# Gene expression of ABC-transporters in the fungal biocontrol agent *Clonostachys rosea* in response to anti-fungal metabolites from *Pseudomonas chlororaphis*

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**Swedish University of Agricultural Sciences**
**Department of Forest Mycology and Plant Pathology**
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# Gene expression of ABC-transporters in the fungal biocontrol agent *Clonostachys rosea* in response to anti-fungal metabolites from *Pseudomonas chlororaphis*

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## Abstract:

*Clonostachys rosea* strain IK726 and *Pseudomonas chlororaphis* strain MA342 are two active biological control microbes against many seed and soil-borne plant diseases. A combination mixture between *C. rosea* IK726 and *P. chlororaphis* MA342 may provide additive or synergistic effects on the biocontrol ability of these microbes, due to complementing modes of action. However, it is not known if *C. rosea* IK726 can tolerate the antifungal metabolites produced by *P. chlororaphis* MA342. In the current study, a dual culture assay and mycelial dry weight measurements were used investigate the effect of antifungal metabolites from *P. chlororaphis* MA342 on the growth rate and biomass production of *C. rosea* IK726. Our result showed that *C. rosea* IK726 displayed a high level of resistance to the antifungal metabolites from *P. chlororaphis* MA342. ABC transporters are membrane-spanning proteins that are known as drug efflux pumps in many microbes. Due to the high number of ABC transporter genes in the *C. rosea* IK726 genome, it is suggested that these proteins may be involved in the antibiotic tolerance of *C. rosea*. Therefore, 13 *C. rosea* candidate ABC transporter genes were selected, and the gene expression patterns were monitored using quantitative reverse transcription PCR during the treatment of *C. rosea* IK726 with a culture filtrate from *P. chlororaphis* MA342. The transcript levels of putative multidrug resistance (MDR) gene *ABC4267* and mitochondrial peptide transporter gene *ABC4987* were induced during 2-6 hours after treatment with MA342 culture filtrate, compared to the non-treated control, but after 24 hours the expression decreased to normal levels. The putative siderophore transporter gene *ABC3069* was induced after 2 and 24 hours of culture filtrate treatment. Expression levels of the putative pheromone transporter gene *ABC2419* and the pleiotropic drug resistance (PDR) gene *ABC3525* were induced after 2 hours. Expression of putative secondary metabolite transporter genes *ABC592* and *ABC3918*, MDR gene *ABC3260* and PDR gene *ABC4345* were induced after 6 hours and then decreased to normal levels. Treatment with *P. chlororaphis* MA342 filtrate had no influence on the expression levels of the putative pheromone transporter gene *ABC2210*, the secondary metabolite transporter genes *ABC3433* and *ABC2700*, or the PDR gene *ABC3189*. The induction of both PDR and MDR genes may be involved in an early stage of detoxification in *C. rosea* IK726. The up-regulation of the putative mitochondrial *ABC4987* may be a response to an increased ATP consumption caused by the ATP-driven ABC transporters in *C. rosea* IK726. The induction of *ABC3069* may be related to Fe limiting conditions in the MA342 culture filtrate. Induction of

*ABC592* and *ABC3918* may imply that *C. rosea* secretes compounds that inhibit MA342 enzymes present in the culture filtrate. We conclude that *C. rosea* IK726 possess sufficient mechanisms to tolerate antifungal compounds from MA342. Certain ABC transporter genes related to different important cellular functions were significantly induced by the treatment with *P. chlororaphis* MA342 culture filtrate. This result indicates that ABC transporters may play a role in tolerance/detoxification mechanisms in *C. rosea* IK726.

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# 1. Introduction

## 1.1 A potential combination: IK726 and MA342

*Clonostachys rosea* strain IK726 and *Pseudomonas chlororaphis* strain MA342 were both identified in a Nordic programme for screening and identification of biological control microbes of seed or soil-borne plant diseases (Knudsen *et al.*, 1997). For simplicity, these two microbes are referred to as IK726 and MA342 in the current work.

Like many other biocontrol fluorescent pseudomonads, MA342 protects crops from seed and soil-borne pathogens via induction of systemic resistance reactions in the plant, competition for space and nutrients and through antibiosis (Hökeberg, pers. com.). A polyketide, 2,3-deepoxy-2,3-didehydrorhizoxin (DDR) is determined as an essential antifungal component in MA342 (Hökeberg, 1998). Several other secondary metabolites expressed by biocontrol fluorescent pseudomonads are identified as broad spectrum antibiotics, including 2,4-diacetylphloroglucinol (2,4-DAPG) (Keel *et al.*, 1992; Bonsall *et al.*, 1997), phenazine (Phz) and its derivatives (Haas *et al.*, 2003).

*C. rosea* is a mycoparasite of many soil borne pathogens (Schroers *et al.*, 1999). Strain IK726 has a competitive root colonization ability, as well as additional biocontrol action modes such as direct parasitism, competition for nutrients and plant growth promotion (Jensen *et al.*, 2000; Lübeck *et al.*, 2002).

A combination mixture between *C. rosea* IK726 and *P. chlororaphis* MA342 may provide additive or synergistic effects on the biocontrol effect against seed and soil-borne pathogens, due to complementing modes of action. However, it is not known if *C. rosea* IK726 can tolerate the antifungal metabolites produced by *P. chlororaphis* MA342.

## 1.2 Fungal ABC transporters

### 1.2.1 The function of fungal ABC transporters in microbial interactions

The fungal ATP-binding cassette (ABC) transporter proteins act as active efflux pumps and are

often regarded as the first line of defense against diverse natural and synthetic antifungal compounds (Del Sorbo *et al.*, 2000; Wolfger *et al.*, 2001). Fungi with different lifestyles have evolved various types of efflux pumps as an adaptation to diverse ecological habitats (de Waard *et al.*, 2006). Fungal ABC transporters are involved in active efflux of a broad range of endogenous and exogenous toxicants. The latter includes antibiotics produced from other microbes, synthetic fungicides and plant defense compounds (Del Sorbo *et al.*, 2000; Coleman *et al.*, 2009). Expression of the ABC transporter gene *BcatrB* in the phytopathogenic fungus *Botrytis cinerea* is induced by phenazine and 2,4-GAPG producing *Pseudomonas* indicate that BcatrB is involved in fungal-bacterial interactions (Schoonbeek *et al.*, 2002). Another study show that the BcatrB efflux pump plays a key role in the non-degradative defence of *B. cinerea* in the early response to the 2,4-DAPG antibiotic, which thereby enables *B. cinerea* to activate a more efficient enzymatic 2,4-DAPG degradation mechanism (Schouten *et al.*, 2008). BcatrB is also involved in fungicide and phytoalexin efflux (Schoonbeek *et al.*, 2001; Stefanato *et al.*, 2009). In the model fungus, *Saccharomyces cerevisiae*, it is shown that a broad range of drugs induced the expression of pleiotropic drug resistance (PDR) ABC transporter genes (Bi *et al.*, 2010). In the biocontrol fungus *Trichoderma atroviride*, the ABC transporter Taabc2 is essential for tolerance of several different chemical stresses, including soil-borne plant pathogen derived mycotoxins and fungicides (Ruocco *et al.*, 2009). In addition, several ABC transporters in *T. atroviride* are up-regulated in the presence of either pathogens or plant tissue, which suggests that these ABC transporters are biocontrol factors that may contribute to the antagonistic ability and root colonization ability of *T. atroviride* (Marra *et al.*, 2006; Woo *et al.*, 2006; Hermosa *et al.*, 2012).

### 1.2.2 The ABC protein superfamily

The ABC protein superfamily is a highly abundant group of integral membrane proteins, which is present in both prokaryotes and eukaryotes (Higgins, 1992). The majority of ABC proteins serve as the primary active transporters, known as ABC transporters, which utilize the hydrolysis of ATP to drive the transmembrane import or export (Higgins *et al.*, 1986; Driessen *et al.*, 2000). ABC transporters involved in cellular import are so far only found in prokaryotes, while ABC exporters are expressed in all living cells (Hollenstein *et al.*, 2007a). Certain periplasmic substrate binding proteins facilitate substrate translocation of the ABC importers (Higgins *et al.*, 1985; Locher, 2009). Certain ABC proteins are also involved in some

cytoplasmic processes and in their regulation (Dassa *et al.*, 2001), including DNA repair (Husain *et al.*, 1986) and cell division (Gill *et al.*, 1986).

As a group, ABC transporters can translocate a wide range of substrates including sugars and other polysaccharides, vitamins, amino acids, peptides, heavy metals, proteins and lipids (Higgins, 1992). The first ABC transporter was discovered in the bacterium *Salmonella typhimurium*, which function as a histidine importer (Higgins *et al.*, 1981; Higgins *et al.*, 1982). Because certain ABC transporters are efflux pumps against exogenous toxics (Del Sorbo *et al.*, 2000), they are partly responsible for multidrug resistance (MDR) during chemotherapy of bacterial infections and cancer (Chang, 2003; Lage, 2003). ABC transporter mediated PDR is becoming an important issue in antifungal therapeutic drug development (Van Bambeke *et al.*, 2000; Monk *et al.*, 2008).

### 1.2.3 The structure and substrate transport of ABC transporters

All eukaryotic ABC transporters share a similar architecture: at least one nucleotide-binding domain (NBD) and several transmembrane domains (TMDs). A typical eukaryotic ABC transporter (full-size) consists of a pair of TMDs and a pair of NBDs fused into a single polypeptide, arranged into forward $[TMD\text{-}NBD]_2$ or reverse $[NBD\text{-}TMD]_2$ configuration. Half-size transporters consists of one NBD fused to a TMD (Higgins, 1992; Bauer *et al.*, 1999; Hollenstein *et al.*, 2007a). There are also examples of ABC transporters that do not fit the typical architecture such as the yeast transporter Adp1p, which has a novel [TMD-NBD-TMD] topology (Holland *et al.*, 2003; Iwaki *et al.*, 2006). Some yeast ABC proteins lack TMDs, such as Caf16p and YDR061W that consists of one and two NBDs respectively (Dassa *et al.*, 2001; Kovalchuk *et al.*, 2010).

The NBD is a domain that participates in ATP hydrolysis to drive the substrate translocation. The NBD is composed of several conserved motifs: Walker A (P-loop) and Walker B (Walker *et al.*, 1982); a signature motif (LSGGQ or C-loop) located upstream of the Walker B (Schneider *et al.*, 1998); a Q-loop located between the Walker A and the signature motif with a helical structure that makes contact to the intracellular loop (IL) of the TMD. A hydrophobic ATP-binding pocket is formed at the interface between the Walker A, walker B and C-loop (Lamping *et al.*, 2010).

TMDs contain 12 conserved transmembrane (TM) helices that provide a translocation pathway. Many Intercellular loops (IL) and extracellular loops (EL) extend outside the TMD boundary, which may contribute to substrate transport and specificity (Lamping *et al.*, 2010).

Based on ABC transporter crystal structures, a translocation model called "ATP switch model" is proposed. The translocation presumably starts when the substrate binds to the cavity (high affinity substrate binding site), which is within the TMDs and open to the cytosol. The substrate binding causes dimerization of two NBDs that close the inner gate and trap two ATP molecules. Binding of the ATP cause the ATP-binding pocket to close and the TMD conformation converts from the inward-open to the outward-open position through the interaction of the Q-loop and IL (Higgins *et al.*, 2004; Hollenstein *et al.*, 2007b; Ward *et al.*, 2007; Locher, 2009). When the cavity of TMD opens outward, the substrate is released from the cavity (low affinity site) near the outer membrane (Chang, 2003). Hydrolysis of ATP is regarded as the restoring force of the reversion of the transporter to the inward-open conformation. Binding of the substrate triggers the conformational change of the TMDs to directly facilitate substrate translocation (Dawson *et al.*, 2007; Ward *et al.*, 2007).

### 1.2.4 The classification of fungal ABC transporters and candidate genes of IK726

Based on a phylogenetic analysis of fungal ABC proteins (Kovalchuk *et al.*, 2010), a classification scheme is proposed that fungal ABC proteins can be divided into 9 subfamilies, from A to H plus a group of "non-classified" ABC proteins. Unlike the ABC protein from groups A to H, the "non-classified" subfamily members only contain NBDs and lack transmembrane domains. Several characterized transporters from subfamily B (full-size) with a $[TMD\text{-}NBD]_2$ topology and subfamily G with a reverse topology $[NBD\text{-}TMD]_2$ are involved in MDR and PDR of exogenous toxic compounds (Coleman *et al.*, 2009). Some transporters from subfamilies B (half-size) and C are also involved in detoxification.

## 2. Objective

The objective of this study is to (1) investigate whether *C. rosea* IK726 can tolerate toxic metabolites produced by *P. chlororaphis* MA342, and (2) investigate gene expression of ABC transporter genes in IK726 in response to antifungal metabolites from MA342. Dual culture and mycelial biomass measurement are used to estimate the influence of antifungal metabolites of MA342 on the growth rate and the biomass of IK726. Gene expression of 13 putative ABC transporter genes in IK726 are monitored using quantitative PCR during the treatment of IK726 with culture filtrates from MA342. These 13 genes include five putative members of subfamily B that may be associated with MDR, siderophore and pheromone transport, three subfamily G members putatively involved in PDR, one member of the half-size subfamily B putatively involved in mitochondrial peptide transport and four subfamily C members presumably involved in secondary metabolite transport.

# 3. Materials and methods

## 3.1 Strains and cultural practice

*C. rosea* IK726 (offered by Dan Funck Jensen, Department of Forest Mycology and Plant Pathology) was cultured on potato dextrose agar (PDA, BD Difco) plates at 25℃. A stock culture of *P. chlororaphis* MA342 was offered by Annika Gustafsson, Lantmannen BioAgri AB. MA342 was cultured on vegetable peptone agar (VPA, Thermo Scientific Oxoid) plates at 25℃. *Microdochium nivale* (causal agent of snow mold disease) was offered by Magdalena Grudzinska-Sterno, Department of Forest Mycology and Plant Pathology. *M. nivale* was cultured on PDA plates at 25℃.

## 3.2 In vitro sensitivity assays

### 3.2.1 Dual culture assay

Two confrontation assays were conducted: IK726 and MA342, *M. nivale* and MA342. In each assay, IK726 (or *M. nivale*) and MA342 (or sterile water as a control) were confronted in dual cultures in 9 cm diameter Petri dishes of PDA or VPA. A MA342 colony was suspended in 1 ml sterile water in a 1.5 ml Eppendorf tube and used as stock inoculum. Agar plugs (4 mm diameter) were excised from the edge of fungal colonies (IK726 or *M. nivale*) and positioned 6 cm apart from the bacterial streak (2.5 cm) of MA342 (or sterile water) in 9 cm diameter Petri dishes. Three technical replicates were included in each confrontation treatment. All plates were incubated at 25℃ in darkness. The growth of IK726 and *M. nivale* in each plate was measured as the distance between the inoculation point to the hyphal front after 8, 10, 13 and 18 days. Percentage growth rate reduction by MA342 was calculated as (1-(growth in MA342 plates / mean growth in control plates))*100.

### 3.2.2 Fungal biomass measurement

Erlenmeyer flasks (250 ml) containing 100 ml vegetable peptone broth (VPB, Thermo Scientific Oxoid) or potato dextrose broth (PDB, BD Difco) were inoculated with MA342. The liquid cultures were incubated on a rotary shaker at 120 rpm at room temperature for 2 days, after which the bacteria were removed by filtration. IK726 and *M. nivale* were inoculated in

flasks (3 replicates) containing 100 ml of MA342 culture filtrate. As comparison, Erlenmeyer flasks containing 100 ml fresh VPB or PDB were inoculated with IK726 or *M. nivale* as control. Inoculated flasks were incubated in growth chamber at room temperature for 4 days. The fungal biomass of IK726 and *M. nivale* was collected by filtration through a Buchner funnel and transferred into 15 ml centrifuge tubes. Any MA342 contaminated flasks were excluded from the fungal biomass harvest. Tubes with fungal biomass were dried at 60℃ for 12 hours, after which biomass dry weight (DW) was determined.

## 3.3 Gene expression of ABC transporters in IK726

### 3.3.1 Induction of ABC transporter gene expression in IK726

Culture filtrate of MA342 was prepared as described in section 3.2.2 two days before the experiment. IK726 was cultured in 100 ml PDB for 7 days, harvested by filtration and placed in 100 ml MA342 culture filtrate or fresh VPB as control treatment. Each treatment had five replicates. After 2, 6 and 24 hours of incubation, IK726 mycelium was harvested by filtration. Harvested samples were frozen in liquid nitrogen and then dehydrated in a vacuum freeze dryer until RNA isolation.

### 3.3.2 RNA isolation, quality analysis and cDNA synthesis

Total RNA of IK726 biomass sample from each treatment in Section 3.3.1 was extracted by using the RNeasy Plant Mini Kit (Qiagen). All RNA samples were treated by DNase I (Fermentas) to remove residual traces of DNA. Concentration and purity of the RNA samples were determined using a NanoDrop spectrophotometer (Thermo Fisher Scientific), while RNA integrity was assessed with Agilent Bioanalyzer (RNA 6000 Nanochip, Agilent Technologies). RNA was reverse transcribed to cDNA (iScript Reverse Transcription Kit, Bio-Rad) and then diluted 5 fold. Genomic DNA contamination in RNA samples was assessed by including a no-reverse transcriptase control.

### 3.3.3 Quantitative PCR (qPCR) primers and qPCR conditions

Gene-specific primers (Table 1) for quantitative PCR assays were obtained from Magnus Karlsson (Department of Forest Mycology and Plant Pathology). Primer efficiency values

were determined by amplification of serial dilutions of IK726 genomic DNA (offered by Magnus Karlsson). A primers pair targeting the β-tubulin gene (*Tub*) was selected as a housekeeping gene in IK726 (Mamarabadi *et al.*, 2008).

Table 1. qPCR primers used for monitoring IK726 ABC transporter gene expression

| Sub-Families | Primer Pairs | Sequence 5' – 3' | Sequence 3' -5' | Annealing temp ℃ | Amplicon Size (bp) |
|---|---|---|---|---|---|
| Group B | 3260 F/R | TCAAAGGAAACCGGGCAGAAT | AGACGGCTTGGACAGGGTTAGAGA | 60 | 142 |
| | 4267 F/R | CTGGCCGAGATGACTTGGGTAAAT | AGGTCCGGGCTGAATGTCTGTT | 60 | 191 |
| | 3069 F/R | TCGCGGTCAAGGAGGATACTA | CCAGAGACGGGCGAATGAG | 60 | 159 |
| | 2210 F/R | GGACAAAGACGCCCACTCG | GGTTCGTCCACTTCGGTTCCT | 60 | 117 |
| | 2419 F/R | GAAGCGCTCATCCCCCACTG | GCGGTTCGGATTTGACGGATAG | 60 | 192 |
| Group B (Half-size) | 4987 F/R | GATGGCTCGCGATTGCTCTC | AGTAAGGCCGAAAAGTTTGATGTCT | 60 | 134 |
| Group C | 592 F/R | GACCCGAGTATCTGGAGCAAACA | TCGGGACCCAAGTAGAATGAGC | 60 | 106 |
| | 3918 F/R | CAACCACGCGACTCACCATTC | ATAACAACGAGGCGGCAAGATAGA | 60 | 141 |
| | 2700 F/R | GAAGCGGAAAACAATAGCAAGACAA | AGGTAGGGAAAGGTGAAAAGGAAGG | 60 | 170 |
| | 3433 F/R | AAGGGGTGTTTGCTCTGGGCTAAG | GGAGAGGGCGGCGATAACGA | 60 | 127 |
| Group G | 3523 F/R | GCAGTACTTTGAGGAGCTGGGTTTC | GGGCTTCGGAGGCGTCTTATTCT | 60 | 129 |
| | 3189 F/R | CAGGCCGAGTCCATATTGTCTTCT | TGCTCCAGGGGCGTTGA | 60 | 152 |
| | 4345 F/R | GGTTCCCGACTTCCTCACTTCAA | CGGGGGACCTGGCTCTCG | 60 | 81 |

### 3.3.4 Relative quantification of ABC transporter gene expression in IK726

QPCR reactions were performed in an iQ™5 Multicolour Real-Time PCR Detection System (Bio-Rad) using the Maxima® SYBR Green qPCR Master Mix (Fermentas) and following the protocol: one cycle of 95°C for 5 min, 40 cycles of 95°C for 10 s, 60°C for 30 s and 72°C for 30

s. Five µl of the cDNA solution described in Section 3.3.2 was added as template for each 20 µl volume PCR reaction. Two negative controls were performed including either using sterile water or no-reverse transcriptase control to replace the cDNA template. Auto calculated cycle threshold (Ct) values were used and melt curve analysis was performed after the run. Relative quantification of ABC transporter gene expression from IK726 was achieved using the $2^{-\Delta\Delta Ct}$ method (Livak *et al.*, 2001) with the β-tubulin gene as an internal control. The gene expression levels of ABC transporters from IK726 exposed for 2 hours in fresh supplemented VPB were used as reference treatment. Gene expression data from the three different sampling time points, 2 hours, 6 hours and 24 hours was compared statistically (treated vs. control) using the Student's t-test. P-values below 0.05 were considered significant.

# 4. Results

## 4.1 Dual culture

After 8 days of incubation, *M. nivale* was significantly more inhibited by MA342 than IK726 (Fig.1, P<0.001; P<0.01) on both VPA and PDA medium. Growth of IK726 was reduced by 8% compared with its optimal growth capacity on VPA, and with 26% on PDA. On both VPA and PDA, the growth of *M. nivale* was reduced by MA342 with more than 50% compared with its optimal capacity.

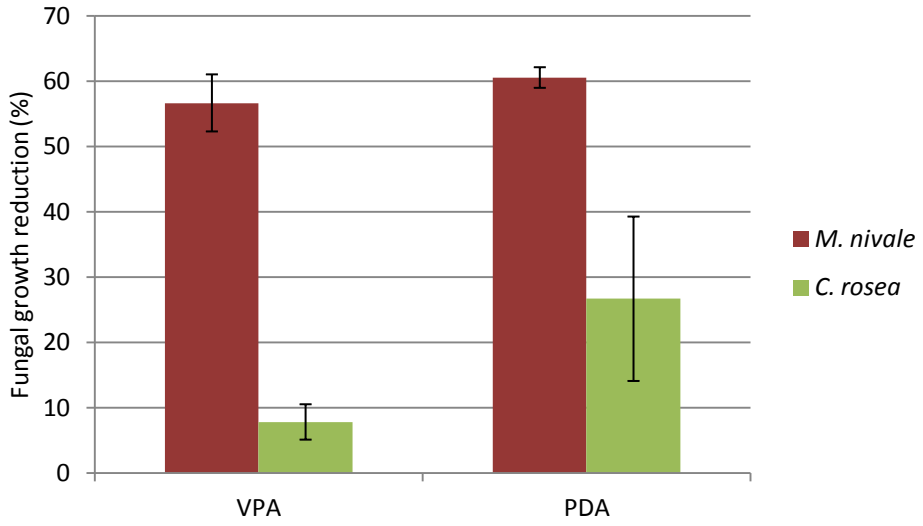



Fig.1 Different effects of *P. chlororaphis* MA342 on the fungal growth of *C. rosea* IK726 and *M. nivale* respectively after dual culture for 8 days on potato dextrose agar (PDA) and vegetable peptone agar (VPA). The fungal growth reduction percentage (y-axis) by MA342 was calculated as (1-(growth in MA342 plates / mean growth in control plates))*100. Two different media (x-axis) were used for the dual culture. Error bars represent the standard deviation of three biological replicates.

## 4.2 Fungal biomass measurement

Because of problems with bacterial contaminations, only parts of valid data were obtained. The experiment demonstrated that growth of *M. nivale* was completely inhibited (P<0.01) in the VPB culture filtrate of MA342, while growth of IK726 was not significantly inhibited

(P=0.21) in the PDB culture filtrate of MA342.

## 4.3 Gene expression analysis

### 4.3.1 RNA quality analysis

Good quality of RNA samples is essential for downstream qPCR gene expression measurements. Our Bioanalyser assay revealed that 12 randomly chosen RNA samples contained sufficiently intact RNA for subsequent cDNA synthesis and quantitative PCR (Fig.2).

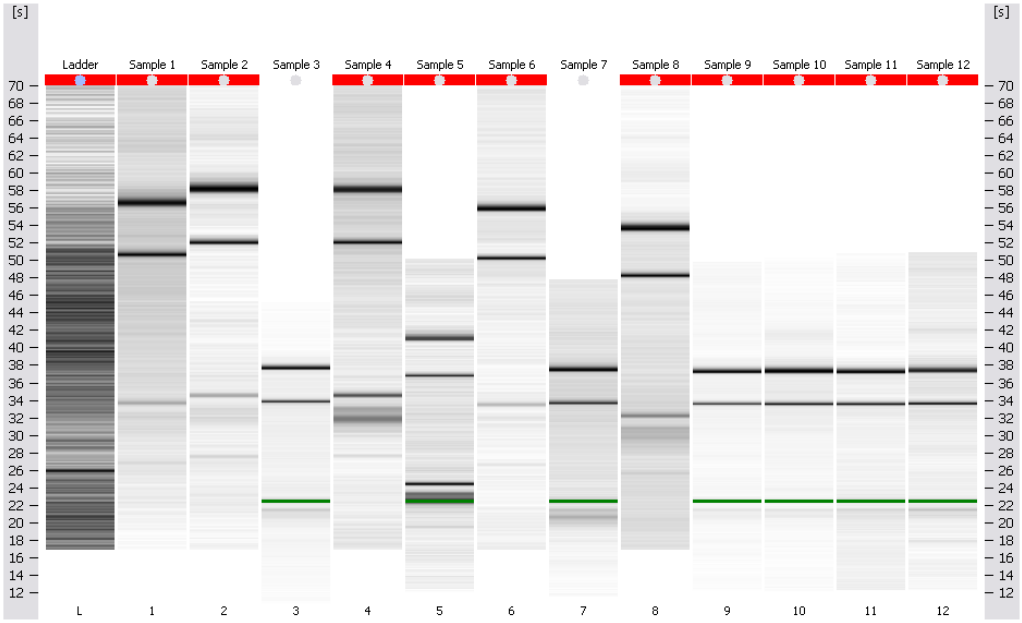



Fig.2 Virtual gel image of total RNA from 12 MA342 treated or non-treated IK726 samples, using gel electrophoresis in an Agilent Bioanalyzer system.

### 4.3.2 Subfamily B

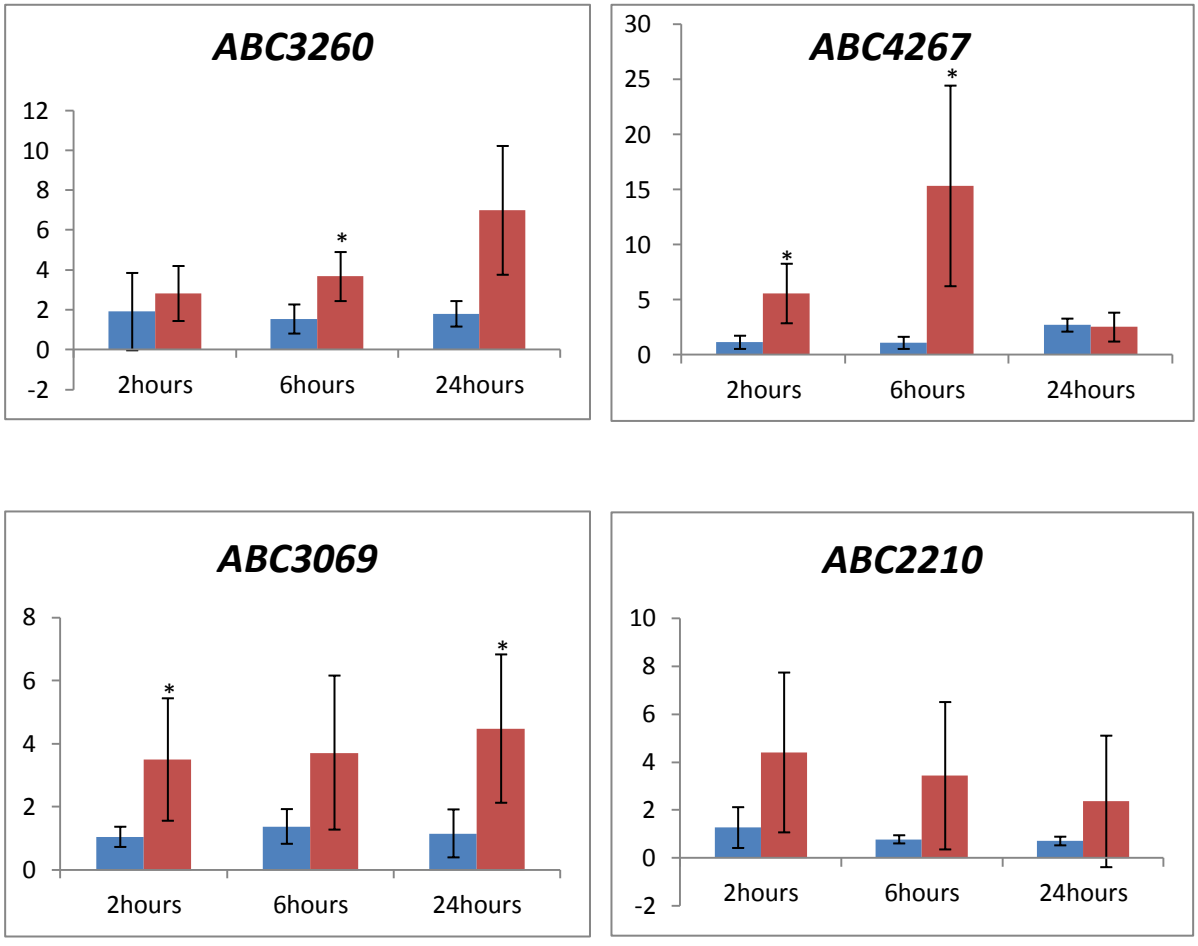
1) The expression of the putative MDR gene *ABC3260* increased 2 fold after 6 hours of exposure of MA342 culture filtrate (Fig.3.1). Another putative MDR gene (*ABC4267*) was induced after 2 and 6 hours of culture filtrate treatment, reaching a 10-fold increase at 6 hours, followed by a decrease to the control level after 24 hours (Fig.3.1).

2) The putative siderophore transporter gene *ABC3069* was induced 4 fold at 6 and 24 hours

of treatment. Another putative siderophore transporter gene ABC2210 was not significantly induced by the MA342 culture filtrate (Fig. 3.1).

3) A putative pheromone transporter gene (*ABC2419*) was significantly up-regulated after 2 hours treated but then decreased to the same level as the non-treated control (Fig 3.1).

4) The expression profile of the putative mitochondrial peptide transporter gene *ABC4987* showed a similar expression pattern as *ABC4267* (Fig 3.1). Gene *ABC4987* was up-regulated during 2-6 hours treatment, reaching a 10 fold increase at 6 hours, followed by a decrease to control levels after 24 hours.



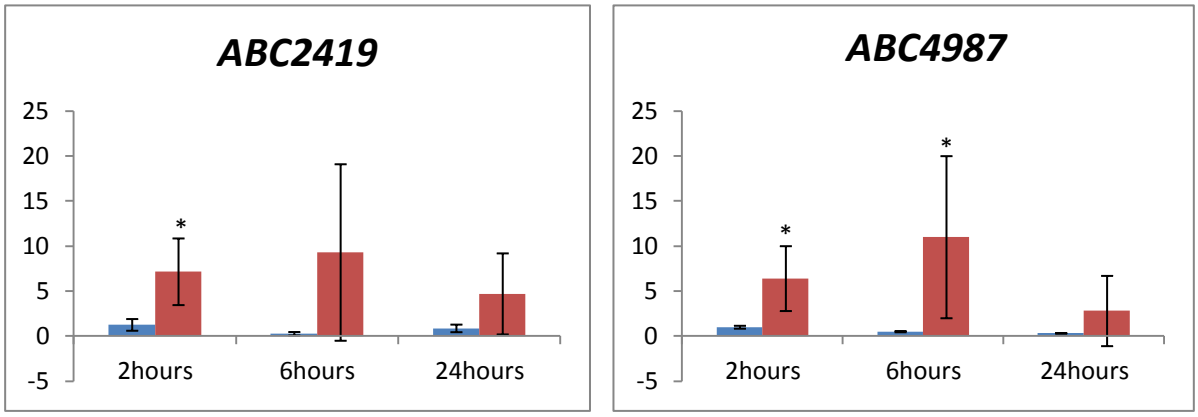
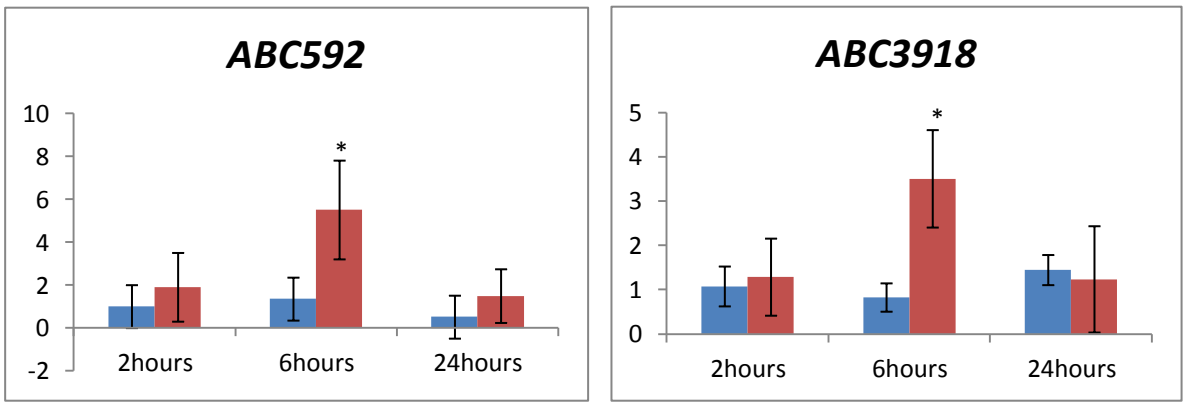



Fig.3.1 Expression profiles of six subfamily B ABC transporter genes in *C. rosea* IK726 during mycelium grown in the culture filtrates of *P. chlororaphis* MA342. Gene expression fold change (y-axis) was calculated with the $2^{-\Delta\Delta Ct}$ method. The blue bars represent the control treatment with fresh vegetable peptone broth (VPB), while the red bars represent the MA342 culture filtrate treatment. Error bars represent the standard deviation of five biological replicates. An asterisk indicates a significant difference ($P \leq 0.05$) between the treated and non-treated treatments at same time point according to Student's t-test.

### 4.3.3 Subfamily C

The expression of the putative secondary metabolite transporter genes *ABC592* and *ABC3918* was up-regulated 3-6 fold after 6 hours of exposure to the culture filtrate (Fig 3.2). In contrast, no significant changes in expression of *ABC3433* and *ABC2700* were detected during 24 hour experimental period (Fig 3.2).



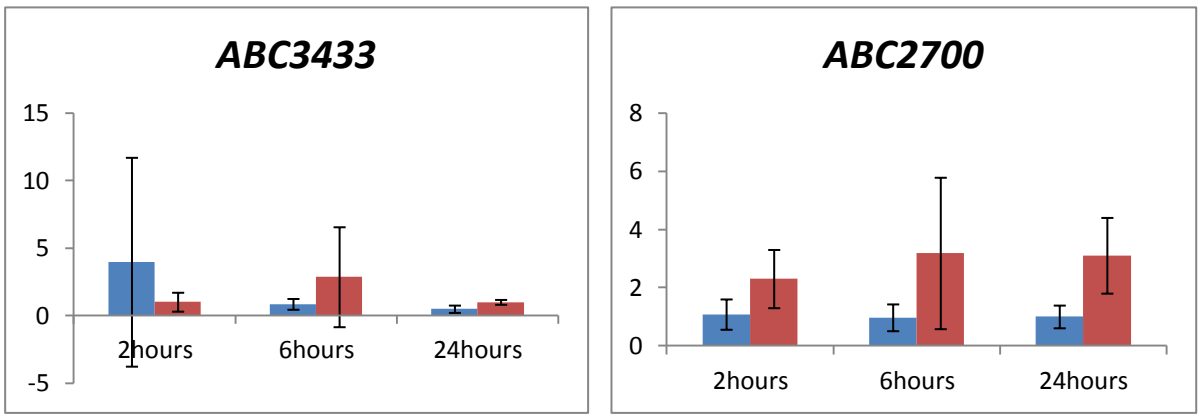
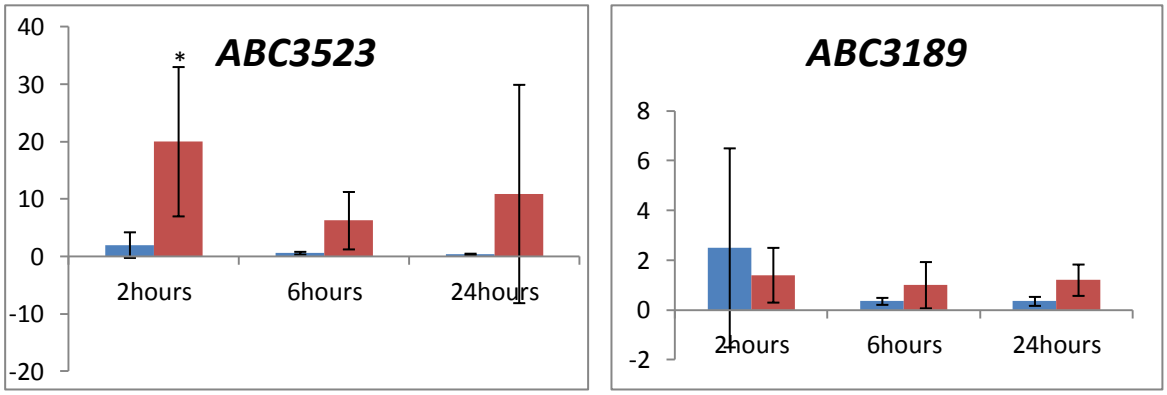



Fig.3.2 Expression profiles of four subfamily C ABC transporter genes in *C. rosea* IK726 during mycelium grown in the culture filtrates of *P. chlororaphis* MA342. Gene expression fold change (y-axis) was calculated with the $2^{-\Delta\Delta Ct}$ method. The blue bars represent the control treatment with fresh VPB, while the red bars represent the MA342 culture filtrate treatment. Error bars represent the standard deviation of five biological replicates. An asterisk indicates a significant difference ($P \leq 0.05$) between the treated and non-treated treatments at same time point according to Student's t-test.

### 4.3.4 Subfamily G

All three genes in subfamily G are putatively involved in PDR. The expression of *ABC3523* was significantly up-regulated (over 10 fold) after 2 hours of treatment and then down-regulated during 6-24 hours (Fig.3.3). No significant difference in the expression level of *ABC3189* between the treated and non-treated samples was detected over the experimental period. A 2 fold up-regulation was observed for *ABC4345* at 6 hours comparing with the level at 6 hours non-treated control.



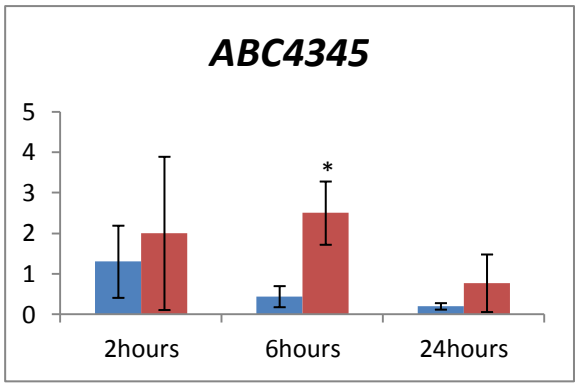



Fig.3.3 Expression profiles of three subfamily G ABC transporter genes in *C. rosea* IK726 during mycelium grown in the culture filtrates of *P. chlororaphis* MA342. Gene expression fold change (y-axis) was calculated with the $2^{-\Delta\Delta Ct}$ method. The blue bars represent the control treatment with fresh VPB, while the red bars represent the MA342 culture filtrate treatment. Error bars represent the standard deviation of five biological replicates. An asterisk indicates a significant difference ($P \leq 0.05$) between the treated and non-treated treatments at same time point according to Student's t-test.

# 5. Discussion

## 5.1 In vitro sensitivity assay

*C. rosea* IK726 was significantly less inhibited by *P. chlororaphis* MA342 than the pathogen *M. nivale* in dual cultures, which shows that IK726 is highly tolerant to metabolites produced by MA342. This trait of IK726 indicates that IK726 possess a mechanism of antibiotic tolerance, not found in *M. nivale*. The biomass of *C. rosea* IK726 harvested from the liquid culture was slightly reduced by *P. chlororaphis* MA342. Similar result was also observed that the slight non-target growth inhibition of plant beneficial fungi in the case of the co-inoculant of *Trichoderma atroviride* and *P. fluorescent* (Lutz *et al.*, 2004). But in the research of Lutz et al, it was suggested that the slight growth inhibitory of biocontrol fungal might be compensated by the induction of genes involved in biocontrol activity. In another case of *Clonostachys* sp. and *Bacillus subtilis* (Li *et al.*, 2005), more *C. rosea* chlamydospores were induced by antibiotic metabolites *from B. subtilis*, which may improve the biocontrol duration and survival of *C. rosea*. The feasibility and biocontrol efficacy of strain mixtures are not only depended on compatible utilization of nutrients, minerals and spaces (Lutz *et al.*, 2004) but are also influenced by signaling pathways between different biocontrol microbes.

## 5.2 Gene expression analysis

### 5.2.1 Subfamily B

1) The transcript profiles of putative MDR transporter genes *ABC4267* and *ABC3260* showed that these genes were rapidly induced (within 6 hours) in response to the MA342 culture filtrate. This pattern is consistent with the function of drug efflux pumps in the early stage of detoxification that supposedly decrease the intracellular toxic concentration rapidly. The repression of *ABC4267* at 24 h may suggest that enough ABC transporter protein is made at this stage and/or that other, yet uncharacterized, more efficient detoxification mechanisms are activated at this stage as in the case of the BcatrB efflux pump in *B. cinerea* (Schouten *et al.*, 2008). For ABC transporters, outward translocation of every single toxic substrate consumes two ATP molecules. Such high energy cost means that ABC transporters are not an ideal long-term detoxification mechanism.

2) The induction of the putative siderophore transporter gene *ABC3069* may be related to iron-limiting conditions in the MA342 culture filtrate. *Pseudomonas* sp. is known to produce high amounts of various siderophores (Matthijs *et al.*, 2007; Youard *et al.*, 2007) to compete with many other microbes in iron-limiting conditions (Kloepper *et al.*, 1980). The 48 hours preculture of MA342 in the VPB used for preparation of the culture filtrate may have reduced the Fe availability considerably. The induction of *ABC3069* in IK726 may be a response to Fe deficiency in the culture filtrate due to siderophore release by MA342. Details about siderophore synthesis in MA342 are still limited.

3) Induction of the *ABC4987* expression, a putative mitochondrial peptide transporter, coincides with the expression of MDR transporter gene *ABC4267*. It is possible that the ATP dependent ABC transporters consume large amount of ATP during translocation of MA342 metabolites in the culture filtrate treatment, which lead to more synthesis of mitochondria to produce more energy.

### 5.2.2 Subfamily C

Induction of genes *ABC592* and *ABC3918*, putatively involved in secondary metabolite transport, at 6 hours treatment may indicate that IK726 produce secondary metabolites in

response to MA342 metabolites or proteins in the culture filtrate. For example, *Clonostachys sp.* is known to produce and secrete cyclic pentapeptides, argidin and argifin, which act as potent inhibitors of chitinases (Arai *et al.*, 2000). It is possible that induction of *ABC592* and *ABC3918* is related to the secretion of compounds that inhibit MA342 enzymes present in the culture filtrate. In addition, some *Clonostachys sp.* possesses an efficient enzymatic detoxification mechanism to convert Zearalenone (ZEN) mycotoxin, produced by *Fusarium* sp. to a non-toxic product (Takahashi-Ando *et al.*, 2002; Takahashi-Ando *et al.*, 2004). The induction of *ABC592* and *ABC3918* in IK726 may also be related to the secretion of degradation products from yet unknown detoxification processes.

### 5.2.3 Subfamily G

The transient up-regulation of putative PDR genes *ABC3523* and ABC*4345* was observed at 2 hours and 6 hours treatment respectively. The similar gene expression patterns observed in both putative PDR and MDR transporter genes may suggest that both group B and G ABC transporters contribute to a rapid antibiotic efflux mechanism in *C. rosea* IK726. Co-regulation of several ABC transporters are reported from another mycoparasitic species, *T. atroviride,* in the presence of fungal pathogens or plant tissue (Marra *et al.*, 2006; Woo *et al.*, 2006; Hermosa *et al.*, 2012).

### 5.2.4 Improvement of the experimental set-up

Large variation between replicates was obtained for expression levels for certain genes using the current experimental set-up. One possible reason for this may be differences in the levels of antibiotic metabolites between different MA342 culture filtrates, as neither the antibiotic levels nor the bacterial titer were normalized. One way to optimize the experimental set-up can be to pool culture filtrate from different flask, or to work with purified compounds to minimize the variation between replicates.

## 6. Conclusion

The aim of the present study was to provide a preliminary exploration of the feasibility of combining two biocontrol microbes, *C. rosea* and *P. chlororaphis*. The sensitivity assay demonstrated that *C. rosea* IK726 possess sufficient mechanisms to tolerate antifungal compounds from MA342. Certain ABC transporter genes were significantly induced by the treatment with *P. chlororaphis* MA342 culture filtrate. This result indicates that ABC transporters may play a role in tolerance/detoxification mechanisms in *C. rosea* IK726. Further functional analyzes are necessary to improve our understanding of the function of ABC transporters during the interaction between these two biocontrol microbes.

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