could help to avoid or to reduce the intensive application of pesticides and minimize their residues in the crop. However, despite intensive investigations and practical approaches (Boland and Kuykendall, 1997; Elad et al., 2001), this type of biological control remains marginally understood.
The investigation of the relationship between the antagonist, the host plant and the pathogen could offer a new approach for understanding the mechanism of biological control of plant diseases.

Materials and Methods
Plant propagation, inoculation of non-pathogenic microbial antagonists, and isolation of intercellular washing fluid (IWF) from *Malus domestica* were done as previously reported by Kürkcüoglu et al. (2004). The non-invasive determination of the microbial antagonists as well as the bacterial pathogen *E. amylovora* were performed as described by Gau et al. (2002) and Schmoock (2006), respectively.

Results
The intercellular washing fluid (IWF) of the pathogen susceptible cultivar Holsteiner Cox before and after application of the non-pathogenic bacterium *P. fluorescens* Bk3 to the leaves was investigated in a comparative manner. For the analyses eight-week-old rooted apple plants of *M. domestica* cv. Holsteiner Cox being genetically identical and grown under sterile conditions were used for isolation of the IWF before and after treatment of the plant leaves with the non-pathogenic *P. fluorescens* Bk3. The IWF was isolated by the infiltration-centrifugation technique and subsequently separated on SDS PAGE.
Some of the proteins being increased or decreased (see Fig. 1) by the treatment of *M. domestica* cv. Holsteiner Cox with *P. fluorescens* Bk3 were excised from the gel and digested with trypsin. The obtained peptide fragments were submitted to *de novo* sequencing on a quadrupole/time-of-flight hybrid mass spectrometer and were identified as e.g. chitinase, ß-1,3-glucanase, thaumatin-like protein and ribonuclease. All identified proteins belong to the group of pathogensis-related proteins (PR).

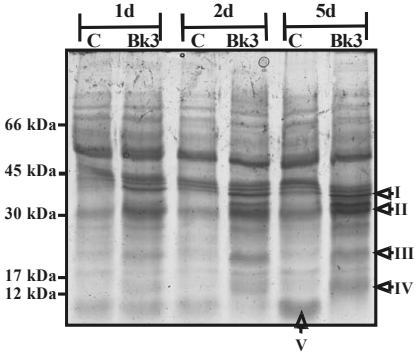

Fig. 1 Separation of apoplastic fluid on SDS-PAGE from *M. domestica* cv. Holstein Cox after application of the non-pathogenic bacterium *P. fluorescens* Bk3 to the leaf surface. C:Control; Bk3 after treatment with *P. fluorescens* Bk3 . Numbered bands were sequenced by ESI Q ToF mass spectrometry and identified with homology search. Band I: ß-1,3-glucanase, band II: thaumatin-like protein/chitinase, band III: ribonuclease-like PR10b protein, band IV: hevein-like protein, band V: non-specific lipid transfer protein.

Further investigations on transcript level revealed the up-regulation of 113 EST clones which also belong to the class of PR-proteins, oxidative stress, and transcripts that code for proteins which have a crucial role at different stages of pathogen recognition and in signalling pathways (Kürkcüoglu et al. 2007).
Moreover applied investigations under field conditions revealed the reduction of apple scab up to 84% when *P. fluorescens* Bk3 was applied to the leaf surface two days before the artificial infection was done (Kürcüoglu, 2006).

In addition to the molecular biological investigations we have developed a method for the non-invasive determination of microbial antagonists or bacterial pathogens on the leaf surface or inside the host plant by using bioluminescence. Therefore the microorganisms were transformed with the *lux*CDABE gene cluster that codes for the two structural genes of the luciferase as well as for the genes of the substrate biosynthesis.
To evaluate the protective function of bacterial antagonists, the fire blight susceptible cultivar Holsteiner Cox was pre-treated with 1 x 10^9 cells of *P. fluorescens* Bk3 two days before the artificial infection with *E. amylovora* occurred. The measurement of the bioluminescence as shown in Fig. 2 revealed that the pre-treated plants are protected against the infection of *E. amylovora* in contrast to the untreated control plants.

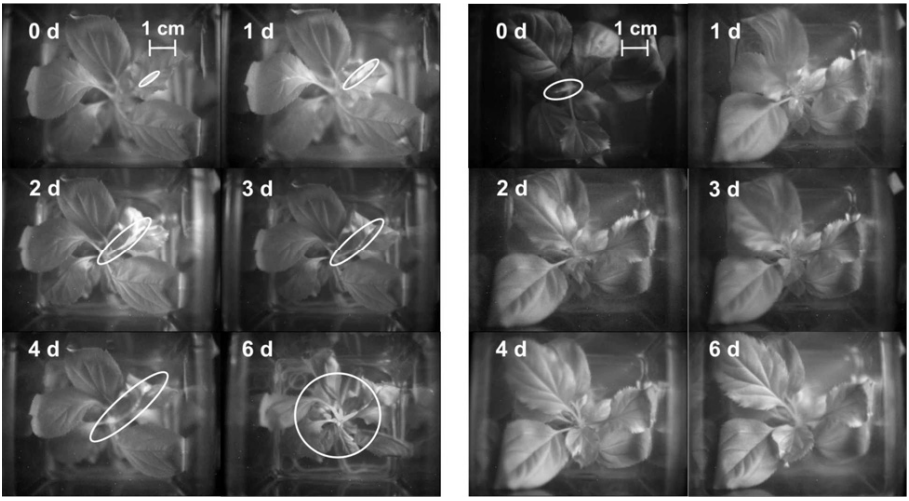

Fig. 2 Determination of *E. amylovora* 222::Tn5-luxCDABE on *M. domestica* cv. Holsteiner Cox. **Left**: Infection of *M. domestica* cv. Holsteiner Cox with 5 x 10^6 Zellen *E. amylovora* 222::Tn5-luxCDABE. **Right:** Infektion of *M. domestica* cv. Holsteiner Cox with 5 x 10^6 cells of *E. amylovora* 222::Tn5-luxCDABE two days after pre-treatment with 1 x 10^9 cells of *P. fluorescens* Bk3. (Schmoock, 2006). *E. amylovora* is marked with a white ellipse.

Moreover, the application of the non-pathogenic bacterium *P. fluorescens* Bk3 causes no visible morphological change of the plants (data not shown).

Discussion

The results presented here could be interpreted by suggesting that a pre-treatment of apple cultivars with *P. fluorescens* Bk3 leads to a induced resistance against infections by *V. inaequalis* or *E. amylovora* due to the fact that PR proteins are expressed before the infection with the pathogen. Such a pre-treatment of apple cultivars with non-pathogenic bacteria can eventually replace excessive treatment of apple trees with pesticides.

Acknowledgements

S.K. expresses her gratitude to the Deutsche Bundesstiftung Umwelt for financial support. For sequencing proteins by ESI Q-ToF mass spectrometry we are very thankful to Dr. M. Piotrowski, (Ruhr University Bochum, Germany).

References

Agrios, G.N. (2005). Plant Pathology. 5th edition. Academic Press Ltd, San Diego, California, USA.

Boland, G. J., Kuykendall, L. D. (1997). Plant-microbe interactions and biological control. Dekker, New York.

Elad, Y., Freemann, S., Monte, E. (eds.) (2001). Biocontrol Agents: mode of action and interaction with other means control. IOBC/wprs Bulletin 24, 1-376.

Gau, A.E., Dietrich, C., Kloppstech, K. (2002). Non-invasive determination of plant-associated bacteria in the phyllosphere of plants. Environmental Microbiology 4, 744-753.

Köller, W., Smith, F.D., Reynolds, K.L., Wilcox, W.F., Burr, J.A. (1995). Seasonal changes of sensitivities to sterol demethylation inhibitors in *Venturia inaequalis* populations. Mycol. Res. 99, 689-692.

Kürkcüoglu, S., Piotrowski, M., Kloppstech, K., Gau, A.E. (2004). Up-regulation of Pathogenesis-related Proteins in the apoplast of *Malus domestica* cv. Holsteiner Cox after application of a non-pathogenic bacterium *Pseudomonas fluorescens* Bk3 to the plant phyllosphere. Z. Naturforsch. 59c, 843-848.

Kürcüoglü S (2006) Eine Strategie zur biologischen Kontrolle von Pflanzenkrankheiten durch den Einsatz natürlicher, nicht pathogener mikrobieller Antagonisten am Modellsystem *Malus domestica* (Apfel)*/Venturia inaequalis* (Apfelschorferreger)/ *Pseudomonas fluorescens* Bk3 (mikrobieller Antagonist) PhD thesis, University Hannover, Germany.

Kürkcüoglu, S., Al-Masri A.N., Degenhardt, J., Gau, A.E. (2007). Characterisation of the difference in the transcriptional level of *Malus domestica* before and after application of the non-pathogenic bacterium *Pseudomonas fluorescens* Bk3. J. Exp. Botany 58, 733-41.

MacHardy, W.E. (1996). Apple Scab- Biology, epidemiology and management. APS Press, St. USA.

Schmoock, S. (2006) Infektion und Ausbreitung des Feuerbranderregers *Erwinia amylovora* in An- und Abwesenheit des bakteriellen Antagonisten *Pseudomonas fluorescens* Bk3 auf verschiedenen Apfelkultivaren. Diploma thesis, University Hannover, Germany.

Spurr, H. W., Knudsen, G.R. (1985). Biological control of leaf diseases with bacteria. In: Biological control on the phylloplane (Windels, C. E. Lindow, S. E. Eds.), Am. Phytophathol. Soc. Press, St. Paul, MN, pp. 45-62.