UNIVERSITÀ DI PISA

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Gene expression analysis of ABC- and MFS-transporters in the fungal biocontrol agent *Clonostachys rosea*

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A me stesso (2)

“Bimbo, per imparare si comincia dal basso: inizia a lavare i tubetti da collezione!”

a Mau

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1. Introduction

1.1 Biopesticides: a short description

The adverse environmental impact due to massive use of chemical pesticides in agricultural practices was the main driving force by which biological control researches were intensified over the last three decades. Chemical residues could affect both quality and safety of food and feed. Thus, there was a need to develop new biological disease control measures with less adverse impacts (Jensen *et al.*, 2007). Biopesticides could be defined as naturally occurring substances to control pests by nontoxic mechanisms. They are living organisms (natural enemies) or their products (e.g., phytochemicals, microbial products), which can be used for the management of pests injurious to crop plants (Usta, 2013). To achieve efficient biocontrol, the organism must normally be alive and active at the sites where there is to control the target pathogens. BCAs should be robust like many chemicals when they are exposed to biotic and abiotic factors such as plant-, soil- and environmental factors. The advantages of using biopesticides are based on the following factors:

- Target specificity, designed to affect only one specific pest or a few target organisms;

- Environmental sustainability; often effective in very small quantities and often decompose quickly, thereby resulting in lower exposures.

Some of the most common fungal BCAs already commercialized are ‘Root Shield’ (*Trichoderma harzianum*), ‘Supresivit’ (*T. harzianum*), ‘BinabT’ (*T. harzianum* + *T. polysporum*), ‘Trichodex’ (*T. harzianum*), ‘Tricho-Dry’ (*Trichoderma* sp.), ‘GlioMix’ (*Gliocladium catenulatum*), ‘ContansWG’ (*Coniothyrium minitans*) and 'Remedier' (*T. asperellum* and *T. gamsii*) (Jensen *et al.*, 2007).

There are some biological and technical issues to deal with in order to develop biocontrol agents such as isolation, screening and selection of potential biocontrol agents; knowledge of biology of

selected organism and its interaction with the pathogen(s); ecology of the BCA (especially the interactions between biocontrol agent and the non-target organisms); production (liquid or solid fermentation); 'large-scale' production; formulation (shelf life); compatibility with existing technologies and delivery systems; seed treatment (seed coating, biopriming etc.); incorporation and application in growth substrates. Each one of these aspects needs to be evaluated before registration (Jensen *et al.*, 2007).

Risk assessment, field performance evaluation, commercial aspects, the economic importance of the disease to be controlled, cost of development, production, registration and marketing are some of the hindrances to deal with during BCAs registration (Jensen *et al.*, 2007).

1.2 Clonostachys rosea IK726 as biocontrol agent

Clonostachys rosea[1] strain IK726 was found out by adopting a 'hierarchic screening procedure'– an *in planta* screening (Figure 1.0, Knudsen *et al.*, 1997) – wherein barley seeds infested with *Fusarium culmorum* were inoculated with test organisms directly at sowing and the disease index was scored. At a later stage, the best candidates selected - which registered the lowest disease indexes - were tested in large field experiments (Figure 1.1). Strain IK726 was selected following this hierarchical procedure as one of the best candidates among more than 400 fungal isolates screened (Jensen *et al.*, 2007).

[1] *Clonostachys rosea* (Link: Fr.) Schroers, Samuels, Siefert & Gams (Fungi, Ascomycota, Hypocreales, Bionectriaceae). Teleomorph: *Bionectria ochroleuca*.

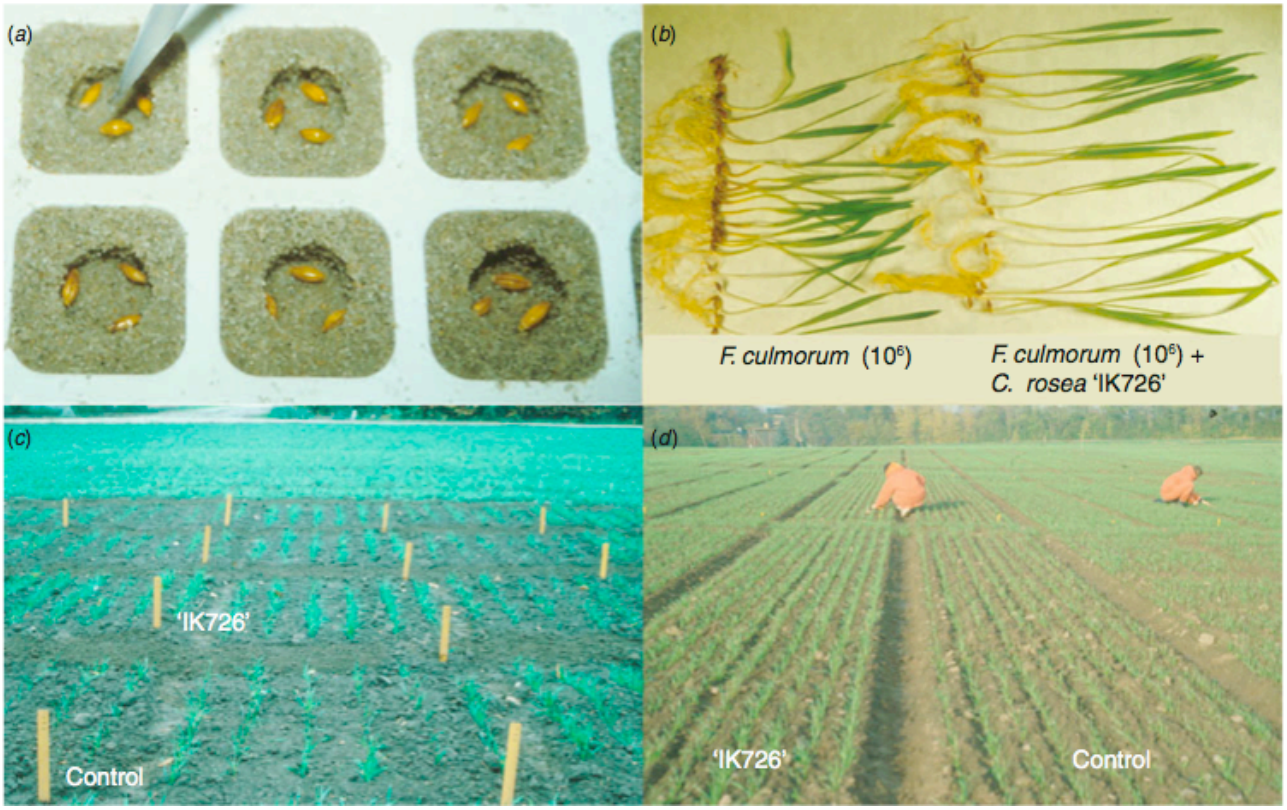

Figure 1.1The hierarchical *in planta* screening procedure used for the selection of IK726. (a) Fungal isolates were screened for antagonistic potential on barley infested with *F. culmorum* and planted in pots with moist sand. (b) The disease index was scored 19 days after sowing. (c) Selected candidates from the sand- screening test were tested in small field plots. (d) The best candidates were tested for biocontrol performance in large field experiments. Photographs supplied by Inge M. B. Knudsen. (Jensen *et al.*, 2007).

With the hierarchical screening method there is no risk of discarding potential antagonists, which do not show any effect *in vitro*. Moreover, mechanisms such as induced resistance and plant growth promotion – which are gaining more and more attention – are not detected using an *in vitro* screening approach such as dual culture tests. Dual cultures screening might be of relevance to select the best candidate among isolates belonging to a specific group of organisms which already have shown to be good candidates in field experiments (Jensen *et al.*, 2007).

C. rosea IK726 has proved to be an effective antagonist in several crops against diseases caused by a range of pathogens, e.g. *Alternaria* spp. (Jensen *et al.* 2004), *Bipolaris sorokiniana* and *Fusarium culmorum* (Knudsen *et al.* 1995), *Pythium* spp. (Møller *et al.* 2003), *Tilletia tritici* (Jensen *et al.*

2001) and *Botrytis cinerea* (Macedo *et al.* 2012). Despite these evidences, *C. rosea* IK726 has not been commercialized yet.

Information about biological and technical issues on BCA registration has been collected on *C. rosea* IK726 after its selection. For example, IK726 can be produced using both liquid and solid state fermentation, even if solid fermentation resulted in conidia with better survival than those produced in liquid fermentation (Jensen *et al.* 2002). Coating barley seeds with freshly harvested conidia of *C. rosea* IK726 showed a high efficacy in the biocontrol of both *F. culmorum* and *B. sorokiniana* using dosages above 10^4 cfu – colonies forming units - per seed (Jensen *et al.* 2000). Furthermore, the best shelf life, more than one year, was obtained using solid state fermentation lasting over 20 days, followed by rapid drying and storing conidia at 20°C or below in sealed bags together with a desiccant such as blue silica gel (B. Jensen *et al.*, unpublished data).

The knowledge of the effects of pesticides commonly applied in crop production on biocontrol fungi is of paramount importance for their exploitation in Integrated Pest Management, as different fungi have different sensitivity to pesticides of the same chemical class or same mode of action (Macedo *et al.* 2012).

C. rosea IK726 has been shown to be compatible with both several insecticides and other chemical compounds in concentrations used in seed technologies such as seed coating and pelleting, and several fungicides as well (Danisco Seed, unpublished data). Moreover, a mixture of *C.rosea* strain 47 and/or *Trichoderma atroviride* strain 312 with thiram and triticonazole – two fungicides known to be effective against *F. culmorum* - applied to wheat seeds, was able to control *Fusarium culmorum* artificially inoculated to wheat seedlings in growth chambers. In the field, the antagonists applied along with triticonazole or thiram at 1/10 of the field dose to seeds naturally infected by *F. culmorum*, gave a disease control comparable to that of triticonazole at full field dose (Roberti *et al.*, 2006). Figure 1.2 shows symptomatic/asymptomatic wheat spikes infected by Fusarium Head Blight (FHB) species complex.

Figure 1.2 *F. culmorum* is a causal agent – together with other *Fusarium* species – of the 'Fusarium Head Blight' (FHB or scab, brown spikes in figure), which affects cereals and causes yield losses and accumulation of mycotoxins.

Since *C. rosea* could be an endophyte for several crops, it is worth focusing the attention on the effects of contact fungicides on spore germination, as after germination the fungus penetrates the leaves, where contact fungicides do not arrive.

C. rosea IK726 has proved to be effective against the seedborne pathogens *Alternaria* spp. when introduced at the beginning of carrot seed priming - called 'biopriming' - and the field emergence was improved significantly as well (Jensen *et al.* 2004). *C. rosea* IK726 can be incorporated in soil or in greenhouse substrates - such as sphagnum peat or composted plant material - in order to control soilborne pathogens (Jensen *et al.*, 2007). Using GFP-technology, it could be possible to follow the germination of spores of *C. rosea* IK726 in sphagnum peat, on leaf surfaces and seed coats without the addition of nutrients (Jensen *et al.* 2004; 2007).

Moreover, *C. rosea* IK726 has never shown negative effects on plant growth – on the contrary, a plant growth promotion effect on barley has been observed – and it can stimulate soil enzyme

activity and soil microbiota, especially culturable pseudomonas and Gram-positive bacteria (Johansen *et al.* 2005).

Beneficial effects of *C. rosea* are based on nutrient competition, mycoparasitism, antibiosis and induced resistance (Yu & Sutton, 1999; Sutton *et al.*, 1997). It has also been shown that the fungus produces 'Cell Wall Degrading Ezymes' (CWDEs) - such as chitinases and endoglucanases - which were demonstrated to play a significant role in successful biocontrol of *C. rosea* IK726 against *B. cinerea*. The activity of *C. rosea* against *B. cinerea* was assessed both *in vitro* - by co-cultivation of *B. cinerea* and *C. rosea* – and on detached and wounded strawberry leaves showing the inhibition of *B. cinerea* in all pathogen–antagonist interactions (Figure 1.3, Mamarabadi *et al.* 2008).

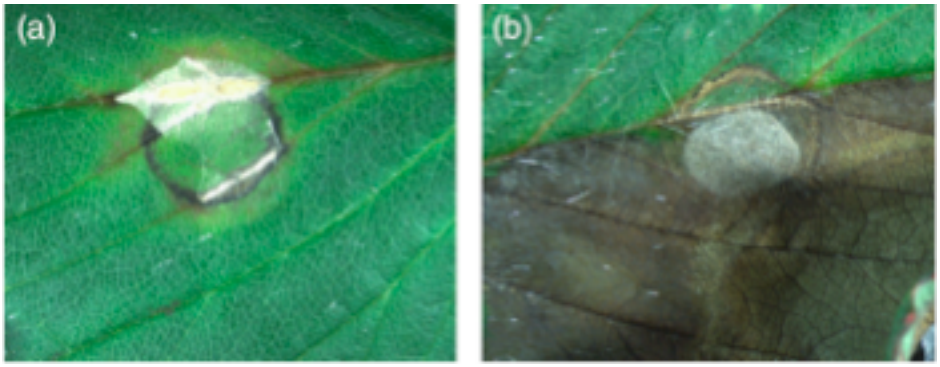

Figure 1.3 Detached strawberry leaves 1 week after inoculation with *Botrytis cinerea*. (a) The leaf was inoculated with *Clonostachys rosea* 24h before application of the pathogen. (b) No antagonist was applied to the leaf where *B. cinerea* caused a large necrotic lesion (Mamarabadi *et al.* 2008).

Real-time reverse transcriptase (RT-PCR) technique was used to analyze gene expression both *in vitro* and in strawberry leaves. The exochitinase gene *cr-nag1* along with the endochitinase *cr-ech42* and *cr-ech37* genes play a significant role during interaction between *C. rosea* and *B. cinerea* since they were up-regulated both *in vitro* and in strawberry leaves. The same assay shows that the enzymes were up-regulated when *B. cinerea* is active – that is when *B. cinerea* is established on the leaf or in the medium before *C. rosea*. In this case, the antagonist is triggered to release more enzymes. When *C. rosea* is established before *B. cinerea* or the fungi are co-inoculated on leaves or in growing media, *B. cinerea* is inhibited and subsequently killed – probably by antibiosis as Authors suggested - or inhibited by substrate competition. At the end, *C. rosea* releases enzymes in

order to necrotrophically utilize the pathogen (Mamarabadi *et al.* 2008). Gene expression analysis represents a fundamental tool to analyze what happens during two- or three-way interactions, but transcript profile patterns might be subject to changes depending on both biological material and the set-up of the assay..

Deoxynivalenol (DON) and Zearalenone (ZEA) [Figure 1.4 shows the molecular structure of the two mycotoxins] are two mycotoxins commonly produced by fungi included within the Fusarium Head Blight (FHB) species complex – with *F. graminearum*, *F. culmorum* as the predominant. DON is a potent protein synthesis inhibitor, which binds eukaryotic ribosomes compromising protein translation. Moreover, DON may has an additional role besides being a virulence factor since it repressed the activity of the cell wall degrading enzyme N-acetyl-beta-D-glucosaminidase chitinase in the biocontrol fungus *Trichoderma atroviride* (Lutz *et al.*, 2003). ZEA is a non-steroidal mycoestrogenic toxin linked both to infertility and cancer. Even if its role is not clarified yet, it was proposed that ZEA synthesis increases competitiveness with other fungi sharing the same niche (Reddy *et al.*, 2010; Zinedine *et al.*, 2007; Utermark and Karlovsky, 2007).

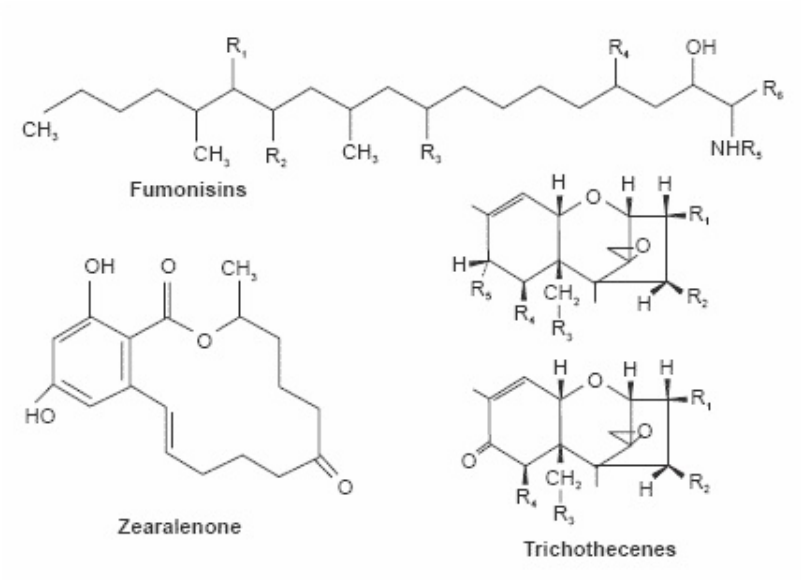

Figure 1.4 Zearalenone (ZEA) and Trichothecens (such as Deoxynivalenol, DON) molecular structure. http://images.engormix.com/e_articles/myco_binding01.gif

Kosawang and colleagues (2014) clarified the molecular basis underlying the capability of *C. rosea* IK726 to tolerate both DON and ZEA. Authors proposed that the metabolic readjustment was a major factor in DON tolerance for *C. rosea* IK726. The DON-induced cDNA library revealed that CYP450, diacyl- glycerol o-acyltransferease and pyruvate decarboxylase were significantly expressed during DON interaction at 72 hai (hours after inoculation), as well as encoding enzymes involved in the triglyceride synthesis pathway (energy reservoir) and stress-response proteins - such as heat shock proteins (Hsp70 and Hsp90 subunit, which prevent protein aggregation and degradation). Thus, the increased need of cellular energy is to produce proteins to compensate those destroyed by DON (Kosawang *et al.*, 2014).

Besides *zhd101* (zearalenone hydrolase, an enzyme which cleaves off one of the lactone rings in the backbone of ZEA, reducing its toxicity), which was expected in ZEA-induced cDNA library, a significantly expression of two ABC-G transporters – ABCG29 and ABCG5 – was noticed after 2 hours of exposure to ZEA. These ATP-Binding-Cassette transporters (whose features will be discussed hereafter, see 'ATP-Binding Cassette (ABC) and Major Facilitator Superfamily (MFS) proteins') belong to group G, which consists of members sharing relevant functions on xenobiotic/drug transport. Further gene expression analysis supported the EST (Expressed Sequence Tag) redundancy data. While *zhd101*, *abcg29* and *abcg5* expression was trigged 2 hours after inoculation with ZEA - and it decreased swiftly at later time points – the same target genes were induced to a lesser extent in DON interaction (Kosawang *et al.*, 2014).

It has not been proposed a unique role (if it be so) for these two ABC transporters yet, whether they are expressed before ZEA degradation – in this case their expression would be promoted by ZEA itself – and/or if the activity of the enzyme ZHD101, whose degradation product is 1-(3,5-dihydroxyphenyl)-6′- hydroxy-1-undecen-10′-one, would trigger their expression due to the toxic activities of the degradation product. However, this study shows that *C. rosea* IK726 acts with a

specific mechanism to withstand ZEA – by efflux of ZEA and/or its digested products. (Kosawang *et al.*, 2014).

1.3 ATP-Binding Cassette (ABC) and Major Facilitator Superfamily (MFS) proteins

Selective transport of most solutes and specific substrates across the lipid bilayer is mediated by a great variety of transport proteins, including transporters and channels, which are present in every single cell (Yan N, 2013). 'Multiple Drug Resistance' (MDR) identifies those mechanisms involved in pumping of solutes/substrates out of the cell. There are two main classes of efflux pumps responsible for fungal drug resistance, each with a different pumping mechanism and source of energy: ATP-binding cassette (ABC) proteins - primary transporters that use the hydrolysis of ATP as source of energy - and Major facilitator superfamily (MFS) pumps - secondary transporters that utilize the electrochemical gradient across the plasma membrane to translocate substrates. Both classes of pumps are integral membrane proteins with distinctive functional domains: ABC pumps contain nucleotide-binding domains (NBDs) while both ABC- and MFS- transporters contain transmembrane domains (TMDs) that confer substrate specificity (Lamping *et al.*, 2010). Whereas ABC transporters can transport macromolecules such as proteins, complex carbohydrates and phospholipids, the MFS channels can transport only smaller substrates (e.g., ions). This is likely due to differences in dimension and basic architectural features between these superfamilies. ABC superfamily and MFS account for nearly half of the solute transporters encoded within the genomes of microorganisms (Pao *et al.*, 1998).

1.3.1 ATP-Binding Cassette superfamily: ATP-binding cassette (ABC) proteins are one of the largest protein families and the number of its members is expanding rapidly while genomes sequencing. ABC proteins are present in every living cell, ranging from Archaea and Bacteria to higher eukaryotes. The main characterized function of ABC proteins is represented by ATP-

dependent transport of a broad range of substrates across biological membranes. However, the role of ABC proteins is not limited to active transport but deal with other functions such as ion channels, receptors, mRNA translation and ribosome biogenesis. The structure of a typical ABC transporter includes two nucleotide-binding folds (NBFs) and two transmembrane domains (TMDs). There is just one such unit (one NBF/TMD) in 'half-size' transporters, which likely function as either hetero- or homodimers. The ABC proteins not involved in membrane transport generally lack the TMDs (Kovalchuk and Driessen, 2010). The NBFs in 'full-size' transporters are involved in cooperative ATP binding and hydrolysis while the TMDs usually operate in pairs and each usually includes six putative a-helical transmembrane segments. The whole molecular mass of a full-size ABC transporter is approximately 160 kDa. Several steps are involved in such transportation:

- Binding of substrate to a high-affinity binding site in the TMD that is usually considered to be open to the cytosol or the inner leaflet of the membrane;

- Binding of efflux substrate triggers a conformational change in the NBDs: this allows NBD dimerization in the presence of ATP;

- The conformational change opens the substrate-binding pocket to the extracellular space and the substrate is released;

- After substrate release, hydrolysis of the bound ATP returns the transporter to its original conformation and the transport cycle can be repeated. Thus, binding of ATP, and not its hydrolysis, is considered the power stroke of substrate transport (Lamping *et al.*, 2010).

The identification of ABC proteins within genome sequences is relatively easy thanks to the conserved motifs 'Walker A' and 'Walker B' boxes, separated by about 120 amino acid residues, and the 'ABC signature motif' situated between the two Walker boxes. In order to adopt a common classification scheme, all eukaryotic ABC proteins have been divided into eight major subfamilies –

ranging from A to H – according with the 'Human Genome Organization' (HUGO) scheme, as shown in Figure 1.5:

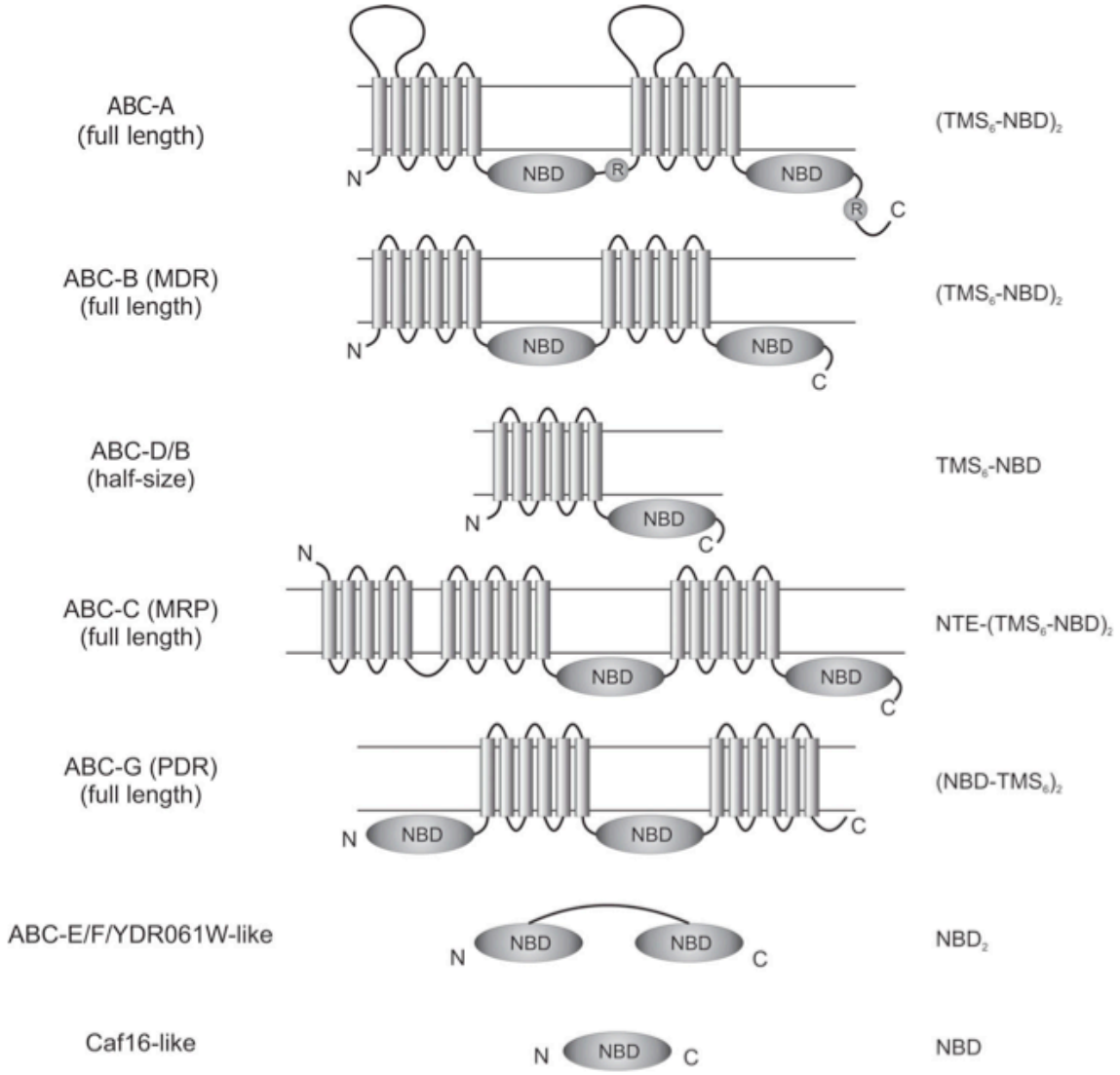

Figure 1.5 Predicted topology and domain organization of different subfamilies of fungal ABC proteins. NBD, nucleotide-binding domain; NTE, N-terminal extension; TMS, transmembrane segment. (Kovalchuk and Driessen, 2010)

This subdivision was based on sequence comparison of NBF motifs (Kovalchuk and Driessen, 2010). Authors analyzed a representative set of fungal genomes – ranging from saprophytic species, animal pathogens to plant pathogens - whose genome sequences were publicly available. In total, 27 species representing 5 phyla and 18 orders of fungi were analyzed. In general, the ABC proteins

per genome varied by more than 5-fold between different species and the highest number was found in members of the subphylum *Pezizomycotina*.

Few ABC proteins are known to be required for cell viability. Actually, *Saccharomyces cerevisiae* has only three essential ABC proteins, Yef3p, Arb1p, and Rli1p, none of which is involved in transport. *S. cerevisiae* was the first eukaryote whose genome was sequenced and, thus, a complete list of ABC proteins is available. However, the number of essential ABC proteins might be larger in filamentous fungi since Kovalchuk and Driessen (2010) found a significantly reduced set of ABC proteins within the order of *Saccharomycetales* (both *S. cerevisiae* and *Saccharomyces pombe*) especially compared to the subphylum *Pezizomycotina* (in details, the highest number of ABC proteins were found in *Aspergillus* species and *Gibberella zeae*). Thus, several ABC proteins present in Ascomycetes/Basidiomycetes are missing from *Saccharomycetales* genome. So far, this is the main reason for the lack of functional information about ABC proteins in fungal species.

ABC proteins have undergone a significant diversification after the divergence of fungal phyla (Chytridiomycetes, Zygomycetes, Ascomycetes, and Basidiomycetes). The great variety of ABC proteins observed in the fungal genomes is likely due to gene duplication processes as well as gene loss events. However, the number of ABC proteins in the genomes of Basidiomycetes tends to be lower than in those of Ascomycetes. In effect, the phylum *Ascomycota* is following an increasing trend in ABC proteins number. This is especially evident within the subphylum *Pezizomycotina*, whereas *S. pombe* and *Saccharomycetales* have a significantly reduced set of ABC proteins. Furthermore, *Pezizomycotina* contains the most diverse sets of ABC proteins, with several groups of proteins specific for this subphylum (Kovalchuk and Driessen, 2010).

Marra and colleagues (2006) found that in different *Trichoderma* spp. some ABC transporter genes were up-regulated during three-way interactions among various plants and fungal pathogens, which may support both antagonistic activity and root colonization. However, the information about physiological functions of ABC transporters is still scarce and mainly restricted to multidrug

resistance. Nowadays, the ABC proteins involved in multidrug resistance in human pathogens such as *Candida albicans*, *Aspergillus fumigatus*, *Cryptococcus neoformans* as well as *Emericella nidulans* (anamorph *Aspergillus nidulans,* a model filamentous fungus) and *Magnaporthe grisea* (plant pathogen) have been functionally characterized (Kovalchuk and Driessen, 2010).

ABC-C subfamily: ABC-C proteins are full-length transporters found in all major groups of eukaryotes. Most of them contain an additional N-terminal hydrophobic region (Figure 1.5). Phylogenetic analysis revealed seven clusters. Some ABC-C transporters are involved in the detoxification of toxic compounds by means of their extrusion from the cell or sequestration in the vacuole – the members of cluster VI are related to *S. cerevisiae* Ycf1p (YDR135C) and Bpt1p (YLL015W) proteins, which conjugate drugs with anionic compounds such as glutathione. Interestingly, one of these clusters - group III - is specific for both *Pezizomycotina* (single gene) and Basidiomycetes (two genes). Remarkably, at least four out of the seven *Aspergillus nidulans* proteins belonging to this subfamily are associated with secondary metabolism clusters, meaning that such transporters are involved in the export of secondary metabolites (Kovalchuk and Driessen, 2010).

ABC-G Subfamily: In the ABC-G transporters subfamily, the nucleotide-binding domain precedes the transmembrane domain: this unique feature distinguishes this subfamily from the others. Several of these transporters ('full-length' ABC-G transporters) are linked to pleiotropic drug resistance (PDR) phenomena, contributing to the export of various hydrophobic molecules, sterol uptake – as well as translocation of various lipid molecules - and anaerobic growth. Their massive expansion in fungal genomes apparently occurred after the diversification of the major fungal lineages. The highest number of members of this subfamily was observed in *Aspergillus oryzae* (as many as seventeen instead of two in *S. pombe*). The phylogenetic analysis reveals that ABC-G proteins are the least conserved among fungal ABC proteins, suggesting their rapid evolution after the divergence of the main fungal lineages. Five clusters of ABC-G transporters have been

recognized. All of them, except for Group IV, are restricted to the genomes of Ascomycetes and Basidiomycetes. Cluster I seems to be specific for Ascomycetes, as they were not almost found in Basidiomycetes genomes, with the exception of *Cryptococcus neoformans*. ABC-G genes number ranges from one in *Neurospora crassa* up to eight in *Gibberella zeae*. This group includes transporters with well-known roles in multidrug resistance as for instance the *S. cerevisiae* Pdr5p, Pdr10p and Pdr15p as well as the *C. albicans* Cdr1p, Cdr2p, Cdr3p, and Cdr4p proteins (Kovalchuk and Driessen, 2010).

Ruocco and colleagues (2009) characterized *Taabc2* in *Trichoderma atroviride*, a gene belongs to pleiotropic drug resistance (PDR), suggesting that TAABC2 has a role in the resistance and transport of various toxins and antibiotics. As early as in 1997 de Waard suggested that ABC transporters in Fungi can play a significant role in fungicide sensitivity and resistance to agricultural fungicides maybe caused by mutations causing over- expression of ABC genes. In effect, *Taabc2* mutants were more sensitive than the wild type to dicloran (fungicide), mycotoxins such as aflatoxins B1, B2, and G1, as well as fusaproliferin, indicating a role of this gene in the mechanisms of cell detoxification from xenobiotic compounds (Ruocco *et al.*, 2009). The Authors suggests that *T. atroviride* is made more competitive towards other microbes by *Taabc2* activity, providing tolerance to bacterial or fungal toxins and by supporting the secretion of its own antibiotics. Interestingly, the addition of beauvericin at up to 10 ppm did not interfere with the mycoparasitic and antagonistic activity of the wild-type strain against *B. cinerea* and different *Fusarium* spp. - which are natural beauvericin producers. Remarkably, the expression of *Taabc2* was trigged by the presence of beauvericin. Furthermore, one of the *Taabc2* knock-out mutants is unable to release 6-pentyl-␣-pyrone, a volatile antibiotic compound normally produced by the wild-type strain during mycoparasitism. Thus, knock-out mutants of the *Taabc2* gene strongly reduced the antagonistic activity of *T. atroviride* strain, both *in vitro* and *in vivo*, against several pathogens belonging to Ascomycetes, Basidiomycetes and Oomycetes fungal groups. Overall, these data

indicate that TAABC2 helps *T. atroviride* to successfully antagonize or colonize various pathogens or plants in many different environmental conditions, playing a key role in its biocontrol activity (Marra *et al.* 2006).

1.3.2 Major Facilitator Superfamily (MFS): The Major Facilitator Superfamily (MFS), also called 'uniporter-symporter-antiporter' family, consists of single-polypeptide secondary carriers capable of transporting small solutes in response to chemiosmotic ion gradients. The MFSs were originally believed to function primarily in the uptake of sugars, but further analysis revealed more diverse functions than had been thought previously. Nowadays these proteins are known to be ubiquitous in the membranes of all living cells, and about 25% of prokaryotic membrane transport proteins belong to this superfamily. The MFS represents the largest and most diverse group of transporters and contains more than 10000 sequenced members (Madej *et al.*, 2013; Yan N, 2013).

Pao and colleagues (1998) constructed, exclusively on the basis of the degrees of sequence similarity, a phylogenetic trees based on all recognizable sequenced members of the MFS that had been deposited in the databases at that time. This computational analysis allowed the Authors to divide all the recognized members of the MFS into 17 families. Remarkably, they found that phylogenetic family correlates with function. Thus, each of the families recognizes and transports a distinct class of structurally related compounds. As early as in 1990, Rubin and colleagues had argued that MFS permeases arose by a tandem intragenic duplication event and Pao and colleagues (1998) provided additional statistical evidence in favour of this possibility. This duplication event has generated the 12-trans-membrane (TMS) protein topology from an original 6-TMS unit.

As mentioned above, the MFS transports are able to transport a wide range of substrates including sugars, drugs, several metabolites, amino acids, nucleosides, vitamins, and both inorganic and organic anions and cations. In details, functionally characterized members of families 1, 5, and 7 are specific for sugars; characterized members of families 2 and 3 are specific for drugs and other xenobiotics; and families 4, 6, 8, 9, 11 to 14, and 17 are specific for various classes of anionic

compounds. Furthermore, the only nucleoside permeases in the MFS are found in family 10, and most of the aromatic acid permeases are found in family 15 (Pao *et al.*, 1998).

Of particular interest in this thesis, families 2 and 3 consist of drug efflux systems, which possess 14 and 12 TMSs, respectively. Since these permeases uniformly catalyze drug:H antiport, they are referred to as the DHA14 and DHA12 families, respectively. Members of both families are found in Bacteria and Eukaryotes, and DHA12 family members have also been identified in Archaea. These two families branch off from each other after the initial divergence from the centre of the tree computed by Pao and colleagues (1998), suggesting that they are more closely related to each other than to other MFS families. The range of organisms in which DHA14 family members were found is wider than that for the DHA12 family. Actually, the DHA14 MDR pumps are found in animals as well as in yeasts and in a variety of gram-negative and gram-positive bacteria. For example, a gene belonging to DHA14 family - named 'CaMDR1' and responsible for pump benomyl, cycloheximide, methotrexate and other xenobiotic compounds out of the cell - has already been characterized in *Candida albicans* (Yeast) and its sequence was available on SwissProt database (Pao *et al.*, 1998).

Nowadays, based on phylogenetic analysis, substrate specificity and working mechanism, the MFS transporters are divided into 76 subfamilies in the 'Transporter Classification Database' TCDB (http://www.tcdb.org/). Nearly half of the MFS subfamilies are of unknown or only putative functions. For instance, members of the sugar porter subfamily (TCDB #2.A.1.1) are essential for metabolism and energy homeostasis in Bacteria, Archaea, Fungi, Protozoa, Plants and Animals because they mediate the cellular uptake of glucose and other mono- and disaccharides. The DHA1 and DHA2 subfamilies (drug:H+ antiporters 1/2, TCDB #2.A.1.2/3, previously named as DHA12 and DHA14, respectively) play a major role in multidrug resistance in Bacteria and Fungi (Yan N, 2013).

As to molecular structure, both the N and C termini of an MFS are usually located on the cytoplasmic side of the membrane. A canonical MFS fold comprises 12 TMs that are organized into two folded domains - the N and C domains - each containing six consecutive TMs. The transport cycle phase-by-phase could be described as follows: the 'ligand-free' state has an outward-open conformation while the 'ligand-bound' state - representing the first step – has an outward-facing conformation, which goes from partly occluded to inward-occluded. The last stage is represented by the inward-open conformation, when the substrate has been pumped out the membrane and the transporter is ready for another cycle. MFS proteins contain a single substrate-binding cavity enclosed by the N and C domains and located halfway into the membrane, at a nearly equal distance between the periplasm and the cytoplasm (Yan N, 2013).

Among the MFS transporters with known structures, with the exception of the antiporters GlpT (Glycerol-3-phosphate:Pi antiporter) and EmrD (Drug:H+ antiporter) – belong to OPA and DHA1 MFS subfamilies, respectively - all are proton-coupled symporters, which shuttle substrate by exploiting the energy stored in the proton gradient across the membrane. The substrate and proton are co-transported by proton symporters in the same direction at a fixed stoichiometry. Whereas substrate transport requires alternate exposure of the binding site to either sides of the membrane, translocation of protons involves protonation and deprotonation of certain residues, most frequently Glu/Asp/His and to a lesser extent Lys/Arg/Tyr (Yan N, 2013).

1.4 Role of housekeeping genes in reverse transcription-quantitative PCR (RT-qPCR) normalization data

The high sensitivity of qPCR technique, which enables the detection of nucleic acid level at very low amount, should go at the same speed of an accurate and robust normalization system when performing relative quantification of qPCR data. Normalizing to a stably expressed gene of the

target organism, often called ‘reference’ or ‘housekeeping gene’, is a powerful method for qPCR internal error prevention. When applying mRNA quantification techniques, the multistage process required to extract, to process and to detect mRNA could be source of errors (Steiger *et al.*, 2010). In order to address these issues, reference genes need a stable expression under certain conditions. It should be carefully evaluated whether the transcription profile of putative reference genes is altered or affected by the experimental conditions. If not, results and conclusions could be substantially altered by using the wrong reference gene, leading to misinterpretation of data. However, it has become clear that the ideal internal control gene universally valid, with a constant expression level across all tissues, cells, treatments does not exist (Vandesompele *et al.*, 2002).

For quantitative real-time reverse transcription-PCR (RT-PCR), the most commonly used normalization strategy involves standardization to a single constitutively expressed control gene. Actin, which is widely used as a reference gene, is not always among the most stable ones (Steiger *et al.*, 2010). A study performed in *Saccharomyces* reveals that actin scores at the third best position when tested with NormFinder and geNorm software (Stahlberg *et al.*, 2008) while in *Aspergillus niger* geNorm suggested actin encoding gene as one of the most stable ones, under the tested conditions (Bohle *et al.*, 2007).

Thus, the expression stability of the intended control gene has to be verified before each experiment. It is likely that one or more genes are constitutively expressed across experimental designs restricted to a few tissue types, cell types, treated and untreated, and so forth. This evaluation is composed of two steps: first, to identify which genes are likely candidates; and second, to verify the stability of these candidates (Andersen *et al.*, 2004).

Nowadays, it is generally advisable to include at least two reference genes into the normalization – multiple reference genes approach for normalization is based on the assumption that the variation in a single gene is higher than the variation in the average of multiple genes. Nevertheless, multiple normalization genes have to be measured each time. This may be impractical, particularly when

only few target genes need to be studied, or when limited amounts of RNA are available (Andersen *et al.*, 2004).

For this aim, several algorithms have been set up over last years. The importance of these algorithms is due to the statistical analysis applied to the data - generated by the qPCR experiment - that can affect considerably not only the validity and reliability of the results but also the biological conclusions (Raffaello and Asiegbu, 2013). For example, the software 'geNorm' ranks the genes according to the similarity of their expression profiles by a pairwise comparison (Vandesompele *et al.*, 2002). The Authors calculated the average pairwise variation of a particular gene with each other and denominated it 'M' - the internal control gene-stability measure. Genes with the lowest M values have the most stable expression.

Steiger and colleagues (2010) applied in parallel two different gene-ranking algorithms - NormFinder and geNorm - to calculate the most stable reference gene comparing two experimental setups (a replacement and a bioreactor cultivation approach). Whereas - under bioreactor growth conditions - NormFinder ranked sar1 (GTPase), cox4 (Cytochrome c oxidase subunit IV) and glk1 (Glucokinase) as the most stable genes whereas geNorm indicated glk1/cox4 and sar1. Under replacement cultivation condition sar1, glk1 and tef1a (transcription elongation factor) were the best ones using NormFinder, whereas geNorm ascertained act/sar1 and glk1. However, NormFinder proposes sar1 and cox4 as the best combination (by evaluating the minimum accumulated standard deviation - significance level 0.095 - among genes).

Brunner and colleagues (2008) – trying to extend the knowledge about G protein signalling in *Trichoderma atroviride* – evaluated actin-encoding gene (act1), the glyceral- dehyde-3-phosphate-dehydrogenase-encoding gene (gpd1), the translation elongation factor-encoding gene (tef1), the GTP-binding protein-encoding gene (sar1) and the gene for 28S rDNA as reference genes using 'geNorm' algorithm. All genes were evaluated on different growth conditions: liquid and solid medium (PDB and PDA, respectively); after replacement of *Trichoderma* to liquid medium with

different carbon sources (glucose, glycerol, N-acetylglucosamine, colloidal chitin, or carbon-starvation); dual plate assays when directly interacting with different confrontation partners or when the two fungi were separated by membrane barriers. tef1 was the best reference gene on PDB and PDA; act1 was the best one in liquid media with different carbon sources; sar1 was chosen as the best reference gene for biocontrol conditions – plate confrontation assay (Brunner *et al.*, 2008). Then, it is clear that different experiments and conditions require a proper validation of the best reference genes that has to be included in the data analysis (Raffaello and Asiegbu, 2013).

Although GAPDH (glyceraldehyde-3-phosphate dehydrogenase gene) is one of the preferred reference genes used in many qPCR studies, when Raffaello and Asiegbu (*l.c.*) analysed all the selected genes together with the NormFinder software – within the analysis of the stability of 11 selected reference genes in *Heterobasidion annosum* grown on three different substrates - it was found to be the least stable compared to all the others. Moreover, the analysis of all the reference genes together indicated the Actin gene as the most stable in their conditions. Interestingly, actin was also found stable in the analysis with BestKeeper software (Raffaello and Asiegbu, 2013).

1.5 Two different excel based software: BestKeeper and NormFinder

'BestKeeper' software (Pfaffl *et al.*, 2004. The software is available at http://www.gene-quantification.info/) was tested on experimental data obtained from RNA samples extracted from bovine *corpora lutea* under the *Estrumate* treatment. It can accommodate a maximum of 10 reference genes per analysis and up to ten target genes can be analysed additionally, whereas the earlier presented 'geNorm' software (Vandesompele *et al.* 2002) is restricted to the HKG analysis. BestKeeper's output is represented by descriptive statistics of the derived threshold Cycles (Ct) computed for each putative housekeeping gene, in details: the geometric mean, arithmetic mean, minimal and maximal value, standard deviation, and coefficient of variance. The first estimation of

HKG expression stability is based on the inspection of calculated variations (SD values). According to the variability observed - CV [% Ct] – housekeeping genes can be ordered from the most stably expressed, which has the lowest variation, to the least stable one. Any studied gene, with the SD higher than 1, can be considered inconsistent (Pfaffl *et al.*, 2004). From the genes considered stably expressed, the 'BestKeeper Index' is calculated as the geometric mean of its candidate housekeeping genes Ct values (see equation below), where 'n' is the total number of housekeeping genes included:

$$\text{'BestKeeper Index'} = \sqrt[n]{Ct_1 \times Ct_2 \times Ct_3 \times \ldots\ldots \times Ct_n}.$$

Then, to estimate inter-gene relations of all housekeeping genes pairs, pair-wise correlation analyses are performed. Within each such correlation *Pearson correlation coefficient* (r) and the probability *p* value are calculated (95% level of significance). The same Authors, however, point out that heterogeneous variance between groups of differently expressed genes leads to invalidate the use of *Pearson correlation coefficient*. In effect, low expressed genes – which have Ct values around PCR cycles 30–35 - surely show different variance compared to high expressed genes. Such samples cannot be correlated parametrically.

'NormFinder' software (Andersen *et al.*, 2004; the software is freely available at http://moma.dk/normfinder-software) uses a model-based approach – developed to identify genes suited to normalize quantitative RT-PCR data from colon cancer and bladder cancer - for the estimation of expression variation dealing with systematic differences in the data set like different tissues or strains. Since a gene which shows no expression variation among sample subgroups does not exist, the strategy is based on a mathematical model of gene expression that enables estimation not only of the overall variation of the candidate normalization genes but also of the variation between sample subgroups of the sample set. This approach entails application of a mathematical model to describe the expression values measured by RT-PCR, separate analysis of the sample subgroups, estimation of both the intra- and the inter- group expression variation, and calculation of

a candidate gene ‘stability value’. This approach top ranks the candidates with minimal estimated intra- and inter- group variation, in contrast to the pairwise comparison approach, which selects those genes with the highest degree of similarity of the expression profile across the sample set. The latter approach implies that the candidates with minimal expression variation do not necessarily become top ranked, but top ranks candidates with correlated expression rather than minimal variation. Whereas model-based approach is not significantly affected by candidate genes co-regulation, this represents a major weakness for the pairwise comparison approach (Andersen *et al.*, 2004). The model-based approach – thanks to such a hallmarks - provides a more precise and robust measure of gene expression stability than the pairwise comparison approach does.

2. Aim of the work

The aim of this work was to provide a validation of 6 putative reference genes that could be included in *Clonostachys rosea* IK726 gene expression studies. No data are available about validation of reference genes in *C. rosea*. In the present work, the selection of these genes was based both on published data referred to other biological systems and on the available Expressed Sequence Tags (ESTs) from *C. rosea* IK726. Several growth conditions were taken into account for *C. rosea* IK726, whose mRNA was extracted and retro-transcribed into cDNA and then used as template for Real Time quantitative PCR (RT-qPCR). Two different excel based algorithms – 'NormFinder' and 'BestKeeper' – were chosen in order to analyze expression data and find the 'best' reference gene. From statistical analysis, actin was chosen and used as reference gene for further expression analysis.

In the latter part of this work, the expression level of 8 putative genes encoding ABC- and MFS-transporters – which were selected according to *C. rosea* IK726 ESTs data – was evaluated for different treatments such as fungal-fungal interaction, zearalenone (ZEA, mycotoxin) and four different fungicides. 'Delta-delta method' was adopted as relative quantification method using *actin* as reference gene.

Keywords: *Clonostachys rosea*, ABC transporters, MFS transporters, BestKeeper, NormFinder, RT-qPCR, Relative quantification, Absolute quantification, Reference genes, Fungicides, Zearalenone, Fusarium mycotoxins.

3. Materials and methods

3.1 'Best' reference gene evaluation

3.1.1 Clonostachys rosea IK726: growth condition and DNA extraction

The fungal strain *C. rosea* IK726, isolated from barley root, was maintained in 10% glycerol at -80°C. *C. rosea* IK726 conidia were used for inoculation of PDA (Potato Dextrose Agar, Difco, Detroit, MI). After one week of incubation at 25°C in the dark, two agar plugs were collected and transferred into PDB (Potato Dextrose Broth, Difco, Detroit, MI) flask on a rotary shaker (120 r.p.m.). After 3 days of incubation, the culture was collected and filtered for DNA extraction. The dried mycelium was transferred into 1.5 mL "screw caps" tube containing around ten ceramic balls (2 mm diameter). DNA extraction was performed as follows:

1. Add 600 µl CTAB 3% to each tube;

2. Put the sample into "Precellys 24 Bertin Technologies" shaker to destroy the cells;

3. Leave the sample at 65°C for 30 min. Mix the tubes 2/3 times during this step;

4. Centrifuge for 10 min at 10.000 r.p.m. and transfer the supernatant to a new 1.5 eppendorf tube:

5. Add 600 µl of chloroform and vortex for 10 seconds;

6. Centrifuge at 13000 r.p.m. for 15 min. Transfer the supernatant to a new 1.5 mL eppendorf tube;

7. Add 1 volume of chloroform, vortex and centrifuge at 13000 r.p.m. for 10 min. Transfer supernatant to a new 1.5 mL eppendorf tube;

8. Add 1 volume of isopropanol and mix. Precipitate at -20 °C for 20 min;

9. Centrifuge at 13000 r.p.m. for 20 min. Discard the supernatant and dry the pellet briefly;

10. Wash the pellet by adding 200 µl 70 % ethanol, centrifuge at 13000 r.p.m. for 5 min, discard the supernatant, and let the pellet dry briefly;

11. Dissolve the pellet in 50 µl TE-buffer or MilliQ water and measure the concentration by the NanoDrop ND-1000 spectrophotometer (Thermo Fisher Scientific, Waltman, MA);

12. Store sample at -20°C.

3.1.2 Primers design and evaluation of selected genes

The selection of the following genes was based on information from both literature and *C. rosea* IK726 transcriptomes:

Protein	Gene	Reference
β-Tubulin	tub	Mamarabadi *et al*. (2008) Fems Microbiol Lett 285:101-110
Actin	actin	Brunner *et al.* (2008) Curr Genet 54:283-299
Translation elongation factor 1α	tef1	Brunner *et al.* (2008) Curr Genet 54:283-300
GTPase	sar1	Brunner *et al.* (2008) Curr Genet 54:283-301
Glyceraldehyde 3-phosphate dehydrogenase	gpd1	Brunner *et al.* (2008) Curr Genet 54:283-302
RNA polymerase III transcription factor subunit	sfc1	Raffaello and Asiegbu (2013) Mol Biol Rep 40:4605-4611

The design of the primers for Real-Time PCR assays was done using the PrimerSelect (Lasergene 10 Core Suite DNAstar, Madison, WI) software. The names, sequences, target genes and the size of amplicons for all primers are given in Table 3.1.

Table 3.1 Sequences of the primers designed for "best" reference assessment: "gpd1", "sar1", "sfc1", "tef1", "tub", "actin" genes in *Clonostachys rosea* IK726 (primers were made by Integrated DNA Technologies, Belgium)

Primer name (F, forward; R, reverse)	Sequence (5' – 3')	Target gene	Amplicon size (bp)
gpd1_F	ACCGGCGCCACCTATGTCGT	Glyceraldehyde 3-phosphate dehydrogenase	107
gpd1_R	GCAGAAGGGGCGGAGATGATGA		
sar1_F	GTCAGCGGCGTCGTCTTCCTC	GTPase	171
sar1_R	GGTGGCGCAGCTCGTCCTC		
sfc1_F	CGGGATGTGGGCGAAAGTGAA	RNA polymerase III transcription factor subunit	121
sfc1_R	GAACCGGCGTCTCGTCTCCAG		
tef1_F	GCCCAGGGTGCCGCTTCTTT	Translation elongation factor 1α	110
tef1_R	GCAAGCAATGTGGGCAGTGTGG		
tub_F	GGTCAGTGCGGTAACCAAAT	β-Tubulin	150
tub_R	ACAGCGCGAGGAACATACTT		
actin_F	GTTCTGGATTCCGGTGATGGTGTC	Actin	160
actin_R	TCGGCAGTGGTGGAGAAGGTGT		

Each specific annealing temperature was determined by using DreamTaq Green PCR Master Mix 2x (Thermo Scientific) and DreamTaq DNA polymerase (Thermo Scientific) into polymerase chain reaction mix - using *C. rosea* IK726 genomic DNA as template (1 µl per reaction). For each 20-mL reaction volume a primer concentration of 250nM was used. The amplification program consisted of 1 min initial denaturation (95° C), 30 cycles of amplification [30" at 95° C, 30" 60/62/65°C, 1 min at 72° C] and a final extension of 10 min at 72° C. The run was performed using ΔPCR 96 Well Thermal Cycler (Applied Biosystem), which allows selecting more than one annealing temperature at the same run. The amplification products were separated on 2% agarose (Fluka) gel electrophoresis using SB buffer (disodium tetraborate) as solvent, Nancy-520 dye (Sigma-Aldrich,

1 µl each 50 mL volume gel) and GeneRuler DNA Ladder Mix (Thermo Scientific). The run was performed at 120v for 45'.

3.1.3 Clonostachys rosea IK726 growth conditions

Trying to simulate as much different as possible growth conditions, *C. rosea* IK726 was grown in solid/liquid media, using different carbon sources. Mycelium was collected either from the edge of the colony (for solid medium and fungal-fungal treatments) or by directly harvesting mycelium/germinated conidia for liquid medium based treatments. In details:

- solid media: PDA (Fluka) and Czapek-dox medium (Fluka), added with 1.5% Bactoagar (Saveen Werner AB);

- liquid media: PDB (Difco) and Czapek-Dox broth (Fluka);

- germinated conidia: grown and collected from liquid medium (PDB);

- fungal-fungal interactions: *C. rosea* IK726 vs *C. rosea* IK726 (self-self interaction) and *C. rosea* IK726 vs *F. graminearum* pks wild type (antagonist - pathogen interaction).

Liquid media were inoculated with 2 plugs of *C. rosea* IK726 - grown on PDA for one week (25°C, dark) - in flasks containing 25 mL of medium and incubated at 25°C in the dark on rotary shaker (120 r.p.m.). Mycelium was harvested 6 days after inoculation (d.a.i.), filtrated through sterile paper cloth, transferred to a RNase-free eppendorf tube, immediately frozen in liquid nitrogen and than maintained at -80 °C until RNA extraction.

Conidia from sporulating cultures of *C. rosea* IK726 were harvested in sterile water and diluted in order to obtain a final concentration of 10^{-5} conidia mL^{-1}; successively, 1 mL of conidial suspension was inoculated in 25 mL PDB (Difco) and incubated at 25°C in the dark on rotary shaker (120

r.p.m.) for 1 day. Germinated conidia were harvested and filtered through sterile paper cloth, transferred to a RNase-free eppendorf tube, immediately frozen in liquid nitrogen and than maintained at -80 °C until RNA extraction.

Solid media were inoculated with 1 plug of *C. rosea* IK726 - grown on PDA plates for one week (25°C, dark) - on a sterilized Millipore membrane (Durapore® Membrane Filters, EMD Millipore Corporation, Billerica, MA) laid down on PDA surface, since *C. rosea* IK726 is unable to degrade it. This membrane allows an easier harvesting of the mycelium, avoiding to pick up medium traces that could negatively affect the PCR efficiency. Plates were incubated at 25°C in the dark. The mycelium was harvested - from the edge of the colony - 6 d.a.i. and transferred to a RNase-free eppendorf tube, frozen immediately in liquid nitrogen and than maintained at -80 °C until RNA extraction.

Both *C. rosea* IK726 vs *C. rosea* IK726 and *C. rosea* IK726 vs *F. graminearum* pks wild type interactions were transferred on Vogel's medium (see online supplementary **S.1** for medium composition at http://etd.adm.unipi.it/) using the sterilized Millipore membrane as previously described. For self-self interaction, two *C. rosea* IK726 plugs were inoculated at the same time 3 cm far away each other and incubated at 25°C in the dark whereas in the antagonist - pathogen interaction, one plug of *C. rosea* IK726 was inoculated 4 days before *F. graminearum* pks wild type (due to its faster growth rate). In both cases, mycelium was harvested 1mm before colonies edge contact and transferred to a RNase-free eppendorf tube, frozen immediately in liquid nitrogen and than maintained at -80 °C until RNA extraction.

Each treatment was replicated three times.

3.1.4 RNA extraction, Dnase treatment and RNA quantification

The samples were ground in liquid nitrogen with sterilized and pre-chilled mortar and pestle until a fine powder appeared. For analysis of gene expression *in vitro*, the total RNA was extracted using RNeasy® Plant Mini Kit (QIAGEN) following the protocol described by the manufacturer. Due to the *in vitro* intensive secondary metabolites production by *C. rosea* IK726 during fungal-fungal interactions, it was necessary to extract RNA with a combination of phenol – firstly - and kit - successively; the phenol extraction procedure was performed as follows:

1. Put the sample (grinded mycelia) in a 25 mL Falcon tube;

2. Add 3 mL CTAB (3%) buffer and 2.5 mL phenol/chloroform/isoamylalcohol (24:1:1);

3. Vortex 1 min;

4. Incubate 50°C for 20 minutes;

5. Centrifuge 8000 r.p.m. 15 min (4°C);

6. Transfer upper phase to new tube;

7. Add equal volume of chloroform/isoamylalcohol (24:1);

8. Vortex and mix vigorously;

9. Centrifuge 8000 r.p.m. 5 min (4°C);

10. Transfer upper phase to a new tube;

11. Add equal volume of isopropanol;

12. Incubate at least 1 hour at -20°C;

13. Centrifuge 8000 r.p.m. 15 min;

14. Discard supernatant and dry the pellet briefly;

15. Dissolve in 200µl RNase free water.

Extracted RNA was further cleaned with the DNase I RNase-free (Thermo Scientific) kit in order to avoid contamination with genomic DNA, using the procedure described by the manufacturer. For avoiding RNA degradation, 5 µl RNase inhibitor (Fermentas) were added to each reaction.

Extracted and cleaned RNA was quantified by using a 2100 Bioanalyzer (Agilent Technologies) following the procedure as described by the manufacturer, which gives information about RNA integrity/concentration and undesirable genomic DNA contamination.

3.1.5 cDNA synthesis

At this stage, it was crucial to know RNA concentration for each treatment since we will have performed the analysis without an internal reference gene during RT-PCR assay. As the main aim of this work was to detect the "best" reference genes between those selected, we had to take into account the same starting RNA amount for each treatment before proceeding with cDNA synthesis.

RNA samples were subjected to cDNA synthesis using the iScriptTM cDNA Synthesis Kit (Bio-Rad, CA), following the procedure described by the manufacturer. After this stage, cDNA samples were diluted in 180 µl of RNase-free water and stored at -20°C.

3.1.6 Set-up of Real Time PCR parameters

According to the 'specific annealing temperature' evaluation previously carried out, genes amplification was performed by using DreamTaq Green PCR Master Mix 2x (Thermo Scientific) and DreamTaq DNA polymerase (Thermo Scientific) in polymerase chain reaction mix using *C.*

rosea IK726 genomic DNA as template (1 µl per reaction). In each 20-mL reaction volume, a primer concentration of 250 nM was chosen. The amplification program consisted of 1 min initial denaturation (95° C), 30 cycles of amplification [30" at 95° C, 30" 60/62/65°C, depending to the genes, 1 min at 72° C] and a final extension period of 10 min at 72° C. The run was performed using a ΔPCR 96 Well Thermal Cycler (Applied Biosystem). The agarose (Fluka) 2% gel was made by using SB buffer (disodium tetraborate) as solvent, Nancy-520 dye (Sigma-Aldrich, 1 µl each 50 mL volume gel) and GeneRuler DNA Ladder Mix (Thermo Scientific). The run was performed at 120v for 45'.

Amplified products were purified as follows:

1. Add 1/10 volume of sodium acetate (3M, pH=5.2);

2. Add 2.5 volumes of ethanol 95%;

3. Incubate 2-3 hours at -20°C;

4. Centrifuge 20 minutes at 13000 r.p.m.;

5. Discard the supernatant;

6. Add 200 µl of cold ethanol 70%;

7. Centrifuge 10 minutes at 13000 r.p.m.;

8. Let the pellet dry and dissolve it in 50 µl of nuclease-free water;

9. Measure the concentration (NanoDrop ND-1000 spectrophotometer (Thermo Fisher Scientific, Waltman, MA).

In order to obtain the standard curves - by knowing the concentration of amplified and purified genes, their amplicon length, the nucleotide mean weight and setting the gene copy number each dilution – serial dilutions were made and used as template in RT-PCR for each gene.

The assays were carried out within the iQ5 real-time PCR detection system including the associated iQ5 optical system software from Bio-Rad. All reactions were set up in Bio-Rad 96-well reaction plates. Each reaction was performed in a final volume of 20-mL in the iQ™ universal SYBR® Green supermix from Bio-Rad, containing the SYBR Green I dye as a fluorophor. A primer concentration of 250 nM was used for all genes. Each sample reaction contained 5 µl cDNA template. Negative control reactions contained sterile water replacing the cDNA template. Each reaction was repeated three times. PCR cycling parameters were 95 °C for 5 min initial denaturation, followed by 40 cycles of denaturation at 95 °C for 10 s, annealing at 60/62/65 °C for 30s (according to the 'specific annealing temperature' previously evaluated) and extension at 72 °C for 30s. After the 40 cycles, melting curves were acquired continuously, collecting the fluorescence data by increasing the temperature from 55°C up to 95 °C with 0.5 °C per second, whereas the temperature was maintained for 10 s.

The output of RT-PCR is represented by 'Ct' (threshold cycle) values determined from a log–linear plot of the PCR signal (Ct is an exponential term) versus the cycle number. Ct indicates the fractional cycle number at which the amount of amplified target reaches a fixed threshold. The standard curve was made using $\log_{10}$ copy number versus corresponding Ct value (a mean between technical replicates) and efficiency of each primer was calculated as follow:

$$E = 10^{(-1/slope)}$$

It would be necessary taking into account the primer efficiency at the statistical analysis' time since the used software requires this data.

3.1.7 Gene expression analysis

After dilution 1:5 in RNase-free water, cDNA samples were used as template in RT-PCR (5 µL per reaction). Expression analysis was performed within the iQ5 real-time PCR detection systems including the associated iQ5 optical system software from Bio-Rad - using SsoFast™ EvaGreen® supermix from Bio-Rad containing the EvaGreen dye as a fluorophor. All 20-mL reactions were set up in Bio-Rad 96-well reaction plates. A primer concentration of 200 nM was used for all genes. Negative control reactions contained sterile water. Each reaction had three replicates. PCR cycling parameters were 95 °C for 30s initial denaturation, followed by 40 cycles of denaturation at 95 °C for 5 s, annealing/extension simultaneously at 60/62/65 °C for 30s (according to the 'specific annealing temperature' evaluation carried out previously). After the 40 cycles, melting curves were acquired collecting the fluorescence data continuously by increasing the temperature from 65°C up to 95 °C with 0.5 °C per second where the temperature was maintained for 10 s.

Absolute quantification determines the input copy number of the transcript of interest, relating the PCR signal to a standard curve. Copy number was calculated as follows:

$$10^{(Ct - b)/a},$$

where 'Ct' is a mean value calculated between three technical replicates (for each biological replicates), "b" and "a" are the intercept and slope value – respectively – extrapolated from relative standard curve.

3.1.8 Statistical analysis

Ct values collected from 'best' reference gene evaluation RT-PCR assay – performed as previously described– were statistically analyzed by using two different excel based software: NormFinder (Andersen *et al.*, 2004) and BestKeeper (Pfaffl *et al.*, 2004).

The strategy of NormFinder is based on a mathematical model of gene expression that enables estimation not only of the overall variation of the candidate normalization genes but also of the variation between sample subgroups of the sample set. This approach entails application of a mathematical model to describe the expression values measured by RT-PCR, separate analysis of the sample subgroups, estimation of both the intra- and the inter- group expression variation, and calculation of a candidate gene 'stability value'. This approach top ranks the candidates with minimal estimated intra- and inter- group variation, in contrast to the pairwise comparison approach, which selects those genes with the highest degree of similarity of the expression profile across the sample set.

BestKeeper's output is represented by descriptive statistics of the derived threshold Cycles (Ct) computed for each putative reference gene (geometric mean, arithmetic mean, minimal and maximal value, standard deviation, and coefficient of variance). The first estimation of reference gene expression stability is based on the inspection of calculated variations (SD values). According to the variability observed - CV [% Ct] – reference genes can be ordered from the most stably expressed, which has the lowest variation, to the least stable one. Any studied gene with the SD higher than 1, can be considered inconsistent. From the genes considered stably expressed, the 'BestKeeper Index' is calculated as the geometric mean of its candidate reference genes Ct values (see equation below), where 'n' is the total number of reference genes included:

$$\text{'BestKeeper Index'} = \sqrt[n]{Ct_1 \times Ct_2 \times Ct_3 \times \ldots\ldots \times Ct_n}.$$

Then, to estimate inter-gene relations of all reference genes pairs, pair-wise correlation analyses are performed. Within each such correlation *Pearson correlation coefficient* (r) and the probability *p* value are calculated (95% level of significance).

The same Authors, however, point out that heterogeneous variance between groups of differently expressed genes leads to invalidate the use of *Pearson correlation coefficient*. Low expressed genes

– which have Ct values around PCR cycles 30–35 - surely show different variance compared to high expressed genes. Such samples cannot be correlated parametrically. Thus, BestKeeper approach implies that the candidates with minimal expression variation do not necessarily become top ranked, but top ranks candidates with correlated expression rather than minimal variation. Whereas model-based approach is not significantly affected by candidate genes co-regulation, this represents a major weakness for the pairwise comparison approach (Andersen *et al.*, 2004). The model-based approach – thanks to such hallmarks - provides a more precise and robust measure of gene expression stability than does the pairwise comparison approach. Thus, the selection of the 'best' reference gene, among the selected ones, was based on NormFinder results, which top ranks the candidates with minimal estimated intra- and inter- group variation - in contrast to the pairwise comparison approach, which selects those genes with the highest degree of similarity of the expression profile across the sample set.

3.2 Gene expression analysis of eight selected membrane transporters in *C. rosea* IK726

3.2.1 C. rosea IK726 transcriptomes analysis: selection of the eight predicted genes encoding membrane transporters

The 8 different *C. rosea* IK726 putative genes encoding membrane transporters were selected by analyzing gene expression data from transcriptomes resulting from three different *C. rosea* IK726 growth conditions: *C. rosea* IK726 vs *C. rosea* IK726; *C. rosea* IK726 vs *F. graminearum* pks wild type; *C. rosea* IK726 vs *B. cinerea* B05.10 (Table 3.2). This selection was done through transcriptomes analysis: five genes were selected since over-expressed in *F. graminearum* pks wild type interaction [CROS1_T00006455_1 (called MFS_6455 from now on); CROS1_T00007234_1 (MFS_7234); CROS1_T00000606_1 (UbiH_0606); CROS1_T00002418_1 (MFS_2418);

CROS1_T00010026_1 (ABC-G_0026)]; three genes were selected since over-expressed in *B. cinerea* B05.10 interaction [ROS1_T00012311_1 (MFS_2311); CROS1_T00009923_1 (MFS_9923); CROS1_T00006570_1 (ABC-C_6570)]. The presence of MFS-/ABC- domains was confirmed by *NCBI conserved domain* analysis (Marchler-Bauer *et al.*, 2011; http://www.ncbi.nlm.nih.gov/Structure/cdd/wrpsb.cgi).

Table 3.2: BC, CR and FG indicate *C. rosea* IK726- *C. rosea* IK726, *C. rosea* IK726 - *B. cinerea*, *C. rosea* IK726 - *F. graminearum* interactions, respectively. *UbiH is a tetracycline resistance gene found in two Bacteroides transposons encoding an NADP-requiring oxidoreductase. §PTR2 belongs to the POT (proton-dependent oligopeptide transport) family - proton dependent transporters.

Transporter protein name ("Similar to")	*C. rosea* IK726 protein code	Domain(s)	Expression level		
			BC	CR	FG
TOXA Putative HC-toxin efflux carrier TOXA (*Cochliobolus carbonum*)	CROS1_T00006455_1	MFS	0.2	0.7	485.3
SPBC1271.10c Uncharacterized MFS-type transporter C1271.10c (*Schizosaccharomyces pombe* (strain 972 / ATCC 24843)	CROS1_T00007234_1	MFS	0.9	0.9	709.9
tetX Tetracycline resistance protein from transposon Tn4351/Tn4400 (*Bacteroides fragilis*)	CROS1_T00000606_1	UbiH*	1.6	1.4	121.0
PTR2 Peptide transporter PTR2 (*Candida albicans*)	CROS1_T00002418_1	MFS/PTR2§	32.5	27.0	103.0
CDR4 ABC transporter CDR4 (*Candida albicans*)	CROS1_T00010026_1	ABC-G	0.5	0.5	2.8
SPAC1002.16c Uncharacterized transporter C1002.16c (*Schizosaccharomyces pombe* (strain 972 / ATCC 24843)) AED:0.06	CROS1_T00012311_1	MFS	16.4	1.8	2.5
SPAC1002.16c Uncharacterized transporter C1002.16c (*Schizosaccharomyces pombe* (strain 972 / ATCC 24843)) AED:0.07	CROS1_T00009923_1	MFS	36.9	7.5	11.7
YCF1 Metal resistance protein YCF1 (*Saccharomyces cerevisiae* (strain ATCC 204508 / S288c))	CROS1_T00006570_1	ABC-C (MRP)	2.7	0.7	0.4

'Transporter protein name (similar to)'; according to NCBI database analysis; '*C. rosea* IK726 protein' refers to the matches obtained by blasting selected transporters protein sequences within *C. rosea* IK726 genome; 'Domain(s)': it was necessary to perform an NCBI conserved domain research in order to verify the presence of the relative conserved domain; 'Expression level' specifies the relative gene expression ratio among the three different interactions previously described.

3.2.2 Evaluation of primers' specificity

The design of the primers for real-time PCR assays was done using the PrimerSelect (Lasergene 10 Core Suite DNAstar, Madison, WI) software. The primer names, sequences, *C. rosea* IK726 protein code and the size of amplicons for all primers are given in Table 3.3:

Table 3.3 Sequences of primers designed for "gene expression analysis" of the 8 selected *C. rosea* IK726 transporter genes (primers were made by TAG Copenhagen A/S, Denmark).

Primer name (F, forward; R, reverse)	**Sequence (5' – 3')**	***C. rosea* IK726 protein code**	**Amplicon size (bp)**
MFS_6455_F	GCGCTGTGTACTACCTTGCCATCTG	CROS1_T00006455_1	195
MFS_6455_R	ATTCCGCCGCCCACACTACATA	CROS1_T00007234_1	
MFS_7234_F	GCCCTTTTCCGCTTGCTATGG	CROS1_T00000606_1	171
MFS_7234_R	ACCGCGCTCGTGAGTGAAGTAAAT	CROS1_T00002418_1	
UbiH_0606_F	GCCCTGGGTCTAGCTCCGTGTT	CROS1_T00010026_1	189
UbiH_0606_R	TCCGCATATCCGCCGAAGAAT	CROS1_T00012311_1	
MFS_2418_F	CCTCGCCTACGCTCTCCCTCTTA	CROS1_T00009923_1	158
MFS_2418_R	ACCTTGGCGTTTCCGTTCTCG	CROS1_T00006570_1	
ABC-G_0026_F	GCCCAATATCGTGCCCAAGTCA	CROS1_T00006455_1	145
ABC-G_0026_R	GAAGCGCCAGCAATCAACATCTC	CROS1_T00007234_1	
MFS_2311_F	CGCCCCGATGCTCATTGTTACTAC	CROS1_T00000606_1	171
MFS_2311_R	GGAAGCAGGGGCGATGTTGTTA	CROS1_T00002418_1	
MFS_9923_F	AGATATGATACCCCCGATGCCAGAT	CROS1_T00010026_1	155
MFS_9923_R	TCCGTTGCGAGACCGATGTTTC	CROS1_T00012311_1	
ABC-C_6570_F	GCGCTGCCACTCCCGTTCT	CROS1_T00009923_1	171
ABC-C_6570_R	CAAGTCGCCGCAGCAAGGT	CROS1_T00006570_1	

The specific annealing temperature was determined – for each primers pair - using DreamTaq Green PCR Master Mix 2x (Thermo Scientific) and DreamTaq DNA polymerase (Thermo

Scientific) in polymerase chain reaction mix using either *C. rosea* IK726 genomic DNA or *F. graminearum* pks wild type and *B. cinerea* B05.10 genomic DNA as template (1 µL per reaction), in order to verify if the amplified genes belong to *C. rosea* IK726 exclusively. In each 20-mL reaction volume a primer concentration of 250 nM was chosen. The amplification program consisted of 1 min initial denaturation (95° C), 30 cycles of amplification [30" at 95° C, 30" 60°C, 1 min at 72° C] and a final extension period of 10 min at 72° C. The run was performed using Applied Biosystems 2720 thermal cycler. The agarose (Fluka) 2% gel was made by using SB buffer (disodium tetraborate) as solvent, Nancy-520 dye (Sigma-Aldrich, 1 µl each 50 mL volume gel) and GeneRuler DNA Ladder Mix (Thermo Scientific). The run was performed at 120v for 45'.

3.2.3 Experimental scheme

All *C. rosea* IK726 cDNA samples used for 'gene expression analysis', with the exception of those from *C. rosea* IK726 vs *C. rosea* IK726 and *C. rosea* IK726 vs *F. graminearum* pks wild type interactions (produced and utilized previously for 'best' reference gene assessment), were already available. All of them were used as templates in RT-PCR assay. The cDNA samples were produced by the following treatments:

Fungal – fungal interactions:

- *C. rosea* IK726 vs *C. rosea* IK726;
- *C. rosea* IK726 vs *B. cinerea* B05.10;
- *C. rosea* IK726 vs *F. graminearum* pks wild type.

Zearalenone treatment:

- mycotoxin (ZEA)
- control (methanol)

Fungicides treatment:

- Chipco Green (Bayer)
- Cantus WDG (NM Bartlett Inc.)
- Apron XL (Syngenta)
- Amistar (Syngenta)
- control (water)

In all 'fungal-fungal" interactions *C. rosea* IK726 was grown on Vogel's medium and its mycelium was collected 1 mm before contact between the edges of the colonies. Czapek-dox medium (Fluka) was used to grow *C. rosea* IK726 for both 'zearalenone' and 'fungicides' treatments. The fungicides' active principle concentrations were added in the media as follows: Apron (mefenoxam, 2 μg/mL), Amistar (azoxystrobin, 7.5 μg/mL), Chipco Green (iprodione, 250 μg/mL) and Cantus (boscalid, 2000 μg/mL). Since the fungicides were dissolved in water, 'control' plates were made adding water instead of fungicide, while – for the same reason – control plates were made adding methanol in 'zearalenone' interaction. Mycelia were collected 2 hours after inoculation either for 'zearalenone' or 'fungicides' interactions.

If interested about fungicides details, please see online supplementary **S.2**.

Three biological replicates were made for 'fungal-fungal' interactions whereas five biological replicates were made for both 'zearalenone' and 'fungicides' treatments.

3.2.4 Gene expression analysis

Actin was chosen as 'reference gene' – after 'best' reference gene assessment – and its expression level was tested together with the *C. rosea* IK726 transporter genes selected. Expression analysis was performed within the iQ5 real-time PCR detection systems including the associated iQ5 optical

system software from Bio-Rad - using SsoFast™ EvaGreen® supermix from Bio-Rad containing the EvaGreen dye as a fluorophor. All 20-mL reactions were set up in Bio-Rad 96-well reaction plates. A primer concentration of 200 nM was chosen for all genes. The cDNA samples from different treatments were used as template (5 µL per reaction). Negative control reactions replacing the cDNA template contained sterile water. Each reaction had three replicates. PCR cycling parameters were 95 °C for 30s initial denaturation, followed by 40 cycles of denaturation at 95 °C for 5 s, annealing/extension simultaneously at 60 °C for 30s (according to the 'specific annealing temperature' evaluation carried out previously). After the 40 cycles, melting curves were acquired collecting the fluorescence data continuously by increasing the temperature from 65°C up to 95 °C with 0.5 °C per second where the temperature was maintained for 10 s.

Relative quantification was achieved using the 'delta delta method' with the actin-encoding gene as reference gene, as previously mentioned. Normalization to an endogenous reference provides a method for correcting results for differing amounts of input RNA. The Ct value for each gene was measured and the expression level of the genes in the different samples was calculated by the formula:

$$(Ct_{\text{target}} - Ct_{\text{actin}}) - (Ct_{\text{mean target calibrator}} - Ct_{\text{mean actin calibrator}}) = \Delta\Delta Ct;$$

Please note: $Ct_{\text{mean target calibrator}} - Ct_{\text{mean actin calibrator}}$ ('ΔCt') represents the 1x expression of the target gene normalized to actin.

Deriving the equation that describes the exponential amplification of PCR and presuming maximal and identical real-time amplification efficiencies of both target and reference genes (Livak and Schmittgen, 2001), the 'expression level' is expressed by

$$2^{-\Delta\Delta Ct}.$$

For the treated samples, this value represents the x-fold change in gene expression normalized to actin (reference gene) and relative to the untreated control used as 'calibrator'. In our case, cDNA

samples from *C. rosea* IK726 vs *C. rosea* IK726 interaction ('fungal-fungal treatment') together with untreated control referred to 'zearalenone', and 'fungicides' treatments were used as calibrator.

3.2.5 Statistical analysis

Whereas BestKeeper can analyse up to 10 target genes, as well as accommodate a maximum of 10 reference genes per analysis, NormFinder – which is more suitable than BestKeeper to determine the 'best' reference gene – does not allow the analysis of target genes. This is the reason why the expression data were analysed by means of analysis of variance (ANOVA).

Gene expression data (expression level, $2^{-\Delta\Delta Ct}$) were used for two-way analysis of variance (ANOVA), assuming growth condition and kind of genes as independent variables. Statistical analysis was performed using SySTAT 10. Pair-wise comparisons were made using the Tukey's method at the 95 % significance level.

4. Results

4.1 'Best' reference gene (HKG) evaluation

4.1.1 Evaluation of primers' specificity

Figure 4.1 shows amplification obtained at 60°C for each candidate gene, resulting in a single amplification product of the expected length: tef1 (A, 110bp), gpd1 (B, 107bp) and sfc1 (C 121bp). For sar1 (D) unspecific amplification was obtained, with the most intense band longer than the expected one (171 bp).

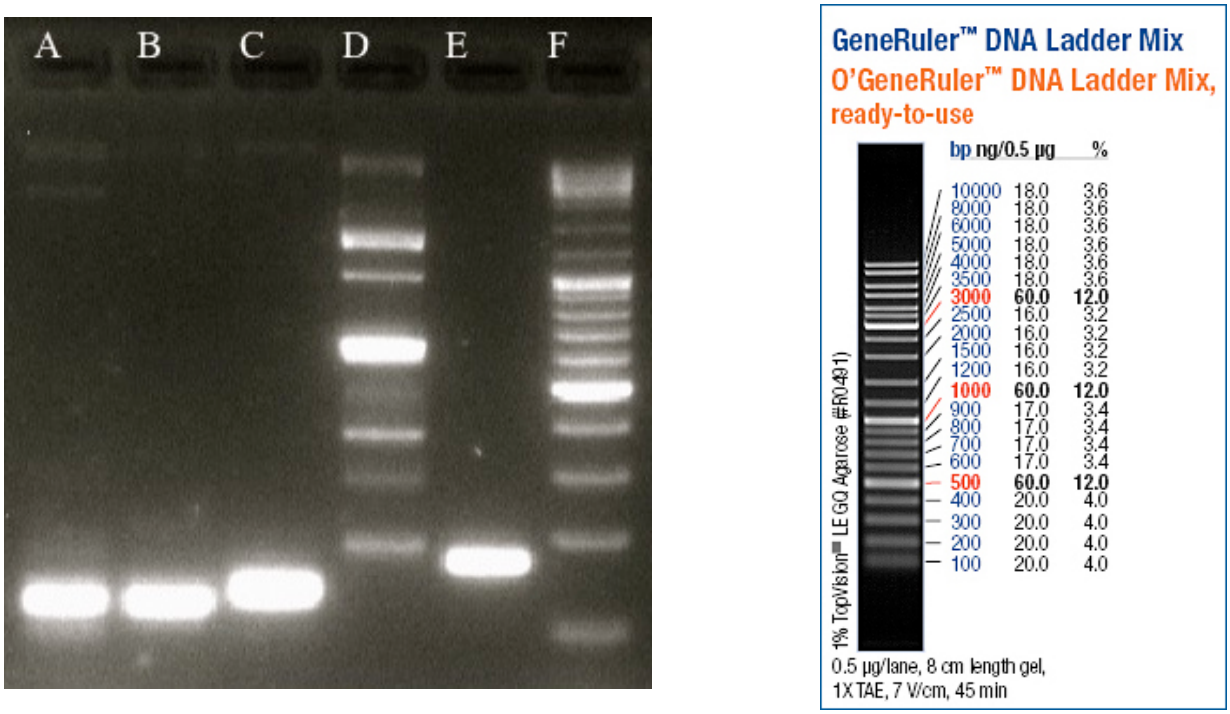

Figure 4.1 Elctrophoresis gel: tef1 (A), gpd1 (B), sfc1 (C), sar1 (D) and GeneRuler DNA Ladder Mix (F). On the right, ladder profile (www.thermoscientificbio.com). Column E refers to another amplified product not included in this test.

From previous PCR test, 60°C was chosen as annealing temperature for both actin and β-tubulin primers (data not shown).

PCR was repeated evaluating both 62°C and 65°C as annealing temperatures, in order to verify if at an increasing annealing temperature corresponded a specific amplification for tef1, gpd1 and sfc1 and, at the same time, to find out the specific annealing temperature for sar1. At 62°C, a specific amplification was achieved for all genes except sar1, which gave positive results at 65°C, as shown in Figure 4.2:

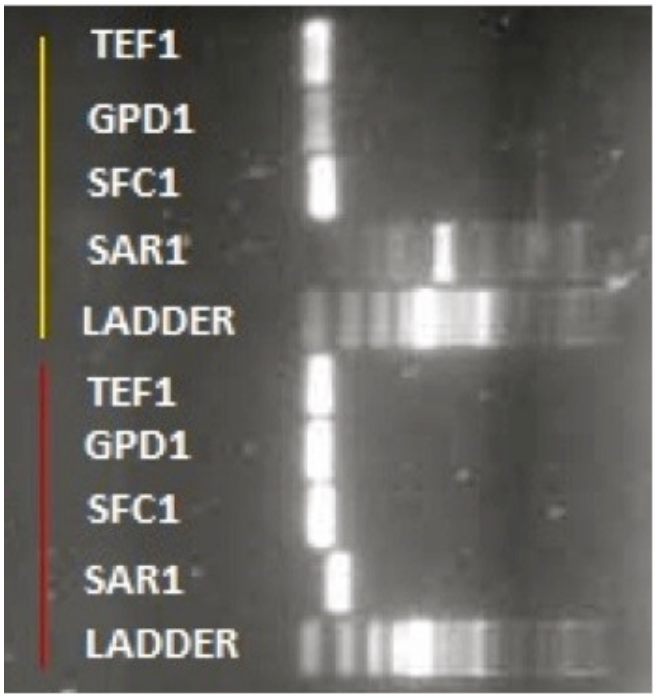

Figure 4.2 Amplification products at 62°C (yellow strip) and 65°C (red strip) annealing temperature. The ladder is the same as shown in Figure 4.1.

According to our results, the following annealing temperatures were chosen:

- actin and β-tubulin: 60°C;

- tef1, gpd1 and sfc1: 62°C;

- sar1: 65°C.

4.1.2 RNA quantification

The evaluation of both quality and quantity of extracted RNA - after Dnase treatment - was done by using 2100 Bioanalyzer (Agilent Technologies) that provides sizing, quantification and quality control of RNA no detecting single strand DNA (differently to the use of NanoDrop, which is not able to give information about RNA integrity). In Figure 4.3 some Bioanalyzer's outputs are shown:

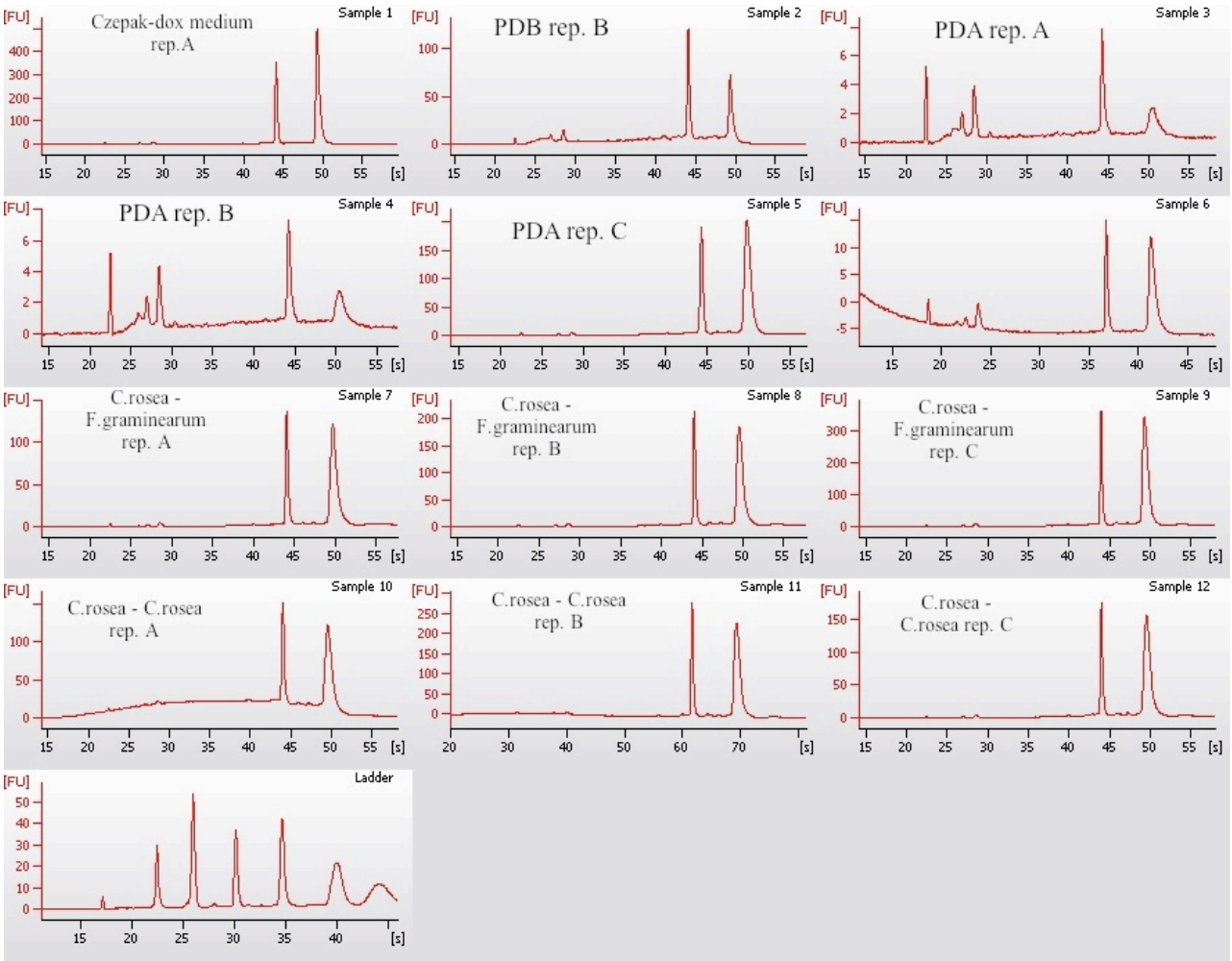

Figure 4.3 'Fluorescence units' [FU] are shown on y-axis, which are proportional to the amount of RNA detected; 'time' [s] is shown on x-axis.

The first peak – which appears 40-45s after the beginning of the run – corresponds to the 18S rRNA subunit, while the second one – which appears about 5s later – corresponds to the 28S rRNA

subunit. Ribosomal RNA (rRNA) represents approximately the 95% of total RNA, whereas the other kinds of RNA (such as mRNA) are almost undetectable in these graphs since the fluorescence is normalized to the rRNA one. Furthermore, all the samples showed good RNA quality and any genomic DNA trace has been found. Rarely, a jagged baseline was found before and/or between the ribosomal peaks, which means a partial degradation of RNA, without affecting our further analysis. Table 4.1 showed RNA concentration (3 biological replicates per treatments):

Table 4.1RNA concentration (ng/µl) per treatment.

RNA concentration (ng/µl)	**biological replicate 1**	**biological replicate 2**	**biological replicate 3**
Germinated conidia	99	134	169
Czepak-dox medium	664	88	75
Czepak-dox broth	91	83	84
PDB	59	370	79
PDA	35	40	37
C. rosea IK726 – *C. rosea* IK726	1232	591	347
C. rosea IK726 – *F. graminearum*	263	394	664

4.1.3 cDNA synthesis

In order to use the same RNA amount for each sample, 525 ng of RNA for each sample was subjected to cDNA synthesis. RNA concentrations are shown in Table 4.2:

Table 4.2 'cDNA synthesis': the calculation for the 'reaction mix' was done according with the manufacturer.

	iScriptTM cDNA Synthesis kit (Bio-Rad)					
Sample name (Rep)	**RNA concentration (ng/µl)**	**RNA (µl)**	**Reaction mix (µl)**	**Transcriptase (µl)**	**Water (µl)**	**Final volume (µl)**
PDA (1)	35	15.0	4.0	1.0	0.0	20
PDA (2)	40	13.1	4.0	1.0	1.9	20
PDA (3)	37	14.2	4.0	1.0	0.8	20
PDB (1)	59	8.9	4.0	1.0	6.1	20
PDB (2)	370	1.4	4.0	1.0	13.6	20
PDB (3)	79	6.6	4.0	1.0	8.4	20
Czapak-dox medium (1)	664	0.8	4.0	1.0	14.2	20
Czapak-dox medium (2)	88	6.0	4.0	1.0	9.0	20
Czapak-dox medium (3)	75	7.0	4.0	1.0	8.0	20
Czapak -dox broth (1)	91	5.8	4.0	1.0	9.2	20
Czapak -dox broth (2)	83	6.3	4.0	1.0	8.7	20
Czapak -dox broth (3)	84	6.3	4.0	1.0	8.8	20
Germinated conidia (1)	99	5.3	4.0	1.0	9.7	20
Germinated conidia (2)	134	3.9	4.0	1.0	11.1	20
Germinated conidia (3)	169	3.1	4.0	1.0	11.9	20
C. rosea - *C. rosea* (1)	1232	0.4	4.0	1.0	14.6	20
C. rosea - *C. rosea* (2)	591	0.9	4.0	1.0	14.1	20
C. rosea - *C. rosea* (3)	347	1.5	4.0	1.0	13.5	20
C. rosea –F. graminearum (1)	263	2.0	4.0	1.0	13.0	20
C. rosea –F. graminearum (2)	394	1.3	4.0	1.0	13.7	20
C .rosea –F. graminearum (3)	664	0.8	4.0	1.0	14.2	20

4.1.4 Standard curves by RT-Real Time PCR of candidate reference genes

In order to create standard curves, a RT-Real time PCR was performed using amplified candidate reference genes RNA as template (3 technical replicates for each dilution). Data – corresponding to the 'Ct' value per reaction (Table 4.3) - were computed in order to obtain a calibration curve for each gene.

Table 4..3 Ct mean value is shown for each dilution. Empty cells (-) refer to those dilutions whose Ct mean value was not included for creating calibration curve. If interested in raw Ct values data, see online supplementary **S.3**.

	Ct mean					
Copies number	**sar1**	**gdp1**	**sfc1**	**tef1**	**actin**	**ß-tubulin**
3000000000	6.21	5.78	5.89	6.09	5.33	11.37
300000000	9.73	8.93	9.24	9.45	8.55	14.94
30000000	13.08	12.2	12.38	12.8	11.90	18.62
3000000	17.01	16.34	16.08	16.68	15.46	22.06
300000	20.33	19.42	19.44	19.79	18.73	25.42
30000	23.69	22.52	23.06	23.36	22.12	28.87
3000	26.75	25.73	25.71	-	25.71	32.42
300	-	29.12	29.45	-	-	35.39

The following standard curves were made by using the Ct values, obtained as previously described:

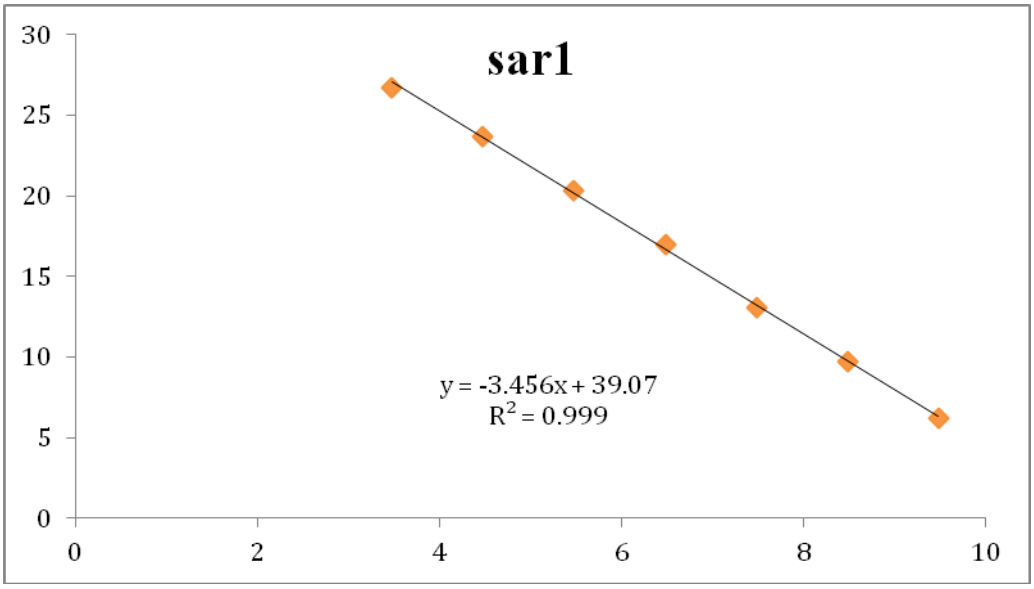

Figure 4.4 Standard curve for sar1.

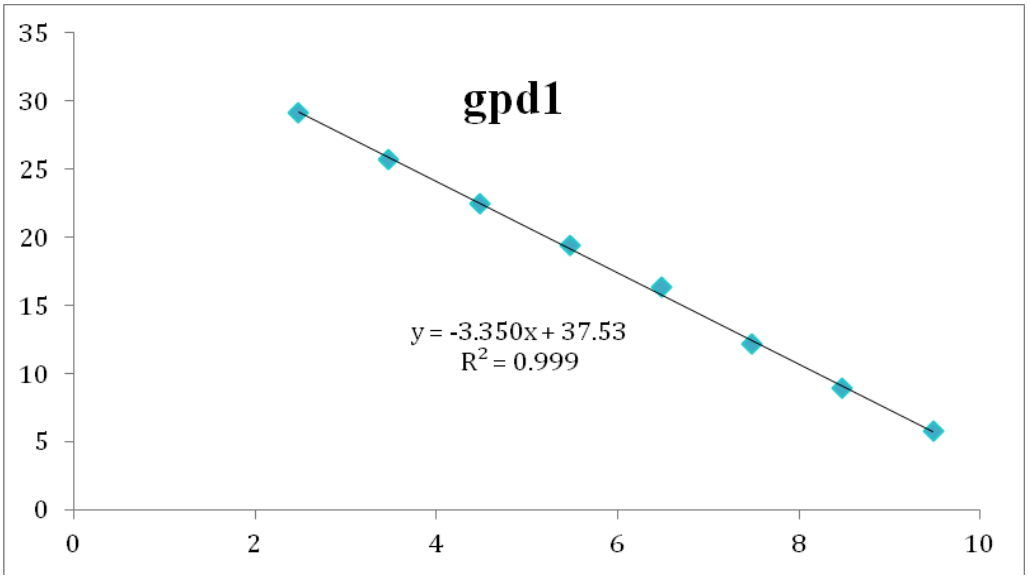

Figure 4.5 Standard curve for gpd1.

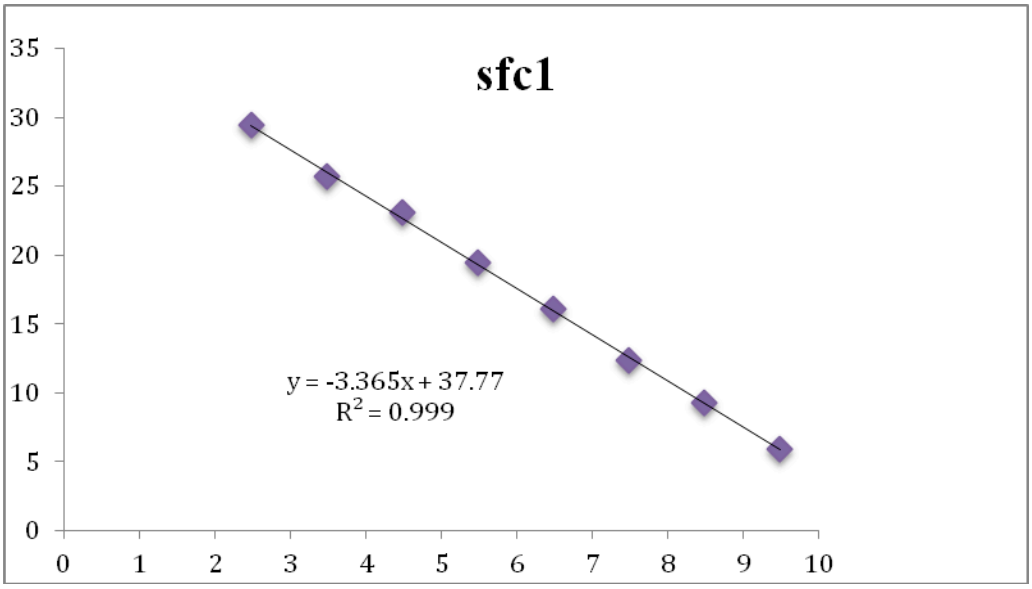

Figure 4.6 Standard curve for sfc1.

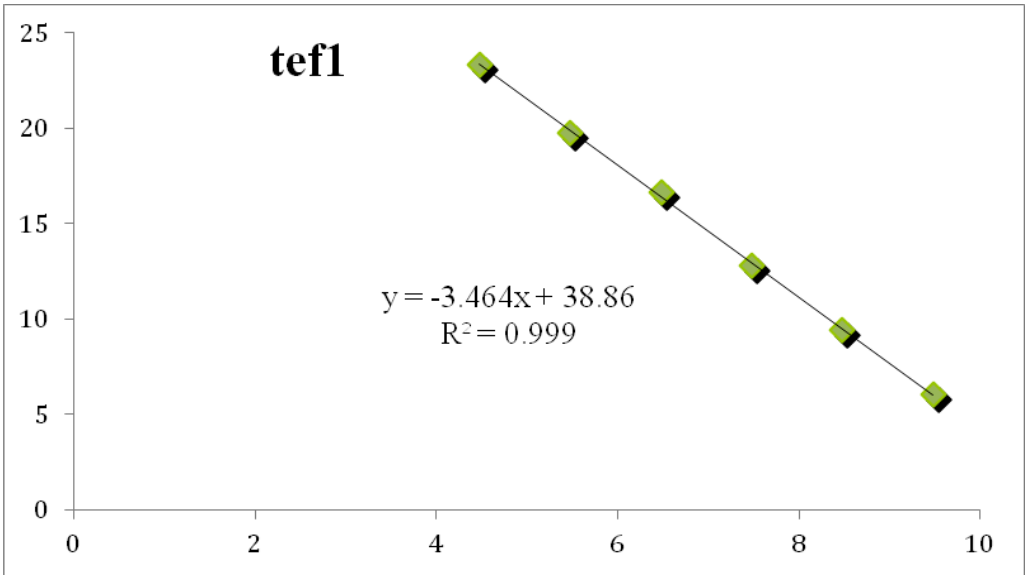

Figure 4.7 Standard curve for tef1.

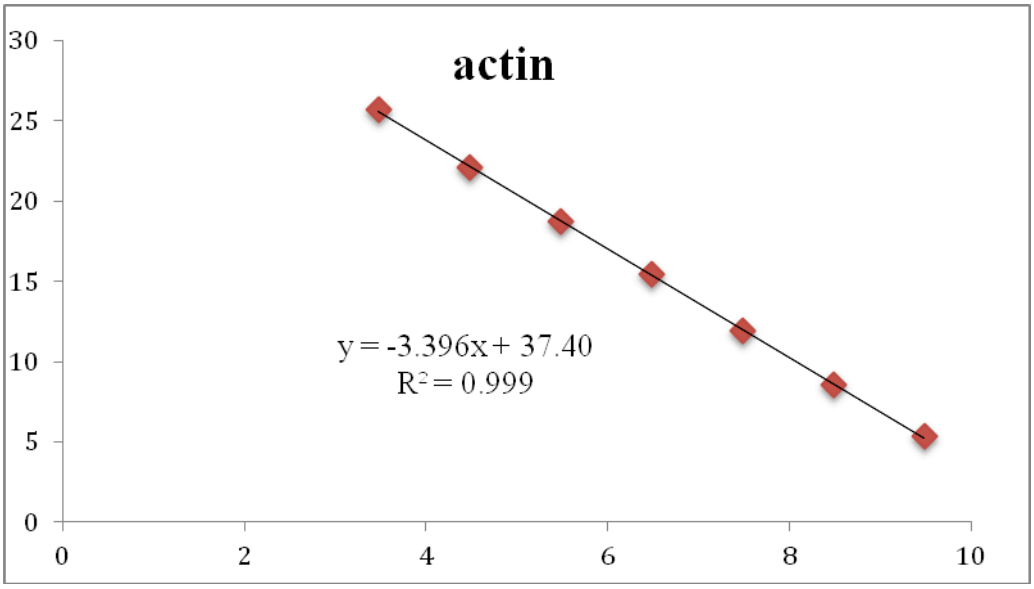

Figure 4.8 Standard curve for actin.

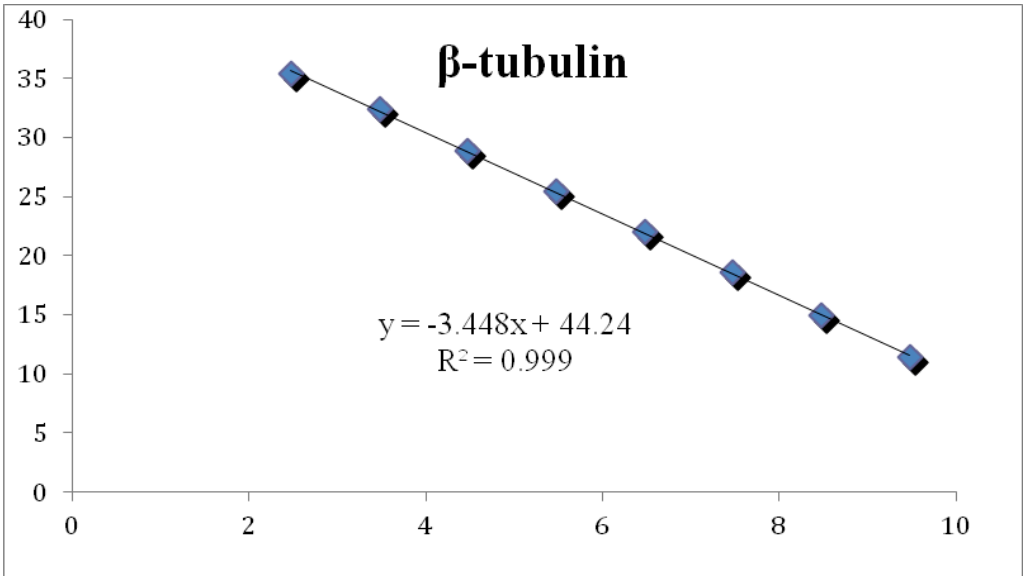

Figure 4.9 Standard curve for ß-tubulin.

In order to calculate genes' copy number, the following formula was applied:

$$E = 10^{(-1/slope)},$$

Where E corresponds to 'Real Time PCR efficiency', calculated for each gene, as summarized in Table 4.4:

Table 4.4 Real Time PCR efficiency (E) for each gene.

	sar1	gdp1	sfc1	tef1	actin	ß-tubulin
primer efficiency (%)	94	98	98	94	97	94

In addition, a melting curve analysis was performed, which resulted in a single specific product for each gene (Figure 4.10). Melting temperatures were: 82.7°C (actin), 83.5°C (gpd1), 86.5°C (sar1), 81°C (sfc1), 83.5°C (tef1) and 84.5°C (tub). No primer-dimers were generated during the RT-PCR assay. As example, 'sar1 melt curve peak chart' is shown below:

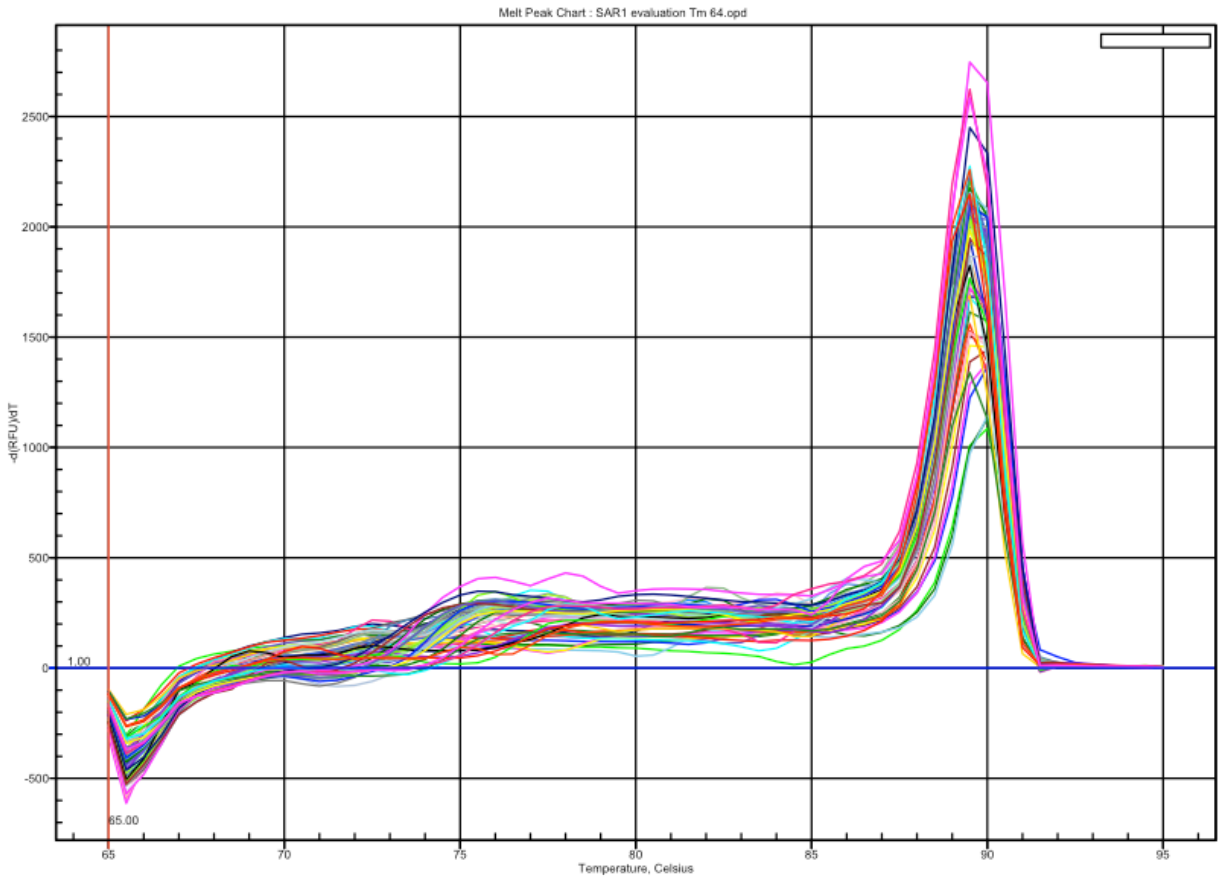

Figure 4.10 sar1 melt curve peak chart' from RT-PCR assay. The 'relative fluorescence unit' (RFU) is shown on y-axis, while temperature on x-axis.

4.1.5 Gene expression analysis at different growth condition

The same starting RNA amount was used (525ng as previously specified) for each growth condition (treatment) before cDNA synthesis. Assuming a theoretical 100% of reaction efficiency, all RNA was retro-transcribed into cDNA. Then, by diluting each cDNA sample - in 20µL of a cDNA synthesis reaction volume – with 180 µL of nuclease-free water (200µL final volume) a final cDNA concentration of 2.625 ng/µL was reached. For this RT-PCR assay, an additional dilution was necessary for reaching a cDNA concentration of 0.525 ng/µL. Since 5µL of these cDNA dilutions were added each RT-PCR reaction, the amount of template was 2.625ng, without cross the 'template limits' suggested by the manufacturer (from 50fg up to 50ng).

Each gene showed specific amplification in every growth condition. The ‘ß-tubulin melt curve peak charts’ – which had an annealing temperature of 60°C – is shown below as example:

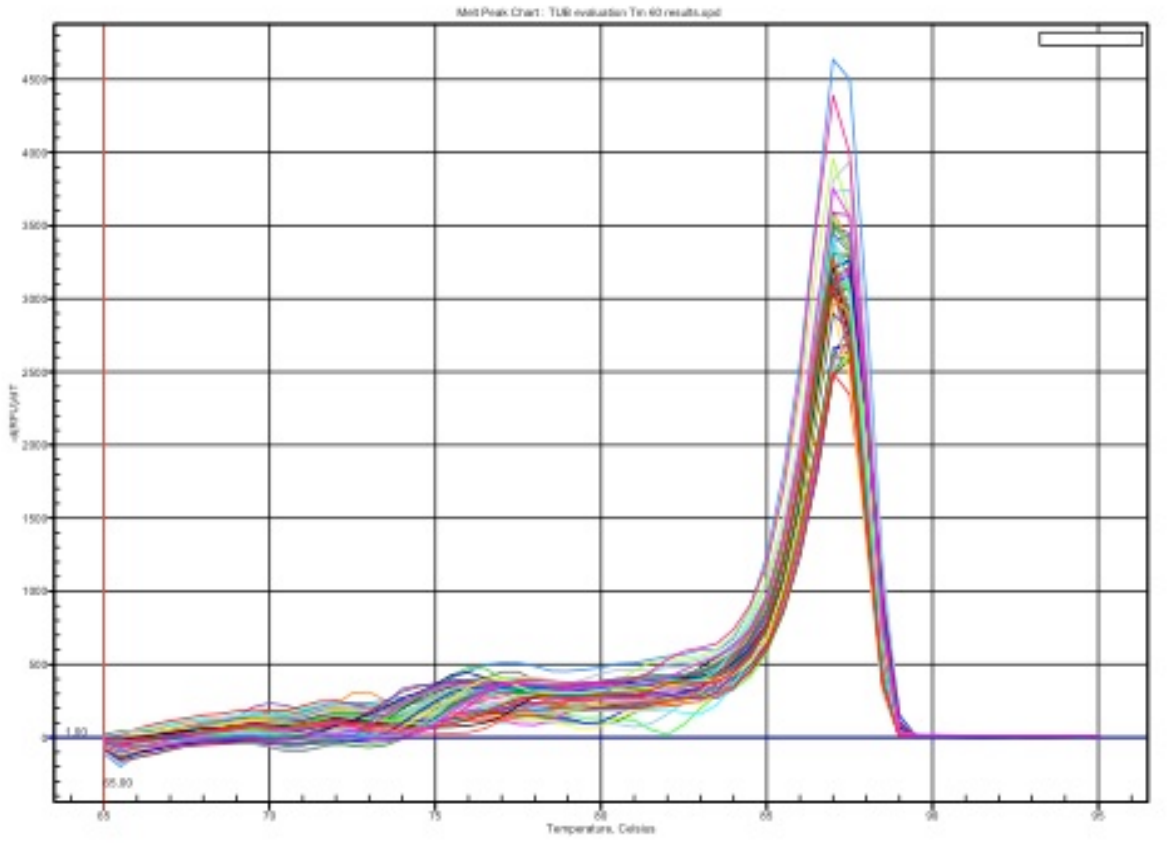

Figure 4.11 tub1 melt curve peak chart. The ‘relative fluorescence unit’ (RFU) is shown on y-axis, while temperature on x-axis.

The collected Ct values were used both as input data set for NormFinder and BestKeeper excel based software (see online supplementary **S.4** for raw Ct values) and for absolute quantification.

4.1.6 'Absolute quantification'

Copy number per treatment was calculated as follows:

$$10^{(Ct - b)/a},$$

where 'Ct' is a mean value calculated using three biological replicates, "b" and "a" are the intercept and slope value – respectively – extrapolated from standard curves. Table 4.5 shows the 'mean copy number' – the average was calculated out of three biological replicates – for each growth condition:

Table 4.5 Gene copy number for each growth condition**.**

Growth condition	sar1	gpd1	sfc1	tef1	actin	ß-tubulin
PDA	23	6439	14	6105	1087	70753
PDB	75	8893	15	13388	2054	111590
Czepak-dox medium	702	74697	51	119092	23781	1085316
Czepak-dox broth	223	31481	22	23105	4118	283635
Geminated conidia	276	18796	19	62765	8982	354895
C. rosea - C. rosea	296	8154	46	27024	8354	643155
C. rosea -F. graminearum	777	16544	61	66858	15204	1250448

4.1.7 Statistical analysis

As described in Materials and Methods, Ct values and RT-PCR primer efficiency (E) were subjected to statistical analysis by using 'NormFinder' and 'BestKeeper'. Data (represented by Ct values) were divided in different subgroups of analysis as follows:

- All growth conditions: all seven growth conditions together (three biological replicates each);

- All growth conditions but Czepak-dox medium, whose expression level was strangely the highest in most genes. Particularly, its expression level was quite different from Czepak-dox broth ones.

- PDA, PDB, germinated conidia: all based on Potato Dextrose media (please note: conidia were germinated in PDB);

- Fungal-fungal interactions: *C. rosea* IK726 – *C. rosea* IK726 and *C. rosea* IK726 – *F. graminearum* pks wild type interactions were statistically treated alone due to their nature.

While NormFinder approach top ranks the candidates with minimal estimated intra- and inter-group variation, BestKeeper approach – based on the pairwise comparison - selects those genes with the highest degree of similarity of the expression profile across the sample set. Table 4.6 shows the NormFinder output:

Table 4.6 NormFinder output. The 'stability value' represents the systematic error introduced when using that particular gene.

NormFinder	All growth conditions	All but Czapek-dox medium	PDA. PDB. germinated conidia	fungal-fungal interactions
Best gene	ACTIN	ACTIN	ACTIN	GPD1
Stability value	0.372	0.375	0.378	0.044
Best combination of two genes	SFC1 and ACT	SFC1 and ACT	GPD1 and ACT	GPD1 and ACT
Stability value for best combination of two genes	0.411	0.403	0.140	0.065

Actin was found to be the best reference gene in all cases but ‘fungal-fungal’ interaction subgroup, even if it could be used combined with gpd1.

BestKeeper results for all subgroups are shown in the following Tables 4.7, 4.8, 4.9, 4.10:

Table 4.7 BestKeeper results for 'all treatments'.

All growth conditions	**sar1**	**gpd1**	**sfc1**	**tef1**	**actin**	**tub**	**BestKeeper index**
n	21	21	21	21	21	21	21
GM [Ct]	31.18	23.45	32.97	23.41	24.67	25.18	26.55
AR [Ct]	31.24	23.49	32.98	23.46	24.72	25.24	26.59
Min [Ct]	28.55	20.79	31.37	20.79	21.99	23.03	24.10
Max [Ct]	35.93	25.65	34.70	26.27	28.14	28.83	29.53
SD [± Ct]	1.60	1.05	0.80	1.27	1.40	1.42	1.19
CV [% Ct]	5.11	4.48	2.43	5.39	5.65	5.64	4.48

n: number of samples; GM [Ct]: the geometric mean of Ct; AM [Ct]: the arithmetic mean of Ct; Min [Ct] and Max [Ct]: the extreme values of Ct; SD [± Ct]: the standard deviation of the Ct; CV [% Ct]: the coefficient of variance expressed as a percentage on the Ct level.

BestKeeper vs.	**sar1**	**gpd1**	**sfc1**	**tef1**	**actin**	**tub**
coeff. of corr. [r]	0.975	0.811	0.845	0.945	0.988	0.952
p-value	0.001	0.001	0.001	0.001	0.001	0.001

The correlation between each candidate HKGs and the BestKeeper index is calculated by the *Pearson correlation coefficient* (r) and the p-value (95% level of significance).

Table 4.8 BestKeeper results for all treatments except Czepak-dox medium.

All but Czepak-dox medium	**sar1**	**gpd1**	**sfc1**	**tef1**	**actin**	**tub**	**BestKeeper index**
n	18	18	18	18	18	18	18
GM [Ct]	31.49	23.83	33.11	23.73	25.01	25.47	26.86
AR [Ct]	31.54	23.85	33.12	23.77	25.05	25.53	26.89
Min [Ct]	28.68	22.24	31.48	21.57	22.96	23.03	24.98
Max [Ct]	35.93	25.65	34.70	26.27	28.14	28.83	29.53
SD [± Ct]	1.59	0.81	0.76	1.14	1.25	1.37	1.11
CV [% Ct]	5.03	3.41	2.29	4.81	4.97	5.38	4.13

n: number of samples; GM [Ct]: the geometric mean of Ct; AM [Ct]: the arithmetic mean of Ct; Min [Ct] and Max [Ct]: the extreme values of Ct; SD [± Ct]: the standard deviation of the Ct; CV [% Ct]: the coefficient of variance expressed as a percentage on the Ct level.

BestKeeper vs.	**sar1**	**gpd1**	**sfc1**	**tef1**	**actin**	**tub**
coeff. of corr. [r]	0.985	0.725	0.824	0.930	0.984	0.952
p-value	0.001	0.001	0.001	0.001	0.001	0.001

The correlation between each candidate HKGs and the BestKeeper index is calculated by the *Pearson correlation coefficient* (r) and the p-value (95% level of significance).

Table 4.9 BestKeeper results for 'PDA, PDB, germinated conidia'.

PDA,PDB, germinated conidia	**sar1**	**gpd1**	**sfc1**	**tef1**	**actin**	**tub**	**BestKeeper index**
n	9	9	9	9	9	9	9
GM [Ct]	32.75	24.20	33.80	24.22	25.86	26.68	27.66
AR [Ct]	32.80	24.22	33.80	24.27	25.90	26.71	27.69
Min [Ct]	30.17	22.90	33.03	21.57	23.55	24.48	25.63
Max [Ct]	35.93	25.65	34.70	26.27	28.14	28.83	29.53
SD [± Ct]	1.56	0.77	0.51	1.30	1.31	1.08	1.02
CV [% Ct]	4.77	3.19	1.51	5.34	5.04	4.04	3.69

n: number of samples; GM [Ct]: the geometric mean of Ct; AM [Ct]: the arithmetic mean of Ct; Min [Ct] and Max [Ct]: the extreme values of Ct; SD [± Ct]: the standard deviation of the Ct; CV [% Ct]: the coefficient of variance expressed as a percentage on the Ct level.

BestKeeper vs.	**sar1**	**gpd1**	**sfc1**	**tef1**	**actin**	**tub**
coeff. of corr. [r]	0.986	0.979	0.657	0.951	0.994	0.956
p-value	0.001	0.001	0.054	0.001	0.001	0.001

The correlation between each candidate HKGs and the BestKeeper index is calculated by the *Pearson correlation coefficient* (r) and the p-value (95% level of significance).

Table 4.10 BestKeeper results for 'fungal-fungal interactions'.

Fungal-fungal interactions	**sar1**	**gpd1**	**sfc1**	**tef1**	**actin**	**tub**	**BestKeeper index**
n	6	6	6	6	6	6	6
GM [Ct]	29.93	23.98	32.03	23.00	23.73	23.78	25.85
AR [Ct]	29.95	23.99	32.03	23.03	23.74	23.79	25.86
Min [Ct]	28.68	23.21	31.48	21.59	22.96	23.03	24.98
Max [Ct]	31.89	25.59	33.17	25.33	25.40	25.24	27.57
SD [± Ct]	0.86	0.61	0.42	0.86	0.59	0.58	0.66
CV [% Ct]	2.86	2.55	1.31	3.75	2.50	2.46	2.57

n: number of samples; GM [Ct]: the geometric mean of Ct; AM [Ct]: the arithmetic mean of Ct; Min [Ct] and Max [Ct]: the extreme values of Ct; SD [± Ct]: the standard deviation of the Ct; CV [% Ct]: the coefficient of variance expressed as a percentage on the Ct level.

BestKeeper vs.	**sar1**	**gpd1**	**sfc1**	**tef1**	**actin**	**tub**
coeff. of corr. [r]	0.95	0.997	0.948	0.987	0.995	0.998
p-value	0.004	0.001	0.004	0.001	0.001	0.001

The correlation between each candidate HKGs and the BestKeeper index is calculated by the *Pearson correlation coefficient* (r) and the p-value (95% level of significance).

In these tables, standard deviation (SD [± Ct]) between Ct values higher than1 are marked in red since the equivalent genes are not considered to be good as reference ones. The stability rank of the reference genes - based on the standard deviation of their Ct values (std dev [±Ct]) – shows that in most cases sfc1 and gpd1 were the best ones, excluding the 'fungal-fungal interaction' subgroup. Surprisingly, in this latter case all genes could fit with our purpose. This was because of the low number of data set, which negatively affected the approach validity. BestKeeper index was calculated for each statistical subgroup and a pairwise *Pearson correlation coefficient* (r), together with a P value, was calculated for each pair 'reference gene/BestKeeper index'. All genes showed a good correlation from 0.66 up to 0.99 with P value < 0.05 (except for sfc1 in potato dextrose based media statistical subgroup). Noteworthy, all 'BestKeeper index' (except for 'fungal-fungal

interactions' statistical subgroup) – which is calculated from the genes considered stably expressed as the geometric mean of its candidate reference genes Ct values – resulted in a standard deviation (SD) value higher than 1, supporting the Authors' warning (Pfaffl *et al*., 2001), who point out that heterogeneous variance between groups of differently expressed genes leads to invalidate the use of *Pearson correlation coefficient*. Low expressed genes – which have Ct values around PCR cycles 30–35 - show different variance compared to high expressed genes. Such samples cannot be correlated parametrically.

According to both statistical analyses, actin was chosen as reference gene for the further expression analysis.

4.2 Expression of eight *C. rosea* IK726 membrane transporters genes

4.2.1 Evaluation of primers' specificity

Specific amplification for every selected gene was first checked assuming 60°C as annealing temperature: three different PCRs were set-up using three different genomic DNA as templates, with '1', '2' and '3' for *C. rosea* IK726, *B. cinerea* B05.10 and *F. graminearum* pks wild type genomic DNA, respectively (Figure 4.12). This was necessary to verify whether pathogens' RNA was accidentally extracted – since they were grown in the same plate together with *C. rosea* IK726 and the mycelium was just harvested 1mm before the contact between the colonies. No amplification was obtained in both cases. Moreover, using *C. rosea* IK726 genomic DNA as template ('1'), specific amplification was achieved for every tested gene – as evident from the single band obtained.

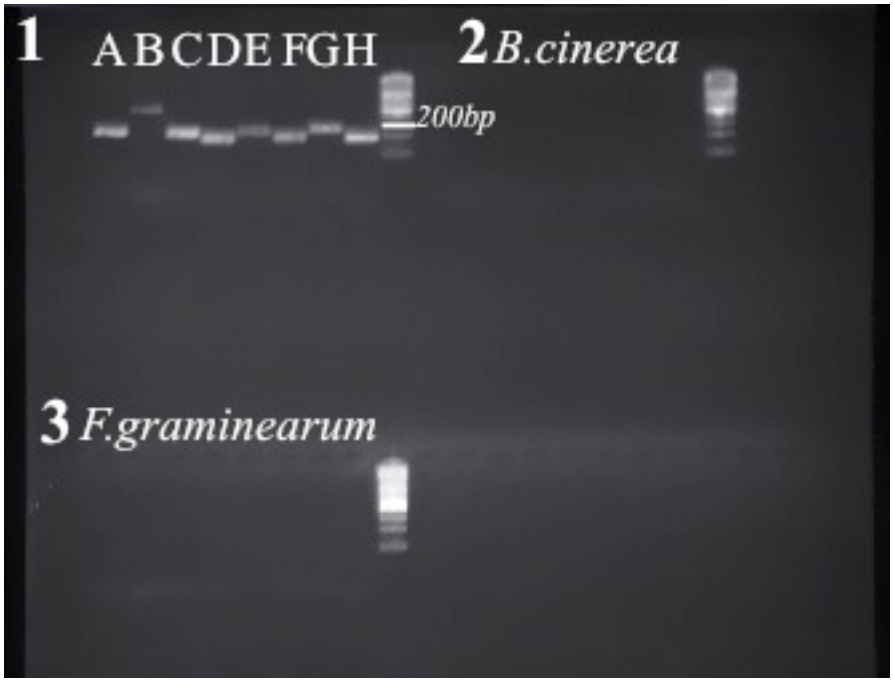

Figure 4.12 Evaluation of primers for the selected genes MFS_6455 (A), MFS_7234 (B), UbiH_0606 (C), MFS_2418 (D), ABC-G_0026 (E), MFS_2311 (F), MFS_9923 (G), ABC-C_6570 (H) in *C. rosea* (1), *B. cinerea* (2) and *F. graminearum* (3).

The MFS_7234 (B) amplicon was longer than expected. This was due to two non-coding regions within the primers pair, resulting on 387 base pairs total length without negatively affect further analysis that have been conducted on cDNA where the amplicon length was the expected one (171 base pairs) as shown in Figure 4.13:

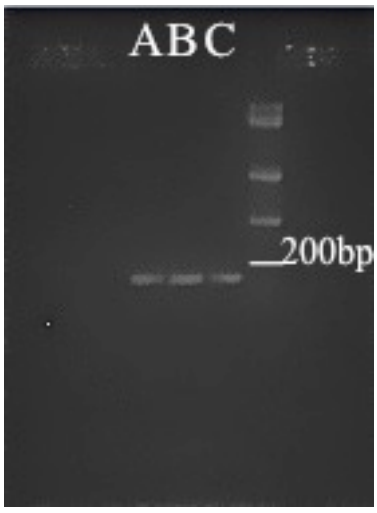

Figure 4.13 Amplification product of the'MFS_7234 gene in *C. rosea* IK726 using cDNA as template.

4.2.2 Gene expression analysis by RT- Real Time PCR: 'Relative quantification' ('delta-delta method')

RT-PCR was performed using *C. rosea* IK726 cDNA as template - as previously described in 'Materials and Methods' – testing eight putative membrane transporters genes. Actin was selected as reference gene, and used to normalize the expression level of target ones. 'Delta-delta method' was used as relative quantification approach, which rapidly allows the expression level evaluation of target genes by applying the following formula:

$$2^{-\Delta\Delta Ct}$$

All tested genes showed different expression pattern when compared each other and within different treatments. In order to go deeper in this snapshot, statistical analysis was necessary.

For each gene, the mean expression level ($2^{-\Delta\Delta Ct}$), calculated out of 3 or 5 biological replicates (depending on the type of treatment), is reported in Tables from 4.11 to 4.18:

Table 4.11 'MFS_6455 relative quantification'. The expression level is calculated by $2^{-\Delta\Delta Ct}$ formula.

MFS_6455		
treatment	**$2^{-\Delta\Delta Ct}$**	**std. dev.**
C. rosea vs *C. rosea*	2.64	2.98
C. rosea vs *B. cinerea*	0.02	0.01
C. rosea vs *F. graminearum*	90.98	35.76
Mycotoxin (ZEA)	2.44	1.54
Mycotoxin control (methanol)	1.02	0.21
Chipco Green	2.83	1.57
Cantus WDG	2.40	1.52
Apron XL	25.45	15.65
Amistar	5.57	5.85
Fungicides control (water)	1.14	0.50

Table 4.12 'MFS_7234 relative quantification'. The expression level is calculated by $2^{-\Delta\Delta Ct}$ formula.

MFS_7234		
treatment	**$2^{-\Delta\Delta Ct}$**	**std. dev.**
C. rosea vs *C. rosea*	2.86	2.89
C. rosea vs *B. cinerea*	0.03	0.00
C. rosea vs *F. graminearum*	122.02	32.14
Mycotoxin (ZEA)	0.00	-
Mycotoxin control (methanol)	1.05	0.41
Chipco Green	0.00	-
Cantus WDG	0.00	-
Apron XL	0.00	-
Amistar	0.00	-
Fungicides control (water)	0.00	-

Table 4.13 'UbiH_0606 relative quantification'. The expression level is calculated by $2^{-\Delta\Delta Ct}$ formula.

UbiH_0606		
treatment	**$2^{-\Delta\Delta Ct}$**	**std. dev.**
C. rosea vs *C. rosea*	1.23	1.01
C. rosea vs *B. cinerea*	0.20	0.03
C. rosea vs *F. graminearum*	19.39	4.76
Mycotoxin (ZEA)	2.66	0.77
Mycotoxin control (methanol)	1.06	0.42
Chipco Green	5.86	1.91
Cantus WDG	1.22	0.79
Apron XL	126.63	25.25
Amistar	3.14	0.74
Fungicides control (water)	1.07	0.45

Table 4.14 'MFS_2418 relative quantification'. The expression level is calculated by $2^{-\Delta\Delta Ct}$ formula.

MFS_2418		
treatment	**$2^{-\Delta\Delta Ct}$**	**std. dev.**
C. rosea vs *C. rosea*	1.03	0.31
C. rosea vs *B. cinerea*	0.53	0.01
C. rosea vs *F. graminearum*	1.30	0.11
Mycotoxin (ZEA)	9.96	18.32
Mycotoxin control (methanol)	2.91	5.30
Chipco Green	2.76	4.12
Cantus WDG	0.33	0.31
Apron XL	3.75	4.64
Amistar	1.26	1.18
Fungicides control (water)	3.50	6.50

Table 4.15 'ABC-G_0026 relative quantification'. The expression level is calculated by $2^{-\Delta\Delta Ct}$ formula.

ABC-G_0026		
treatment	**$2^{-\Delta\Delta Ct}$**	**std. dev.**
C. rosea vs *C. rosea*	1.22	0.98
C. rosea vs *B. cinerea*	0.25	0.03
C. rosea vs *F. graminearum*	1.97	0.66
Mycotoxin (ZEA)	10998.77	4595.89
Mycotoxin control (methanol)	0.80	0.45
Chipco Green	0.00	-
Cantus WDG	0.41	0.62
Apron XL	171.05	25.26
Amistar	0.00	-
Fungicides control (water)	0.12	0.28

Table 4.16 'MFS_2311 relative quantification'. The expression level is calculated by $2^{-\Delta\Delta Ct}$ formula.

MFS_2311		
treatment	**$2^{-\Delta\Delta Ct}$**	**std. dev.**
C. rosea vs *C. rosea*	1.02	0.22
C. rosea vs *B. cinerea*	4.61	1.22
C. rosea vs *F. graminearum*	0.39	0.07
Mycotoxin (ZEA)	1.93	2.35
Mycotoxin control	1.11	0.61
Chipco Green	2.23	3.15
Cantus WDG	3.10	3.74
Apron XL	4.54	4.93
Amistar	1.51	3.37
Fungicides control (water)	0.53	0.76

Table 4.17 'MFS_9923 relative quantification'. The expression level is calculated by $2^{-\Delta\Delta Ct}$ formula.

MFS_9923		
treatment	**$2^{-\Delta\Delta Ct}$**	**std. dev.**
C. rosea vs *C. rosea*	1.07	0.47
C. rosea vs *B. cinerea*	3.86	1.36
C. rosea vs *F. graminearum*	1.13	0.09
Mycotoxin (ZEA)	0.49	1.09
Mycotoxin control (methanol)	0.42	0.66
Chipco Green	0.00	-
Cantus WDG	0.00	-
Apron XL	0.00	-
Amistar	0.00	-
Fungicides control (water)	0.00	-

Table 4.18 'ABC-C_6570 relative quantification'. The expression level is calculated by $2^{-\Delta\Delta Ct}$ formula.

ABC-C_6570		
treatment	**$2^{-\Delta\Delta Ct}$**	**std. dev.**
C. rosea vs *C. rosea*	1.02	0.21
C. rosea vs *B. cinerea*	1.62	0.23
C. rosea vs *F. graminearum*	0.43	0.04
Mycotoxin (ZEA)	0.83	0.39
Mycotoxin control (methanol)	1.03	0.25
Chipco Green	2.23	0.37
Cantus WDG	1.47	0.89
Apron XL	0.76	0.34
Amistar	2.11	0.84
Fungicides control (water)	1.08	0.50

A '0.00' value of expression level corresponds to a 'no expression' of the relative gene. This could mean both the gene was not expressed and/or the RT-PCR amplification cycles were not enough to allow the gene's fluorescence to cross the threshold line. If you are interested about raw Ct values see online supplementary **S.5**.

4.2.3 Statistical analysis

Gene expression data (expression level, $2^{-\Delta\Delta Ct}$) were subjected to a two-way analysis of variance (ANOVA), assuming treatments and kind of genes as independent variables, as described in Materials and Methods. Treatments were follows:

- ‘biological interactions’: fungal-fungal interactions;

- ‘mycotoxin treatment’: zearalenone and its control;

- ‘fungicide treatment’: four different fungicides and their control.

When fungal-fungal interactions and genes were used as sources of variability, both showed a significant P value. Moreover, the interaction between treatment and gene was highly significant:

Table 4.19 ANOVA: expression level was used as dependent variable, whereas treatment (biological interactions *C. rosea* vs *B. cinerea*, *C. rosea* vs *C. rosea* and *C. rosea* vs *F. graminearum* pks wild type) and Gene (MFS_9923, MFS_7234, ABCC_6570, MFS_2311, MFS_6455, UbiH_0606, MFS_2418 and ABCG_0026) were used as independent variables.

Biological interactions					
Source	Type III SS	df	Mean Squares	F-ratio	**p-value**
Treatment	12771.704	2	6385.852	69.807	**0.000**
Gene	16403.309	7	2343.330	25.616	**0.000**
Gene*Treatment	33158.710	14	2368.479	25.891	**0.000**
Error	4208.037	46	91.479		

Results of pairwise comparison (Tukey’s test) in the interaction Treatment x Gene are shown in Table 4.20. There is no significant difference in most of combinations, whereas a significant increasing in expression level was observed for MFS_6455 and MFS_7234 in presence of *F. graminearum*, whose expression levels were also significantly different from each other.

Table 4.20 Pairwise comparison (Tukey's test) of expression level ($2^{-\Delta\Delta Ct}$) of selected genes (MFS_9923, MFS_7234, ABCC_6570, MFS_2311, MFS_6455, UbiH_0606, MFS_2418 and ABCG_0026) in the biological interactions *C. rosea* vs *C. rosea (C. r. – C. r*), *C. rosea* vs *B. cinerea* (C.r. – B. c.) and *C. rosea* vs *F. graminearum* pks wild type (C. r. – F. g.). At different letters correspond values significantly different for P≤0.05.

Biological interactions		
Treatment	**Gene**	**Exp. Lev. ($2^{-\Delta\Delta Ct}$)**
C.r. - B.c.	MFS_6455	0.02[a]
C.r. - B.c.	MFS_7234	0.03[a]
C.r. - B.c.	UbiH_0606	0.20[a]
C.r. - B.c.	ABCG_0026	0.25[a]
C.r. - F.g.	MFS_2311	0.39[a]
C.r. - F.g.	ABCC_6570	0.43[a]
C.r. - B.c.	MFS_2418	0.53[a]
C.r. - C.r.	MFS_2311	1.02[a]
C.r. - C.r.	ABCC_6570	1.02[a]
C.r. - C.r.	MFS_2418	1.03[a]
C.r. - C.r.	MFS_9923	1.07[a]
C.r. - F.g.	MFS_9923	1.13[a]
C.r. - C.r.	ABCG_0026	1.22[a]
C.r. - C.r.	UbiH_0606	1.23[a]
C.r. - F.g.	MFS_2418	1.30[a]
C.r. - B.c.	ABCC_6570	1.62[a]
C.r. - F.g.	ABCG_0026	1.97[a]
C.r. - C.r.	MFS_6455	2.64[a]
C.r. - C.r.	MFS_7234	2.86[a]
C.r. - B.c.	MFS_9923	3.86[a]
C.r. - B.c.	MFS_2311	4.61[a]
C.r. - F.g.	UbiH_0606	19.39[a]
C.r. - F.g.	MFS_6455	90.98[b]
C.r. - F.g.	MFS_7234	122.02[c]

When mycotoxin treatments (zearalenone or its control – methanol) and genes were used as sources of variability, both showed a significant P value as observed for their interaction (Table 4. 21).

Table 4.21 ANOVA: expression level was used as dependent variable, whereas Treatment (mycotoxin) and Gene (MFS_9923, MFS_7234, ABCC_6570, MFS_2311, MFS_6455, UbiH_0606, MFS_2418 and ABCG_0026) were used as independent variables.

Mycotoxin treatment					
Source	Type III SS	df	Mean Squares	F-ratio	**p-value**
Treatment	37865339.511	1	37865339.511	28.696	**0.000**
Gene	2.645E+008	7	37783170.359	28.634	**0.000**
Gene*Treatment	2.645E+008	7	37788999.322	28.638	**0.000**
Error	79171784.013	60	1319529.734		

Results of pairwise comparison (Tukey's test) in the interaction Treatment x Gene are shown in Table 4.22. There is no significant difference in most of combinations, whereas a significant increasing was observed for ABCG_0026 gene in presence of zearalenone.

Table 4.22 Pairwise comparison (Tukey's test) of expression level ($2^{-\Delta\Delta Ct}$) of selected genes (MFS_9923, MFS_7234, ABCC_6570, MFS_2311, MFS_6455, UbiH_0606, MFS_2418 and ABCG_0026) in presence/absence of zearalenone at different letters correspond values significantly different for $P \leq 0.05$.

Mycotoxin treatment		
Treatment	**Gene**	**Exp. Lev. ($2^{-\Delta\Delta Ct}$)**
zea	MFS_7234	0.00[a]
ctrl	MFS_9923	0.42[a]
zea	MFS_9923	0.49[a]
ctrl	ABCG_0026	0.80[a]
zea	ABCC_6570	0.83[a]
ctrl	MFS_6455	1.02[a]
ctrl	ABCC_6570	1.03[a]
ctrl	MFS_7234	1.05[a]
ctrl	UbiH_0606	1.06[a]
ctrl	MFS_2311	1.11[a]
zea	MFS_2311	1.93[a]
zea	MFS_6455	2.44[a]
zea	UbiH_0606	2.66[a]
ctrl	MFS_2418	2.91[a]
zea	MFS_2418	9.96[a]
zea	ABCG_0026	10998.77[b]

When fungicides treatments (Amistar, Apron, Cantus, Chipco and their control - water) and genes were used as sources of variability, both showed a significant P value as for their interactions (Table 4.23).

Table 4.23 ANOVA: expression level was used as dependent variable, whereas Treatment (Apron, Amistar, Cantus, Chipco and control -water) and Gene (MFS_9923, MFS_7234, ABCC_6570, MFS_2311, MFS_6455, UbiH_0606, MFS_2418 and ABCG_0026) were used as independent variables.

Fungicides treatment					
Source	Type III SS	df	Mean Squares	F-ratio	**p-value**
Treatment	51554.164	4	12888.541	303.683	**0.000**
Gene	32433.811	7	4633.402	109.173	**0.000**
Gene*Treatment	128850.108	28	4601.790	108.429	**0.000**
Error	6620.760	156	42.441		

Table 4.24 shows the expression level in presence of different fungicides (Amistar, Apron, Cantus, Chipco and their control - water). There is no significant difference among combinations fungicide/gene but MFS_6455, UbiH_0606 and ABCG_0026 in presence of Apron, whose expression levels were also significantly different to one another.

Table 4.24 Pairwise comparison (Tukey's test) of expression level ($2^{-\Delta\Delta Ct}$) of selected genes (MFS_9923, MFS_7234, ABCC_6570, MFS_2311, MFS_6455, UbiH_0606, MFS_2418 and ABCG_0026) in presence of fungicides At different letters correspond values significantly different for $P \leq 0.05$.

Fungicide treatments		
Treatment	**Gene**	**Exp. Lev. ($2^{-\Delta\Delta Ct}$)**
amistar	ABCG_0026	0[a]
chipco	ABCG_0026	0[a]
amistar	MFS_7234	0[a]
apron	MFS_7234	0[a]
cantus	MFS_7234	0[a]
chipco	MFS_7234	0[a]
ctrl	MFS_7234	0[a]
amistar	MFS_9923	0[a]
apron	MFS_9923	0[a]
cantus	MFS_9923	0[a]
chipco	MFS_9923	0[a]
ctrl	MFS_9923	0[a]
ctrl	ABCG_0026	0.12[a]
cantus	MFS_2418	0.33[a]
cantus	ABCG_0026	0.41[a]
ctrl	MFS_2311	0.53[a]
apron	ABCC_6570	0.76[a]
ctrl	UbiH_0606	1.07[a]
ctrl	ABCC_6570	1.08[a]
ctrl	MFS_6455	1.14[a]
cantus	UbiH_0606	1.22[a]
amistar	MFS_2418	1.26[a]
cantus	ABCC_6570	1.47[a]
amistar	MFS_2311	1.51[a]
amistar	ABCC_6570	2.11[a]
chipco	ABCC_6570	2.23[a]

chipco	MFS_2311	2.23[a]
cantus	MFS_6455	2.40[a]
chipco	MFS_2418	2.76[a]
chipco	MFS_6455	2.83[a]
cantus	MFS_2311	3.10[a]
amistar	UbiH_0606	3.14[a]
ctrl	MFS_2418	3.50[a]
apron	MFS_2418	3.75[a]
apron	MFS_2311	4.54[a]
amistar	MFS_6455	5.57[a]
chipco	UbiH_0606	5.86[a]
apron	MFS_6455	25.45[b]
apron	UbiH_0606	126.63[c]
apron	ABCG_0026	171.05[d]

5. Discussion

5.1 'Best' reference gene evaluation

When performing a quantitative reverse transcription-PCR (RT- PCR) analysis, several parameters need to be controlled to obtain reliable quantitative expression measures. These include variations in initial sample amount, RNA recovery, RNA integrity, efficiency of cDNA synthesis and differences in the overall transcriptional activity in the analyzed tissues/cells (Andersen *et al*., 2004). Normalization to a stably expressed gene of the target organism, often called 'reference' or 'housekeeping gene', is a powerful method to prevent qPCR internal errors. It should be carefully evaluated whether the transcription profile of putative reference genes is altered or affected by the experimental conditions. If not, using the wrong reference gene, leading to a misinterpretation of data, could substantially alter results and conclusions. For example, actin - which is widely used as reference gene - is not always one of the most stable ones (Steiger *et al*., 2010). A study performed in *Saccharomyces* reveals that *actin* scores at the third best position when tested with NormFinder and geNorm software (Stahlberg *et al*., 2008), whereas - in *Aspergillus niger* - geNorm software suggested *actin* encoding gene as one of the most stable ones (Bohle *et al*., 2007). Thus, it has become clear over the years that the ideal internal control gene, universally valid, with a constant expression level across all tissues, cells, treatments does not exist (Vandesompele *et al*., 2002).

The present study was carried out to evaluate the 'best' reference gene, among those selected, as no data has been published before in *Clonostachys rosea*. The expression level of six genes - β-Tubulin (tub); actin; translation elongation factor 1α (tef1); GTPase (sar1); glyceraldehyde 3-phosphate dehydrogenase (gpd1) and RNA polymerase III transcription factor subunit (sfc1) - was evaluated in as much different as possible growth conditions. *C. rosea* IK726 was grown in solid/liquid media, using different carbon sources (PDA, Czapek-dox medium, PDB, Czapek-Dox broth, germinated conidia) and in presence of fungi (vs *C. rosea* IK726 and vs *F. graminearum* pks wild

type). For each growth condition, RT-qPCR assay was set up and data were statistically analyzed by two excel based softwares: 'NormFinder' and 'BestKeeper'. Whereas NormFinder top ranks the candidates reference genes with minimal estimated intra- and inter- group variation, BestKeeper – based on pairwise comparison approach (*Pearson correlation coefficient*) - selects those genes with the highest degree of similarity of the expression profile across the sample set. Furthermore, heterogeneous variance between groups of differently expressed genes leads to invalidate the use of *Pearson correlation coefficient*. Low expressed genes – with Ct values of about 30–35 PCR cycles - surely show different variance compared to high expressed genes. Such samples cannot be parametrically correlated. BestKeeper approach implies that the candidates with minimal expression variation do not necessarily become top ranked, but top ranks candidates with correlated expression rather than minimal variation. Whereas model-based approach is not significantly affected by candidate genes co-regulation, this represents a major weakness for the pairwise comparison approach (Andersen *et al.*, 2004). In our case, BestKeeper top ranked sfc1 and gpd1 encoding genes in most of cases, even though the standard deviation of the 'BestKeeper index' (calculated as the geometric mean of Ct values from the genes considered stably expressed) resulted higher than 1 in all growth conditions, but the 'fungal-fungal' interactions, confirming the weaknesses of the pairwise approach as mentioned above in the text. Remarkably, actin encoding gene was found as the 'best' reference gene in all growth conditions except 'fungal-fungal' interactions where NormFinder suggests to use actin in combination with gpd1.

In the present study, the 'best' reference gene - actin - was selected by NormFinder approach - providing a more precise and robust measure of gene expression stability – as recently done by Steiger and colleagues (2010) in *Hypocrea jecorina* (anamorph: *Trichoderma reesei*).

5.2 Gene expression analysis

The hyperparasitic fungus *Clonostachys rosea* strain 'IK726' has proved to be an effective antagonist in several crops against diseases caused by a range of pathogens, e.g. *Alternaria* spp. (Jensen *et al*. 2004), *Bipolaris sorokiniana* and *Fusarium culmorum* (Knudsen *et al*. 1995), *Pythium* spp. (Møller *et al*. 2003), *Tilletia tritici* (Jensen *et al*. 2001), *Botrytis cinerea* (Macedo *et al*. 2012). *C. rosea* IK726 is able to tolerate relatively high concentrations of many synthetic and natural toxic compounds, including its own antibiotics, and also to act as strong competitor of different microorganisms. This depends on efficient cell detoxification mechanisms supported by a complex system of membrane pumps, as found by Ruocco and colleagues in *Trichoderma atroviride* (2008). Kosawang and colleagues (2014) have recently investigated the capability of *C. rosea* IK726 to tolerate both deoxynivalenol (DON) and zearalenone (ZEA), two mycotoxins commonly produced by fungi included within the Fusarium Head Blight (FHB) species complex. Whereas tolerance to DON was provided by a broad range of genes, from metabolism to transporters ('metabolic readjustment'), two ABC transporters (ABCG5 and ABCG29) may participate in conferring resistance to ZEA together with ZHD101 (zearalenone hydrolase).

Along the lines of results obtained by Kosawang and collagues (2014), the aim of the second part of this thesis was to understand whether the selected membrane transporters, belonging to MFS- and ABC-superfamily, were involved in tolerance to different fungicides as well as to the mycoestrogenic toxin zearalenone. Nonetheless, the expression rate of these genes was also evaluated on different fungal-fungal interactions, since they have been selected by analyzing *C. rosea* IK726 ESTs – relatively to transcriptomes from three different *C. rosea* IK726 fungal interactions (*C. rosea* IK726 vs *C. rosea* IK726; *C. rosea* IK726 vs *F. graminearum* pks wild type; *C. rosea* IK726 vs *B. cinerea* B05.10). Five genes were over-expressed in the interaction with *F. graminearum* pks wild type (MFS_6455, MFS_7234, UbiH_0606, MFS_2418, ABC-G_0026), whereas three genes were over-expressed in the interaction with *B. cinerea* B05.10 (MFS_2311,

MFS_9923, ABC-C_6570). In this work, Real-time RT-PCR assays have been used to study the expression levels of these *C. rosea* IK726 genes in different growth conditions.

The expression rate of MFS_6455 and MFS_7234 were up-regulated when interacting with *Fusarium graminearum* pks wild type. MFS_6455 and MFS_7234 were ~91- and ~122-fold up-regulated, respectively. When performing *Blast n* research (Altschul *et al*., 1997; http://blast.ncbi.nlm.nih.gov/Blast.cgi) using MFS_6455 nucleotide sequence as query several 'hypothetical proteins' shown up, which reveal a MFS domain by means of *NCBI conserved domain* analysis (Marchler-Bauer *et al*., 2011; http://www.ncbi.nlm.nih.gov/Structure/cdd/wrpsb.cgi). On the other hand, some characterized MFS transporters showed up significant alignments with those belonging to 'EmrB/QacA' subfamily – a drug efflux proteins family part of the Major Faciliator Superfamily (MFS), which includes also EmrB from *E. coli*, FarB (antibacterial fatty acid resistance) from *Neisseria gonorrhoeae*, TcmA (tetracenomycin C resistance) from *Streptomyces glaucescens*. Interestingly, this MFS-subfamily was previously classified as family '3' by Pao and colleagues (1998) which includes specific transporters for drugs and other xenobiotics compounds such as a putative aflatoxin efflux pump protein in *Chaetomium thermophilum* var. *thermophilum* strain DSM 1495, a toxin efflux pump in *Aspergillus clavatus* strain NRRL 1 MFS, an efflux pump antibiotic resistance protein in *Talaromyces stipitatus* strain ATCC 10500. Similar results are achieved when performing *blast n* using *C. rosea* IK726 MFS_7234 nucleotide sequence. Significant alignments resulted with a MFS transporter in *Penicillium marneffei* strain ATCC 18224 as well as with a hypothetical protein in *Verticillium albo-atrum* strain VaMs.102 containing MFS transporters conserved domain. We may speculate – based on both gene expression analysis and databases research - that MFS_6455 and MFS_7234 may play a significant role to tolerance towards a broad range of xenobiotic compounds, which may be secreted when *F. graminearum* and *C. rosea* IK726 are growing in the same plate, both by the pathogen and/or antagonist. A biocontrol agent has to deal with the

secretion of its own antibiotics compounds, which may be involved in antagonistic activity, as well as to tolerate bacterial or fungal toxins. Surprisingly, almost no expression of other genes was detected in any of the 'fungal-fungal' interactions assays, not supporting the ESTs data.

One out of the eight selected genes, ABC-G_0026, was extremely and swiftly induced by ZEA-exposure (2 h.a.i.). The expression rate of this gene was ~11111-fold up-regulated 2 hours after ZEA inoculation. According to Kosawang and colleagues (2014), this is the second demonstration suggesting the involvement of the ABC transporters in ZEA resistance. When performing blast n using ABC-G_0026 nucleotide sequence as query, several ABC-G genes showed up from different fungi belonging to Pezizomycotina subphylum: a 'hypothetical protein' in *Nectria haematococca* strain mpVI 77-13-4 (ABC-G conserved domain was found in this protein); a multidrug resistance protein CDR1 in *Verticillium albo-atrum* strain VaMs.102, and an ABC multidrug transporter in *Aspergillus flavus* strain NRRL3357. Even though an unique role for ABC transporters in *C. rosea* has not been proposed yet, whether they are expressed before ZEA degradation and/or if the activity of the enzyme ZHD101 (zearalenone hydrolase) would trigger their expression, the expression rate of ABC-G_0026 confirms that the biocontrol agent uses a specific mechanism to withstand ZEA – by efflux of ZEA and/or its digested products – as previously proposed by Kosawang and colleagues (2014). Both databases research and ABC-G_0026 expression rate (~11111-fold) support the hypothesis that ABC-G transporters may play a key-role in such mechanism.

MFS_6455, UbiH_0606 and ABC-G_0026 were swiftly induced by the fungicide Apron (2 h.a.i.). The expression rate of these genes were ~25-, ~127- and ~171-fold up-regulated, respectively. It is noteworthy that fungicide Apron XL (Syngenta) – whose active ingredient is Metaxil-M - is effective against specific pathogens as *Pythium* and *Phytophthora* spp.. Interestingly, when performing *blast n* with *C. rosea* IK726 UbiH_0606 nucleotide sequence, just one predicted protein in *Trichoderma reesei* strain QM6a showed up a significant alignment, which contains two conserved domains: 'UbiH', a flavoprotein monoxygenase (FMO) involved in energy production

and conversion - which is able to catalyze a remarkable wide variety of oxidative reactions - and NAD(P)-binding Rossmann-like domain (FMO uses NADPH as cofactor). The increased expression rate of 'UbiH' when *C. rosea* was growing in presence of Apron is along with its biological role, since FMOs are involved in metabolism of several pharmaceuticals/pesticides and toxic compounds. Furthermore in Yeast, FMOs are involved in redox cycling of glutathione to maintain the redox state of the cell (Chiba *et al.*, 1995).

As early as in 1997, de Waard proposed that a function of ABC transporters of plant pathogens was protection against plant defence products, accepting these products as substrates, thereby limiting their accumulation in mycelium and avoiding their toxic effects. On the other hand, another function of ABC transporters in pathogenesis could be the secretion of particular pathogenicity factors (toxins, peptides) from plant pathogens. This study represents a further confirmation that ABC transporters may play a significant role not only in plant pathogens but also contributing to resistance to ZEA and other xenobiotic compounds (such as fungicides), as demonstrated for *C. rosea*. MFS transporters are well known to be involved in drug resistance, especially those genes belonging to 'EmrB/QacA' subfamily. In this study we demonstrate, for the first time, the participation of MFSs to fungicide tolerance in *C. rosea*. Remarkably, only Apron-exposure gave an increasing expression of three out of the eight tested genes. Greater information are required to establish whether this is due to different molecular composition of Apron – Metalaxyl-M, N-(2,6-dimethylphenyl)-N-(mehtoxyacetyl)-DL-alanine methyl ester as active ingredient – and/or to its specific mode of action. Metalaxyl is a systemic fungicide used in mixtures as a foliar spray for foliar diseases, such as downy mildew and soilborne diseases caused by *Phytophthora* and *Pythium spp.*, which inhibits the nucleic acid synthesis. The active ingredients of the other fungicides, 'Iprodione' (Chipco), 'Azoxystrobin' (Amistar) and 'Boscalid' (Cantus) act on mycelial growth, preventing the respiration of fungi due to the disruption of electron transport chain (preventing ATP

synthesis), and inhibit growth of new fungal cells as well as blocking the energy production in existing cells by succinate dehydrogenase inhibition, respectively.

All of these fungicides can be applied on different crops in order to control several plant pathogens, from Ascomycetes to Basidiomycetes. Only Amistar and Apron are effective against Oomycetes, such as *Phytophthora* and *Pythium* spp.. No significant expression rate was registered in any of the tested genes, when *C. rosea* was growing in presence of Amistar. Thus, we hypothesize that both different mode of action and molecular structures may contribute to different expression rate.

Surprisingly, the expression level of ABC-C_6570 was not up-regulated in any of the growth conditions, even though the ABC subfamily C – also known as MRP (Multidrug Resistance-associated Protein) - is known to be involved in the detoxification of toxic compounds by means of their extrusion from the cell or sequestration into the vacuole. However, Kovalchuk and Driessen (2010) found that four out of the seven *Aspergillus nidulans* proteins belonging to this subfamily were associated with secondary metabolism clusters. It is likely that such transporters may be involved more into export of secondary metabolites rather than participate to tolerance towards xenobiotic compounds. This could be true also in *C. rosea*, where ABC-C subfamily may contribute to drug resistance to a lesser extent than ABC-G transporters.

Recently, Kosawang and colleagues (2014) investigated the biocontrol activity of *C. rosea Δzhd101* mutants, which were generated through homologous recombination. The mutants were sensitive to ZEA due to the lack of ZEA detoxifying ability, thus they exhibited reduced antagonistic capacity toward the *F. graminearum* ZEA-producing wild type strain as well as partially failed to protect wheat plants against disease caused by the non-ZEA-producing Δ*PKS4 F. graminearum* isolate. Taking into account this latter evidence, the Authors proposed that ZHD101 performs additional functions in *C. rosea* biology than merely ZEA detoxification, such as hyphal growth. In the present study, the expression rate of ABC-G_0026 supports the hypothesis that not only ZHD101 is involved in ZEA-detoxification/tolerance. However, ABC-G_0026 role in biocontrol activity has

not been elucidated yet. The disruption of ABC-G_0026 gene could led to better a understanding of its functional role in the biocontrol fungus *C. rosea*.

6. Conclusions

NormFinder uses a model-based approach for the estimation of expression variation dealing with systematic differences in the data set like different treatments or interactions. We suggest to use this software when evaluating a set of 'putative' reference gene, since the pairwise approach (*Pearson correlation coefficient*) of BestKeeper is invalidated by heterogeneous variance between groups of differently expressed genes. This is the main reason why the 'best' reference gene was selected by NormFinder in our study. According to previous work (Bohle *et al*., 2007), actin resulted the best reference gene among the selected ones. However, such a kind of evaluation should be carried out whenever the experimental conditions - such as biological system, growth condition and set of genes - vary. Otherwise, results and conclusions of the gene expression analysis could be substantially altered by using the wrong reference gene, leading to misinterpretation of data. Furthermore, it has become clear that the ideal internal control gene universally valid, with a constant expression level across all tissues, cells, treatments does not exist (Vandesompele *et al.*, 2002).

Antagonistic fungi and, particularly mycoparasitic species, can tolerate a variety of mycotoxins and antibiotics, including their own, and also some chemical fungicides. Therefore, it is likely that these microbes possess an extensive and effective membrane pump system that actively removes many different harmful compounds from the cell (Ruocco *et al*., 2008).

In the present study, a gene expression analysis performed on the biocontrol agent *C. rosea* IK726 clearly reveals that ABC-G_0026 is sharply expressed during ZEA-exposure, emphasizing its active role in ZEA-tolerance. In addition, this study confirms that the time point chosen for gene expression analysis was accurate. ABC- and MFS-transporters involved in drug resistance (ZEA

and fungicides) are expressed about 2 hours after inoculation. A gene expression analysis performed to later time points could lead to better understanding the expression of these genes in the course of time, even though Kosawang and colleagues (2014) have recently investigated the expression of abcg5 and acbg29 transporters 6 hours after inoculation.

Interestingly, we noticed an increase in expression of two MFS transporters encoding genes (MFS_6455 and MFS_7234) when *C. rosea* grew in the same plate with *F. graminearum* pks wild type. Since mycelium was collected before contact, we may hypothesize that these two transporter proteins may be involved in direct recognition of PAMPs ('Pathogens-Associated Molecular Patterns'), which trigger 'PAMP-Trigged Immunity' (PTI) in plants as well as may contribute to 'fungal-fungal' recognition. In effect, when pathogen overcomes plant defence by means of effector molecules (termed 'virulence factor') - which are mostly small proteins secreted into hosts from pathogens – we speak about 'Effector-Triggered Suppression" (ETS) of PTI; whereas, when plant possesses resistance genes (R) which directly/indirectly detect effectors (termed 'avirulence factor'), it successfully activates defence mechanisms, such as Hypersensitive Response (HR), Programmed Cell Death (PCD). Further information need to be collected to figure out whether this effectors could lead to a *C. rosea* (biocontrol agent) – *F. graminearum* (pathogen) recognition and, thus, activate mechanisms underlying biocontrol activity. Nonetheless, MFS_6455 and MFS_7234 may play a significant role in the secretion of antibiotic compounds, which could contribute to the biocontrol activity against *F. graminearum*.

Finally, UbiH_0606 encoding gene was up-regulated by the fungicide Apron. This gene belongs to flavoprotein monoxygenase (FMO) protein family, which is involved in energy production and conversion, catalyzing a remarkable wide variety of oxidative reactions. The increased expression rate of 'UbiH,' when *C. rosea* was growing in presence of Apron, is coherent with its biological role, since FMOs are involved in metabolism of several pharmaceuticals/pesticides and toxic compounds. Furthermore in yeast, FMOs are involved in redox cycling of glutathione to maintain

the redox state of the cell (Chiba *et al.*, 1995). Thus, *C. rosea* seems to deal with the presence of Apron, metabolizing the fungicide as source of energy. Furthermore, the promising outlook that biocontrol agent *C. rosea* IK726 can be applied along with Apron for crops protection is even supported by the expression rate of ABC-G_0026 and MFS_6455 genes. In any case, further information need to be collected in order to elucidate (i) the reason why only Apron-exposure triggers the expression of three out of eight genes, whether it depends on its molecular structure and/or its specific mode of action, and whether (ii) taking into account other membrane transporters, within *C. rosea* IK726 genome, would lead to the same conclusions.

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"Where the acknowledgements have no name"

I'm writing whence I left. From salty taste
which pushed me. Gratitude is what of purest
I feel.
Gratitude, if I push me beyond and,
I am surprised at it, again.

Words and actions,
they melted into suggestion,
moving inside me.
Adventure's instinct!

Smiles helped
separation and loneliness.

Finally, looks minded gaps,
for either a hello or a farewell.
I painted a circle.

I'm where I left. From my beloved salty taste
full of 'Good',
with no borders and a single language.

To all of You,

THANKS.

Ringraziamenti

"Dove i ringraziamenti non hanno nome"

Scrivo da dove son partito. Dal salmastro
che mi spinse. È gratitudine ciò che di più
puro sento.
È gratitudine se mi spingo oltre e,
di nuovo, mi stupisco.

Parole e gesti,
si fusero in consiglio,
mossero dentro.
Istinto d'avventura!

Sorrisi soccorsero
lontananza e solitudine.

Infine, sguardi colmarono distanze,
per un ciao o un addio.
Dipinsi un cerchio.

Sono dove partii. Dal salmastro a me caro
pieno di 'Bene',
senza confini ed un solo linguaggio.

A tutti voi,

GRAZIE!