Functional characterization of sterol regulatory element transcription factors in the biocontrol fungus *Clonostachys rosea*

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Abstract

Phytopathogens account for a large portion of the yield loss in the world today. Developing sustainable strategies to limit their effect for increasing food safety and security is one of important challenge for agriculture and horticulture production systems. Using biological control is one of the cornerstones in Integrated Pest Management (IPM), in which multiple control strategies are used and with the aim to reduce pesticide use. The biological control agent *Clonostachys rosea* IK726 is a fungal antagonist of many important phytopathogens. The ability of *C. rosea* IK726 to tolerate relatively higher dose of fungicide makes it possible to use it in IPM strategies that includes both BCA and fungicides. To develop a successful IPM strategy, the underlying mechanisms of fungicide tolerance in *C. rosea* need to be further investigated. The role of Sterol Regulatory Element Binding Proteins (SREBP's) in azole tolerance, pathogenesis and hypoxia have recently been studied in numerous fungi and provided further knowledge of the sterol regulatory pathway in fungi. The aim of this study was to identify proteins involved in sterol regulatory pathway and characterize the biological function of SREBPs in *C. rosea* with emphasis on their role in fungicide tolerance, hypoxic resilience, antagonism and biocontrol. Blast search against *C. rosea* genome identified two genes coding for SREBP (named SRE1 and SRE2), one for INSIG (insulin induced-genes) and two for SCAP (SREBP cleavage-activating protein). The result was validated by conserved domain analysis and phylogenetic analysis. Functional characterization of SRE1 and SRE2 in *C. rosea* was performed by generating gene deletion mutants of *sre1* and *sre2*. Gene deletion of *sre1* (Δ*sre1*) resulted in mutants with reduced growth rate (p=0,000331, p= 0,000030, p= 0,011457) on the medium supplemented with 1/60 recommended dosage of proline and (p=0,004100, p=0,000233, p= 0,000169) 4 mM cobalt chloride (hypoxia-mimetic agent) compared to that of the WT, suggesting an increased sensitivity of Δ*sre1* to prothioconazole and hypoxia. However, deletion of *sre2* showed no significant difference in growth rate under the same conditions. Interestingly, the strains Δ*sre1_1* (p=0,045334056) and Δ*sre2_55* (p=0,008146712) increased shoot length significantly compared to WT and the overgrowth of *Δsre1_1* (p= 0,042264) and Δ*sre1_15* (p= 0,020591) was significantly different to WT during dual cultivation. SRE1 and SRE2 likely influence the hyphal structure and pigmentation since this was a consistent observed phenotype within the gene deletion strains.

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Abbreviations

SREBP	Sterol Regulatory Element Binding Protein
SRE1	Sterol Regulatory Element 1 protein
SRE2	Sterol Regulatory Element 2 protein
INSIG	Insulin Induced Gene protein
SCAP	Sterol Cleavage Activating Protein
PCR	Polymerase Chain Reaction
RT-qPCR	Reverse Transcription quantitative PCR
BCA	Biological Control Agent
IPM	Integrated Pest Management
ATMT	*Agrobacterium tumefaciens* mediated transfer
ds	downstream
ups	Upstream
FR	Flanking region
F	Forward
R	Reverse
Hyg	Hygromycin
WT	Wild type
HR	Homologous Recombination
Δ*sre1*	*sre1* gene deletion strain
Δ*sre2*	*sre2* gene deletion strain

Introduction

Producing enough food for a growing population is one of the biggest future challenges for agriculture, and it is estimated that our food production needs to increase by 60% until 2050 to feed 10 billion people (Fedoroff 2015; FAO 2019). The use of industrial fertilizers and pesticides has contributed a lot to the increase in food production since their implementation (Godfray et al. 2010). However, these agrochemicals also come with environmental challenges involving eutrophication and negative effects on non-target organisms. Hence, further increase in crop production must be made without increasing the area of arable land as well as decreasing water and agrochemicals use to have more sustainable agricultural production systems (Fedoroff 2015). At the same time, climate change, loss of arable land and pesticide-resistant pests and pathogens puts additional strain on our current crop production and threaten food security (Fedoroff 2015; IPPC Secretariat 2021). The need to satisfy the global need for food under these circumstances, whilst also implementing environmental and social sustainability, requires multifaceted and integrated globally applied solutions (Godfray et al. 2010).

Food waste and food loss is an issue that has increased in attention the latest decades and minimizing food loss is a resource-efficient way to increase the food available (Gustavsson et al. 2011). The food supply chain starts on the farm and reducing pre-harvest yield loss and post-harvest diseases is one solution to increase the amount of available food.

The amount of yield loss due to pathogens and pests are different depending on the crop and country, but it is estimated that between 31-42% crops is lost annually to insects, diseases and weeds. Out of these losses, 14.1% is caused by plant diseases where fungi constitutes of 80%, which leaves an estimation of 11,28% of all yield loss to be the result of phytopathogenic fungi (Agrios 2005; Anonymous 2017; Savary et al. 2019; Peng et al. 2021b). If post-harvest diseases are accounted for, the percentage rises to 6-12% on average (Agrios 2005). However, post-harvest diseases are particularly prevalent in developing countries and tropical regions, where the losses can reach up to 50% and be particularly devastating (Tripathi & Dubey 2004; Agrios 2005). Hence, food loss due to phytopathogenic fungi is a huge issue, and is translated to losses amounting to more than 200 billion USD each year

and loss of food that would be sufficient to feed 600 million people (Agrios 2005; Anonymous 2017).

1.1 Fungicide resistance

Since the introduction of widespread fungicide usage, fungicide resistance has developed in many plant pathogen species, leaving many fungicide classes unusable today, which compromises plant disease control and food security (Lucas et al. 2015). The development of new fungicides with novel modes of action is limited and may not keep up with the current needs (Bosch et al. 2014). Even combinations of several fungicides may not be efficient in cases where plant pathogens are reported to evolve multi-drug resistance (Kretschmer et al. 2009).

There are a number of different compounds with different modes of actions that are used in fungicides, but one of the most commonly used is azole-fungicides (Price et al. 2015). Azole-fungicides targets a broad group of fungi and is relatively cheap to use which have led to its wide usage (Price et al. 2015). Fungicides containing the triazolinethione derivative prothioconazole was first introduced in 2002 and has since become one of the most commonly used substances in triazole fungicides (Price et al. 2015). Azoles targets the 14α-demethylase CYP51 which is an important enzyme in the ergosterol biosynthesis pathway (Price et al. 2014). Ergosterol is the most abundant sterol in the cell membrane, and is also involved in several other mechanisms such as protein function, fluidity and permeability (Dhingra & Cramer 2017; Rodrigues 2018). However, there have been many instances since then where fungi have developed resistance or exhibit tolerance to prothioconazole (Price et al. 2014).

Succinate dehydrogenase inhibitors (SDHI's) are broad spectrum fungicides that inhibits the respiration of fungi (Peng et al. 2021). Over 20 phytopathogens has reported to have reduced sensitivity or tolerance to SDHI's (Peng et al. 2021). The resistance level are considered medium to high by the Fungicide Resistance Action Committee (FRAC) and can be correlated to point mutations of target genes (FRAC 2020; Peng et al. 2021a). Two of the newest SDHI's are boscalid and fluopyram, and is seen as compounds that have a potential to be used against fungi with known resistance (Peng et al. 2021a).

The first fungicides that were released were multisite inhibitors, acting on several metabolic functions or cellular processes of the fungi. The development of resistance to these fungicides was considerably slower compared to the single site inhibitors that is mainly used today. Because single site inhibitors are often systemic and highly active and efficient, the selection pressure becomes considerably high in fungal populations. Furthermore, resistance towards single site inhibitors can typically be achieved by only a few mutational events resulting in

minor conformational changes of the target protein. The high intensity of selection efficiently selects for pathogen individuals carrying alleles conferring resistance, which results in fast development of resistant populations (Lucas et al. 2015).

The fungicides mode of action is however not the only determining factor during selection. The exposure time and dosage of the selected fungicide can greatly influence the selection pressure in a given pathogen population. A counteracting strategy is to alternate between fungicides with different modes of action and adjusting the dosage and application timing (Milgroom & Fry 1988; Bosch et al. 2014). It is easy to assume that an increased dose and application would lead to a more effective eradication of the pathogen in interest. However, evidence show that increasing the dosage and application rather increase the selection pressure, hence increasing the rate of resistance development. The increased dose and application typically lead to a drastic decrease of sensitive populations, but that in turn results in less competition for the fungicide-resistant strains (Bosch et al. 2014).

The biology and reproductive capacity of the pathogen does also have an influence the rate of resistance development. For instance, the amount of disease cycles each year, and if there is sexual or asexual reproduction can influence the rate in which resistant alleles spread in the population (Hahn 2014; Drenth et al. 2019; Taylor & Cunniffe 2022).

Besides the mentioned target site mutation mechanism of fungicide resistance, there are other types of mutations that can result in lower fungicide efficacy. Another mechanism that can confer resistance to target site fungicides is target site overexpression (TSO). Instead of mutations at the target site, TSO involves mutations or changes in the promoter region which results in an increased transcription of the target gene and thereby increased target protein levels (Hahn 2014; Hu & Chen 2021).

There are phytopathogens that have evolved multi-drug resistance in certain areas, including Sweden. Apart from showing resistance based on target protein modifications, *Botrytis cinerea*, *Zymoseptoria tritici* and *Penicillium digitatum* has also evolved multi-drug resistance efflux pumps that protects the pathogen and limits the efficacy of several fungicides (Hahn 2014). Since many fungicides are either becoming inefficient or are restricted to use in Europe, there is an urgent need to develop alternative strategies for disease control (Lucas et al. 2015).

1.2 IPM and biological control, strategies for sustainable agriculture

Using combinations of different disease management strategies may reduce the high selection pressure resulting in fungicide resistance, as well as reducing the total amounts of chemical fungicides used. In the European Union (EU), integrated pest management (IPM) and restricted use of pesticides are management principles that have been implemented to achieve a long-term sustainable plant protection strategy (The European Commission 2009). IPM typically aims at keeping pest populations below the economical threshold level, whereas pesticides often strives for complete eradication of pests (Stenberg 2017). One of the tools available in IPM is the use of biological control, which is a strategy that use natural enemies to control pests and pathogens (Torres & Bueno 2018). The natural enemy can be a parasitoid, predator, pathogen, antagonist or a competitor to the plant pathogen or pest (Van Driesche & Bellows 1996). The organism that is providing the biocontrol effect is referred to as a biological control agent (BCA). Using BCA as a standalone measure or in integrated management has both proven to be successful (Jensen et al. 2016). The biological control approach aims at reducing disease severity and suppress the pathogen population to limit economical damage, similar to other disease management procedures. Therefore, BCAs should be evaluated to ensure that the organism is safe and also economically sustainable (Köhl et al. 2011).

Using biological control is more common in organic farming where pesticide use is prohibited (Baker et al. 2020). Chemical control can often be viewed as a secure management practise in conventional agriculture, with biological control being more inefficient and expensive and thus neglected as a management strategy. The use of living organisms for biological control typically requires more knowledge about the pathosystem and timing of the application, which may be a hurdle for implementation. However, there are indirect and additional aspects that can be considered in the decision making process (Jensen et al. 2016). As mentioned before, fungicide resistance is becoming more prevalent, and fungicides are losing their potency to many pathogens which weakens the argument for pesticides being the most efficient and economically sustainable, at least in the long term. Chemical control also come with an environmental and social cost, which is harder to translate into economic figures (Menzler-Hokkanen 2006). Incorporating biological control with traditional management procedures and current application technologies could strengthen the incentive to use a BCA whilst also reducing pesticide use, providing sustainable and efficient plant protection.

Another drawback with extensive pesticide use is that it can have a negative impact on populations of natural enemies, thereby reducing the natural biological control ecosystem service (Stenberg et al. 2021). The use of BCAs with a certain level of tolerance towards fungicides can in fact be a beneficial trait and can

increase the potential of combining different control measures. For example, integrated management practices with reduced fungicide use and BCAs from the fungal genus *Trichoderma* has proven efficient (Jensen et al. 2016). Likewise, similar and ongoing studies with the fungal BCA *Clonostachys rosea* indicate its potential success in an integrated approach with pesticides (Jensen F. et al. 2021).

1.3 *Clonostachys rosea* as a biocontrol agent

The ascomycete fungus *C. rosea* was first described in 1907 by Bainier and was later reported as a potent mycoparasite by Barnett & Lilly (1962). Initially, *C. rosea* was named and classified as *Gliocladium roseum*, but reclassified in 1999 (Schroers et al. 1999). However, the name *G. roseum* is considered as a synonym and is sometimes still used in the literature of biocontrol (Jensen F. et al. 2021).

C. rosea can be found worldwide in a range of different climates and soil types which greatly affect its potential to be used as a BCA (Sutton et al. 1997). Strains have been isolated from plant leaves, roots, flowers and plant debris as well as in the soil and from fungi, nematodes and insects (Walker & Maude 1975; Mueller & Sinclais 1986; Verdejo-Lucas et al. 2002; Mendoza Garcia et al. 2003; Nobre et al. 2005; Haarith et al. 2020). Because of its versatility in life strategies and habitats, *C. rosea* is considered to have an ecological generalist lifestyle which is tightly linked to its potency as a BCA (Jensen F. et al. 2021).

The antagonistic ability of *C. rosea* can vary between strains, and many strains are curated for a wide variety of different crops on the market globally (Iqbal et al. 2020; Jensen F. et al. 2021). The strain IK726 has proven to be an effective BCA against some of the most economically damaging plant pathogens (Dean et al. 2012), including *F. culmorum* and *Bipolaris sorokiniana* (Knudsen et al. 1995), *Alternaria* spp. (Jensen et al. 2004), *Zymoseptoria tritici* (Jensen et al. 2019), *Pythium tracheiphilum* (M⊘ller et al. 2003), *Tilletia tritici* (Jensen et al. 2001) and *Botrytis cinerea* (Sutton et al. 1997, 2002).

The antagonistic lifestyle enables *C. rosea* to kill or outcompete other fungi in the soil (Jensen F. et al. 2021). The main strategies *C. rosea* uses is exploitation and interference competition, parasitism and antibiosis (Jensen F. et al. 2021). Antagonistic antibiosis is the disturbance and inhibition of another organisms by another organism with the usage of metabolites (Cook & Baker 1983). The antibiosis of *C. rosea* involves the production of toxic compounds, cell-wall degrading enzymes and secreted proteins to kill their fungal preys (Dubey et al. 2014b, 2020; Karlsson et al. 2015; Fatema et al. 2018). The direct parasitism by *C. rosea* is through infection structures with direct penetration and producing an appressorium like structure (Makkonen & Pohjakallio 1960; Walker & Maude 1975; Li et al. 2002; Karlsson et al. 2015). Apart from being a potent antagonist, *C.*

rosea can also promote plant growth and induce plant defence/resistance against pathogens (Roberti et al. 2008; Lahlali & Peng 2014).

The mycoparasitic interaction between *C. rosea* and plant pathogenic fungi results in exposure of toxic secondary metabolites, produced by *C. rosea* but also by the fungal prey, and tolerance towards these compounds is therefore a very beneficial and necessary trait (Dubey et al. 2014a, 2016; Jensen F. et al. 2021). Tolerance towards particular secondary metabolites by means of cellular efflux as well as detoxification has evolved in *C. rosea*, including the mycotoxin zearalenone (ZEA) produced by several *Fusarium* species as well as phenazine metabolites from *Pseudomonas chlororaphis* (Utermark & Karlovsky 2007; Kosawang et al. 2014; Karlsson et al. 2015).

Managing plant pathogens with both *C. rosea* and fungicides could be possible since *C. rosea* has shown a certain level of tolerance to iprodione (dicarboximides)- and mefenoxam-based fungicides, boscalid (succinate dehydrogenase inhibitors), guazatine, triticonazole, carboxin and thiram (dithiocarbamates) (Roberti et al. 2006; Veloukas et al. 2011; Dubey et al. 2014a; Karlsson et al. 2015). Using an integrated management procedure with *C. rosea* and reduced fungicide use has proven successful against *B. cinerea* in strawberry and against *F. culmorium* in wheat (Roberti et al. 2006; Cota et al. 2009). Depending on the tolerance level of *C. rosea* to the particular fungicide, it may be applied either directly or a few days after *C. rosea* application (Jensen F. et al. 2021).

A common concern of using an antagonistic BCA is that non-target organisms would be subjected to antagonism, toxicity or competition and hence disturbing the ecological stability (Cook et al. 1996; Johansen et al. 2005; Jensen et al. 2016). A detailed analysis on the impact the BCA on soil microbiota is necessary and a part of the requirements for introducing a new BCA (Jensen et al. 2016). Risk assessments which take exposure time and hazard into consideration is necessary to determine if the BCA poses any risk to the environment (Cook et al. 1996).

Greenhouse experiments by Johansen et al. (2005) showed that *C. rosea* IK726 had a stimulating effect on soil microbiota and the soil enzyme activity. The risk of *C. rosea* disturbing the microbiota is hence seen low (Johansen et al. 2005). Seed treatment with *C. rosea* is a common application method for seed- and soil-borne diseases and damping off (Jensen F. et al. 2021).

1.4 Sterol regulatory element binding proteins

In mammals, sterol regulatory element binding proteins (SREBP) are membrane bound transcription factors that regulate cellular cholesterol uptake and synthesis (Espenshade & Hughes 2007). The human genome have two genes that code for three different SREBPs (Bien & Espenshade 2010). All SREBPs have a basic helix loop helix (bHLH) leucine zipper DNA binding domain with an unique tyrosine

residue at the N-terminus that enable the SREBP to bind to SRE (Sterol Regulatory Element) sequences that regulates gene expression (Bien & Espenshade 2010).

When cholesterol levels are sufficient in the cell, SREBP are bound to the sterol-sensing protein SCAP (SREBP cleavage activating protein) at the endoplasmic reticulum (ER) membrane. This binding is allowed by a conformation change of SCAP due to its binding to cholesterol. In this conformation, the SREBPs N and C terminus face the cytosol, with the C terminus bound to SCAP, and with the SREBP transmembrane region bound to the ER membrane (Bien & Espenshade 2010). SCAP further form a complex with the ER-bound protein INSIG (Insulin Induced Gene protein) (Yang et al. 2002).

When cholesterol levels are low, the binding between SCAP and INSIG is disrupted which releases the SREBP-SCAP complex. The complex is then furthered transported to the Golgi apparatus where the N-terminus of the SREBP is released from SCAP by proteolytic cleavage by two proteases (Espenshade & Hughes 2007). The release enable SREBP to travel to the nucleus where it binds to the sterol regulatory element (SRE) which induces gene expression of proteins involved in lipid biosynthesis and uptake (Espenshade & Hughes 2007).

The first fungal homologs of SREBP, INSIG and SCAP, called SRE1 (sterol regulatory element 1), INS1 and SCP1 respectively, were identified in the fission yeast *Schizosaccharomyces pombe*, and was shown to have overall similar functions as in mammals but for ergosterol (Hughes et al. 2005). However, the SRE1 in *S. pombe* had some additional functions compared to mammals. The research in *S. pombe* revealed that SRE1 also had an important part in counteracting azole compounds that targets the ergosterol biosynthesis pathway, as well as to stimulate the transcription of genes that induce hypoxia adaptation (Hughes et al. 2005). Ergosterol biosynthesis requires high levels of oxygen, and as demonstrated in *S. pombe*, SRE1 stimulated its own transcriptions under anaerobic or low oxygen conditions (Todd et al. 2006). SRE1 further induced genes for oxygen-requiring biosynthetic pathways for ergosterol, heme, sphingolipid and ubiquinone (Todd et al. 2006).

SREBPs, INSIG and SCAP was later investigated in several fungal species with diverse life styles such as *Cryptococcus neoformans*, *Candida albicans*, *Magnaporthe grisea*, *Saccharomyces cerevisiae*, *Xanthophyllomyces dendrorhous* and *Aspergillus fumigatus* (Lane et al. 2001; Hughes et al. 2005; Chang et al. 2007; Willger et al. 2008; Bien & Espenshade 2010; Gutiérrez et al. 2019).

The important role of the *Aspergillus fumigatus* SRE1 homolog SRBA during anaerobic conditions was further studied, which revealed that a loss of *SrbA* resulted in a gene deletion strain that was not able to grow in hypoxic conditions (Willger et al. 2008). Adaptation to hypoxic microenvironments is a necessity for pathogenicity in *A. fumigatus*, and the gene deletion strains consequently lost their ability to cause disease, which concluded that *srbA* is essential for pathogenesis

(Willger et al. 2008). Similar to the result in *S. pombe*, *srbA* had an important role in azole tolerance with the gene deletion strain of *A. fumigatus* becoming more sensitive to azole compounds. Gene deletion of *srbA* did also result in abnormal hyphal branching and cell wall plasma membrane interfaces which indicate that *srbA* is necessary for cell polarity and maintaining cell wall processes (Willger et al. 2008).

Apart from SRE1, *S. pombe* contain an additional SREBP-like protein that is called SRE2 (Hughes et al. 2005). Like all SREBPs, SRE2 had the basic helix-loop-helix (bHLH) domain with the unique tyrosine residue (Hughes et al. 2005; Bien & Espenshade 2010). Both SRE1 and SRE2 are predicted to being membrane bound transcription factors because they are predicted to contain two transmembrane helices (Hughes et al. 2005). However unlike SRE1, SRE2 do not have the C-terminal domain that interacts with SCP1 (Hughes et al. 2005). Interestingly, SRE2 is thus not cleaved from SCP1 under low sterol levels which raises questions as to why it would be associated with the membrane when its detainment in the ER is not dependent on SCP1. SRE2 is not as well studied as SRE1, leaving many questions regarding its specific function, regulation and evolutionary history (Bien & Espenshade 2010).

In *S. pombe*, gene deletions of *ins1* and *sre2* have no effect on SRE1 cleavage, which suggests that INS1 is not essential for the ER retention of SCP1 in *S. pombe* (Hughes et al. 2005). Also, research in *C. neoformans* revealed that a homolog of INSIG was absent, and that only the *C. neoformans* homolog of SCAP activated SRE1 in response to low sterol and oxygen levels, similar to its function in *S. pombe* (Chang et al. 2007; Chun et al. 2007). The fungal homologous forms of INSIG and SCAP must have diverged evolutionary considering their function and existence are different between divisions and orders of yeast and filamentous fungi. Proving that further, a homolog to SCAP has not been found in any filamentous fungi (Willger et al. 2008). Only a homolog to INSIG, named INSA, was found in *A. fumigatus*, but the role of INSIG in the absence of SCAP has not yet been studied in filamentous fungi (Willger et al. 2008). This raises the question if there is another sterol sensing factor that regulate SREBPs in filamentous fungi. Since INSIG can bind sterol in mammals, INSA in *A. fumigatus* is a possible candidate among others (Willger et al. 2008; Bien & Espenshade 2010).

1.5 *Agrobacterium tumefaciens*-mediated transformation of fungi

The plant-pathogenic bacteria *A. tumefaciens* is commonly used as a tool for genetical engineering. The bacterium naturally induces tumours in plants by

delivering transferred (T) DNA into the plant cells nucleus. This process can be manipulated and used to insert foreign DNA in fungi as well, by replacing the tumour inducing sequences of the Ti-plasmids DNA to a sequence of interest (Hwang et al. 2017; Hooykaas et al. 2018). *A. tumefaciens* mediated transformation (ATMT) is seen as relatively easy and efficient method to transform fungi, and able to either have a targeted integration of the T-DNA by homologous recombination (HR) or by non-homologous end-joining (NHEJ). When using ATMT on fungi, several conditions must be met to induce transformation (Hooykaas et al. 2018). A phenolic inducer of the virulence genes, commonly acetosyringone, must be added when co-cultivating the fungi and *A. tumefaciens* on solid media (Hwang et al. 2017; Hooykaas et al. 2018). The media should have a pH between 5-6 and the temperature should be between 20-25ºC during the whole co-cultivation for successful transformation of the host (Hooykaas et al. 2018).

1.6 Aim and objectives

In the current work, I investigated the evolution of SRE1, SRE2 and to identify putative INSIG and SCAP homologs in *C. rosea* IK726 and other filamentous fungi from the order Hypocreales, by genome mining and phylogenetic analyses. My aim was to further characterize the function of *sre1* and *sre2* in the fungal BCA *C. rosea* strain IK726 by generating *sre2*- and studying provided *sre1*-gene deletion strains, with emphasis on their role in biological control, fungicide tolerance and hypoxic adaptation.

More specifically, the generated *C. rosea* gene deletion strains will be used in a bioassay with *F. graminearum* and a dual cultivation assay with *B. cinerea* and *F. graminearum* to characterize the function of SRE1 and SRE2 in *C. rosea* antagonism. Furthermore, tolerance of the gene deletion strains towards fungicides and hypoxic are assessed by supplementing their growth medium with the respiratory inhibiting fungicide Boscalid, a sterol biosynthesis inhibiting fungicide containing prothioconazole or $CoCl_2$ as a hypoxia mimetic agent, respectively.

I hypothesise that the gene deletion of *sre1* or *sre2* will have a negative effect on *C. rosea*'s hypoxic adaptation, ability to antagonise and lead to a loss of tolerance to Boscalid and prothioconazole. This is based upon the assumption that SRE1 and SRE2 have a similar role in *C. rosea* as SREBPs in other studied fungi. However, a possibility is that the loss of *sre1* (Δ*sre1)* could lead to an increased expression of SRE2, and similarly that the Δ*sre2* strains lead to an increased expression of *sre1*, resulting in a phenotype that is similar or perform better compared to *C. rosea* WT.

It's plausible that SRE1, SRE2 and INSIG is conserved in the order Hypocreales since orthologs of SRE have been found in both yeast and filamentous fungi,

suggesting that the SREBPs provide an essential function and are conserved through many orders of fungi.

The number of paralogs to SRE1 seem to differ between species studied, with some species having either one or three paralogs of SRE in the genome (Bien & Espenshade 2010). By studying the species phylogeny, it's possible to get a clue when a gene duplication event occurred. This could answer if SRE1 and SRE2 are both conserved within Hypocreales or if similar *sre* duplication events have occurred in separate species. Considering that both the yeast *S. pombe* and *C. rosea* have SRE1 and SRE2, it is likely that other species within Hypocreales have both SRE1 and SRE2 and that SREBPs within Hypocreales are possible orthologs to the SRE in yeast and thus have been conserved within fungi evolutionary.

Materials & method

2.1 Phylogenetic and structural analysis of SREBPs and associated proteins

The sequences of SRE1, SRE2, SCAP and INSIG in *C. rosea* IK726 was gathered from genome version 1 (Karlsson et al. 2015) and genome version 2 (Broberg et al. 2021). The protein structures was analysed using NCBI's conserved domain search (CDS) (Marchler-Bauer & Bryant 2004; Marchler-Bauer et al. 2011, 2015, 2017) and SMART's protein search (Letunic & Bork 2018; Letunic et al. 2021) to confirm that the proteins had similar domains and function as confirmed SREBPs, INSIG and SCAP homologs found in fungi.

The sequences were screened in MycoCosm (Grigoriev et al. 2014) blastp to determine if similar proteins are encoded for in a selected reference species of Hypocreales and Pezizomycotina. The matches from blastp were aligned in Muscle (Edgar 2004) and phylogenetically analysed using the maximum likelihood model in Mega-X version 11 (Tamura et al. 2021).

The phylogenetic analysis was made with the bootstrap method and 500 replications and pairwise deletion with 95% site coverage cutoff of gaps. The Jones-Taylor-Thornton (JTT) amino acid substitution model with gamma distribution (G) was used for INSIG, SCAP and SRE2. The JTT amino acid substitution model with gamma distributed with invariant sites (G+I) was used for SRE1. LG+G for SRE1 in Pezizomycotina. The model for combined tree with SRE1 and SRE2 was JTT +G (108 parameters) and the LG+G+I model was used for SCAP2.

2.2 Strains, primers and media

The fungal strains were provided by the Department of Forest Mycology and Plant Pathology. *Clonostachys rosea* strain IK726 wild type (WT; Karlsson et al. 2015, Broberg et al., 2021) and gene deletion strains derived from it were grown and maintained on potato dextrose agar (PDA; Becton, Dickinson and Company, Franklin Lakes, NJ) medium at 25 ºC and in potato dextrose broth (PDB; Becton, Dickinson and Company, Franklin Lakes, NJ). *C. rosea*s fungal preys *B. cinerea*

strain B05.10 (Staats & van Kan 2012) and *F. graminearum* strain PH-1 (King et al. 2017) was maintained on PDA at 25℃. GM7 medium (Utermark & Karlovsky 2008) was used for the selection of *C. rosea* gene deletion strains and Czepak-dox (CZ) agar medium was used throughout the phenotypical analysis. *Agrobacterium tumefaciens* strain AGL1 (Lazo et al. 1991) was used for ATMT and grown on YEP agar and in YEP liquid media. The expression vector containing the gene deletion cassette was provided by the supervisor. The plasmid DNA from the expression vectors were stored -70 before transforming *A. tumefaciens*.

2.2.1 PCR primers

Primers were designed to amplify approx. 1 kb of 5' -flank (upstream) and 3' –flank (downstream) regions of *sre1* and *sre2*. The primers were made and provided by the supervisor. The PCR primers used to generate *sre2* gene deletion cassette and validation of transformants are presented in Table1.

Table 1: The PCR primers sequences and length, primer melting temperature (TM), amplicon size in bp, and attB sequence.

Primer specifics					
	Sequence	Length	TM ℃	Amplicon size	Primer with attB sequence (in bold)
sre2 ups F	TTTTTGGTTGCGT CATAGCGTCTG	24	60.0		**GGGGACAAGTTTGTACAAAAAAGCAGGCTTATT** TTTGGTTGCGTCATAGCGTCTG
				1237 bp	
sre2 ups R	TCATACGGAGACA CGGCGATTTC	23	59.8		**GGGGACAACTTTGTATAGAAAAGTTGGGTG**TCA TACGGAGACACGGCGATTTC
sre2 ds F	GGTGGCCGGGCTG GAGAT	18	59.3	1089 bp	**GGGGACAACTTTGTATAATAAAGTTGTAGGTGG** CCGGGCTGGAGAT
sre2 ds R	TGGCGAGCTAATC TTAAGGCAGTT	24	57.6		**GGGGACCACTTTGTACAAGAAAGCTGGGTTTGG** CGAGCTAATCTTAAGGCAGTT
sre2 ko F	CAGGAGTTGGAG GCGGAAAT	20	55.5	3274 bp	
sre2 ko R	CGGGCAAATTCCC TTTCGTTAGC	23	61.6		
sre2 qPCR F	CCAAGCAGGGGC AGCAGTTC	20	59.3	207bp	
sre2 qPCR R	TCCCCCTGACGAC GGTTGCT	20	61.2		

Gene deletion was determined correct if the product from the gel electrophoresis was the corresponding kb size as hyg and *sre2* sequences, *sre2*. Primers of *sre2* KO-R and Hyg-F was always used in the first round of PCR, a second validation of

samples that produced a band of the correct size was made with *sre2*-KO-F and Hyg-R primers.

The *sre2* ds F and R, *sre2* ups F and R was used in the gene expression analysis, in the following PCR after RT-qPCR and cDNA was obtained.

2.3 Generation of gene deletion strains

2.3.1 Preparation of chemically competent *A. tumefaciens* cells and plasmid transformation

The preparation of chemically competent *A. tumefaciens* cells was based on Xu & Qingshun (2008). A colony of *A. tumefaciens* was grown in 2 ml of YEP liquid media in a flask and incubated at 28 ºC overnight with shaking at 250 rpm. The cells were then transferred to a flask containing 50 ml of YEP liquid media and was left to incubate for 4 hours with shaking until OD_{600} reached between 0.5-1.0. The culture was chilled on ice in a falcon tube for five minutes before centrifuging the sample at 3000 x g for 5 minutes at 4 ºC. The supernatant was then discarded, and the pellet rinsed with 10 ml of 20mM ice cold $CaCl_2$ to resuspend the cells, always keeping the sample on ice. An aliquot of 0,05 ml of the competent cells was put in pre-chilled 2ml tubes with screw caps. The aliquots were then frozen in liquid nitrogen and stored at -70 ºC.

Before transformation, the competent cells were lightly thawed at room temperature. When thawed, 1 µg of plasmid was added to the 0,05 ml competent cells, and the tube was mixed by gentle shaking. The tube was then thawed in a 37ºC water bath for 5 minutes to provide a brief heat shock to introduce the plasmid to the competent cells. 150µl YEP liquid media was added to the tube, and left incubating at 28 ºC for 2-4 hours with gentle shaking. The competent cells were transferred to two YEP plates containing 50 µg/ml spectinomycin and 50µg/ml rifampicin and left to incubate at 28 ºC for 3 days.

The positive transformants were validated through a restriction analysis by extracting plasmid DNAs from the two colonies using the Gene JET plasmid miniprep kit® (Thermo Scientific) followed by enzymatic digestion (Thermo Fisher Scientific, Waltham, MA) at 37°C for 25 min and agarose gel electrophoresis.

2.3.2 Preparation of *A. tumefaciens* as donor

A single colony of *A. tumefaciens* strain AGL1 containing the gene deletion cassette were inoculated into 10 ml of YEP medium in a flask containing 50 µg/ml

rifampicin (20 µl) and 100 µg/ml Spectinomycin (10ul). The colony was grown at 28 ºC on a rotary shaker at 200 rpm until OD_{600} reached 0,550. The solution with *A. tumefaciens* was centrifuged in a falcon tube at 4000x g for 5 minutes at 21 ºC. The supernatant was removed, and the pellet was re-suspended in 1 ml of liquid IM. The suspension was then centrifuged again as above, and the washing step repeated as well. The pellet was then resuspended in 5 ml of liquid IM. After resuspending, OD_{600} were measured, and the cell density adjusted to 0,21 with liquid IM. Acetosyringone were then added with a final concentration 200µM. The bacterial culture was then incubated at 28 ºC with 150 rpm shaking for 17 hours until OD_{600} reached 0.365.

2.3.3 *A. tumefaciens* mediated genetic transformation

C. rosea IK726 was grown on PDA agar plates at 25 ºC for ~14 days. The plates were exposed to light to initiate conidiation and to be used as recipient. A conidial suspension of *C. rosea* was made in sterile Mili-Q H_2O by harvesting conidia from the plate. The spore concentration was calculated using a Haemocytometer and the concentration adjusted with Mili-Q H_2O to 1 x 10^7 spores/ml. A 1:1 mixture of the *C. rosea* conidial suspension and induced *A. tumefaciens* were made in an eppendorf tube. 200µl of the mixture was then spread on the surface of sterilised cellophane sheets placed on eight IM agar plates containing 200µM Acetosyringone. The plates were then incubated at 24 ºC in dark. The cellophane sheets with mycelia growth were transferred to selection plates after 53 hours of co-cultivation. The selection plates were made using GM7 media supplemented with 200-400 µM cefotaxime and 200µg/ml hygromycin. The plates were left incubating at 25 ºC. >200 transformants appeared as mycelial colonies after 7 days. Pieces of the growing fungal colonies were transferred to new GM7 selection plates containing 200µg/ml hygromycin after 11 days to proceed with validation of the transformation. The selection plates were maintained at 4°C to decrease the growth rate of the colonies.

2.4 Validation of transformants

Each colony from the selection plates was numbered, and a piece of each respective colony were transferred to a fresh PDA selection plate containing 200µg/ml hygromycin, and with one piece of each colony transferred to a flask with liquid PDB medium. The PDA plates were maintained at 24 degrees and the colonies in liquid PDB were grown at 25 ºC for 2-3 days before proceeding with DNA extraction and validation by PCR.

The DNA was extracted by the CTAB (Hexadecyltrimethylammonium bromide) method, using 3% CTAB (Nygren et al. 2008). Roughly 200-300mg of mycelial

sample was dried on a clean paper and placed in a 2ml tube with screwcap, containing 2-3 glass beads and 600µl of 3% CTAB buffer. The samples were homogenised into a slurry with a homogeniser with the settings of 5000 rpm 2x 30 sec with 15 sec intervals. The samples were left incubating at 65ºC for 1-2 hours, vortexing the samples every 15 minutes until thick and DNA clumps was visible. When incubated, 600µl of chloroform was added to each tube and mixed by vortexing. The samples were centrifuged at 13000 rpm for 10 minutes. An upper phase of around 500µl separated by a salt membrane appeared, and the upper phase was transferred to new tubes, making sure not to transfer any of the lower phase or disturbing the membrane to avoid contaminations. The DNA was precipitated by adding 1000µl of isopropanol (2-propanol) and mixing it thoroughly by inverting the tubes several times. The samples were left to incubate for a minimum of 30 minutes at -20ºC. After incubation, the samples were centrifuged at 13000 rpm for 15 minutes. The supernatant was poured out, carefully not spilling out the pellet containing the DNA. The pellet was washed by adding 200µl 70% ethanol and the samples centrifuged for 5 minutes at 13000 rpm. The supernatant was poured out and left out to dry before adding 50-100ml of Mili-Q H_2O to resuspend the pellet.

The DNA concentration was checked for each sample using the Nanodrop spectrophotometer. The samples that contained DNA were saved for PCR and the concentration, if needed, adjusted to ~100 ng/µl with Mili-Q H_2O.

The PCR master mix was made with Dream Taq-DNA polymerase, 2.0mM dNTP and Taq-polymerase following the instructions from the manufacturers (Thermo-Fisher Scientific, Waltham, MA). As well as primer specific Hyg R/F and *sre2*-KO R/F. The PCR conditions were adjusted of sre2 KO-R and Hyg-F primers after a few rounds with 57 ºC to reduce unspecific binding of the primers, an increase of the annealing temperature was set to 60 ºC, table 2. The annealing temperature was left at 57 ºC for sre2-KO-F and hyg-R.

Table 2:PCR conditions using Sre2 KO-R and Hyg-F and sre2-KO-F and hyg-R.

Initial template denaturation	DNA denaturation	Primer annealing	Extension	Final extension	Store
95ºC	95 ºC	60/57 ºC	72 ºC	72 ºC	10 ºC
3 min	30 sec	30 sec x 30 cycles	3 min	5	∞

The products from the PCR were visualized using gel electrophoresis. The gel composed of SB buffer, DNA binding dye Nancy 520 (1 µl/50ml gel) and 1% agarose powder. The solution was heated until the agarose powder was completely dissolved and then put in a tray with a gel-comb in a gel caster. After cooling and

loading the PCR products, the tray was put in the electrophoresis machine at 150 V for 30-40 minutes.

The PCR product was visualized using Bio-rad Gel Doc 2000 (Bio-rad, Hercules, CA) and the transformants that produced an amplicon of the expected size of 2,7 kB were saved and repeated with an additional PCR, but this time using Sre2-KOF and Hyg-R primers to further validate the transformants. The possible positive *sre2* gene deletion strains were saved and proceeded with single spore purification.

The positive transformants were used for two rounds of single spore purification. The gene deletion strains were left to grow on PDA plates to produce conidia, subjecting them to light a few hours per day. After conidiation, conidia were gathered from the plates and diluted in sterilised Mili-Q H_2O to around 10^3 spores/ml. The spore solution of each transformant was then spread on solid PDA plates and left to grow, and colonies started to appear after 3 days. A piece of mycelia developing from single spore was then divided in two with one piece transferred to a PDA plate and the other piece to a PDA plate containing 200µg/ml Hygromycin to validate that the hygromycin resistance was still existing in the strains.

2.4.1 RNA extraction

To further validate the transformation, RNA was extracted from each strain. Firstly, a piece of mycelia from WT and respective single spore purified gene deletion strains were transferred to flasks containing PBD and were left to grow for 4 days at 25°C.

The RNA was extracted with the RNeasy® Plant Mini Kit (50) (Qiagen, Hilden, Germany) as per the protocol, and the RNA concentration of each sample was measured with the Nanodrop spectrophotometer.

The RNA concentration of the samples were adjusted to 50 ng/ul, followed by a DNaseI treatment (Sigma-Aldrich, Saint Louis, MO) to remove DNA contaminants following procedure described by manufacturers. For cDNA synthesis, 15 µl of the DNase treated RNA was transferred to the new tubes and cDNA synthesis was done using iScript cDNA synthesis kit as per protocol (Bio-Rad, Hercules, CA).

The cDNA of the samples was then used in a PCR using the Dream Taq reaction mix mentioned before, but adjusting the DNA to 2 µl of the cDNA and dividing each sample with primers specific to Hygromycin ds-R and F and primers specific to *sre2* ups R and F. The results from the PCR were then used in gel electrophoresis as described before.

2.5 Phenotypic analysis

2.5.1 Treated plates

To test the gene deletion strains and WT's ability to tolerate fungicides Proline and Cantus and hypoxia, different treated Czapek-dox (CZ) agar-plates were made. Plates only containing CZ agar was used as control. For Proline (containing active ingredient prothioconazole) treatment CZ medium was amended with 0.05 µl/ml of Proline® (Bayer CropScience, Monheim am Rhein, Germany) making a solution of 1/60 of the recommended dosage. For Cantus treatment, CZ media amended with 2 mg/ ml of the SDHI fungicide Cantus®, containing the active substance Boscalid, (BASF, Ludwigshafen am Rhein, Germany) was prepared. To induce hypoxic conditions, CZ plates containing concentrations of 2.5 mM and 4 mM $CoCl_2$ was made. Agar plugs of 6 mm from mycelial edge of each gene deletion and WT strains were transferred to the middle of the plates. The diameter of the colonies was measured with a ruler in cm the third day, sixth day, tenth day and 14 days after inoculation. The experiment was performed in four biological replicates.

2.5.2 Dual cultivation

Dual cultivation of the gene deletion strains and WT were made with *C. rosea*'s fungal preys *B. cinerea* and *F. graminearum* on CZ plates as described before in Dubey et al. (2020). In brief, agar plugs of 6 mm from *C. rosea* WT and gene deletion strains was put on the plates close to one corner. After 7 days, 6 mm plugs with *F. graminearum* or *B. cinerea* was put in the opposite corner of respective plate. The mycelial growth of the pathogens and *C. rosea* strains was recorded every day till the mycelial contact of the interacting species. Growth of *C. rosea* strains over *B. cinerea* and *F. graminearum* was recorded from the point of the mycelial contact for five days with a last measurement the tenth day after pathogen inoculation.

2.5.3 Bioassay

To test the biocontrol ability of *sre1* and *sre2* deletion strains, a bioassay using wheat seeding test for fusarium foot rot disease was performed following the procedures described before (Knudsen et al. 1995; Dubey et al. 2014).

For seed coating, winter wheat seeds of the variety Stava were surface sterilised using detergent and 2% NaOCl. A seed coating suspension of 1 x 107 spores/ml sterile Mili-Q H_2O was made by harvested spores from each gene deletion strain and the WT by counting the spores using a Haemocytometer and diluting with sterile Mili-Q H_2O. The spore suspension of each respective strain was used for

seed coating in sterile beakers containing 75+ wheat seeds, and a beaker containing only seeds and sterile Mili-Q H_2O was used for the controls.

Wheat seeds were shown in plastic pots (5 cm x 5 cm x 5 cm) filled with moistened sand (three seeds per pot). An agar plug with *F. graminearum* was placed in each pot, and then covered with sand. Uncoated wheat seeds inoculated with *F. graminearum* and uncoated wheat seeds inoculated with PDA without *F. graminearum* were used as positive and negative control treatments. The experiment was performed in five biological replicates with 15 seeds per treatment.

The pots were arranged in trays and were incubated in a growth chamber with a photoperiod of 12h light with 100-150 μmol m^2/s light intensity, and 12 h dark, 70% ±5 relative humidity, and 15°C temperature. The seedlings were watered with 300 ml water after one week and with 500ml after 12 days. After three weeks in the growth chamber, the plants were harvested and levels of disease were assessed. The disease level was scored on a 0-4 scale (Knudsen et al. 1995; Dubey et al. 2014). 0 = healthy plants with no symptoms, 1 = slightly brown roots/coleoptiles, 2 = moderately brown roots/coleoptiles, 3 = severely brown roots/coleoptiles and 4 = dead plants. To investigate the plant health promotion effect of *C. rosea* strains shoot length and shoot fresh weight of wheat seedlings were measured., The plants were then dried at 40ºC for three days to measure dry weight.

2.6 Statistical analysis

One-way Analysis of Variance (ANOVA) was performed on the bioassay data by using the Data Analysis package from Excel (Microsoft 365®). The data from the treatment and dual cultivation assay was statistically analysed through One way ANOVA using Statistica version 13.0 (TIBCO Software Inc., Palo Alto, CA, United States). Pairwise comparisons were made using the Fisher's method at the 95% significance level.

Results

3.1 Identification and sequence analysis of SRE1, SRE2, INSIG and SCAP

Putative homologs of SRE1, SRE2, INSIG and SCAP in *C. rosea* IK726 was identified by domain organization, sequence validation and phenological analyses.

SRE1 consist of 999 residues in *C. rosea* and have a predicted basic Helix-Loop-Helix (bHLH) domain on the residue interval 232-329 at the N-terminus (Marchler-Bauer & Bryant 2004; Marchler-Bauer et al. 2011, 2015, 2017). To be considered an SREBP, a unique tyrosine residue must be located in the bHLH region that is necessary for SRE-binding and thus extinguish SREBPs from other bHLH proteins (Bien & Epenshade 2010). This unique tyrosine residue was found in *C. rosea*, as well as other putative SRE1 proteins found in Hypocreales (Figure 1). The domain of unknown function (DUF2014) is found in the family of SREBP membrane bound transcription factors and is suggested to be the domain that interact with SCAP (Bien & Epenshade 2010). It is located at the C-terminus on the residue interval 586-845 in *C. rosea* (Figure 1). The SRE1 protein has a sequence identity of 50% to Sre1 found in *S. pombe* according to the JGI-blastp search (Grigoriev et al. 2014).

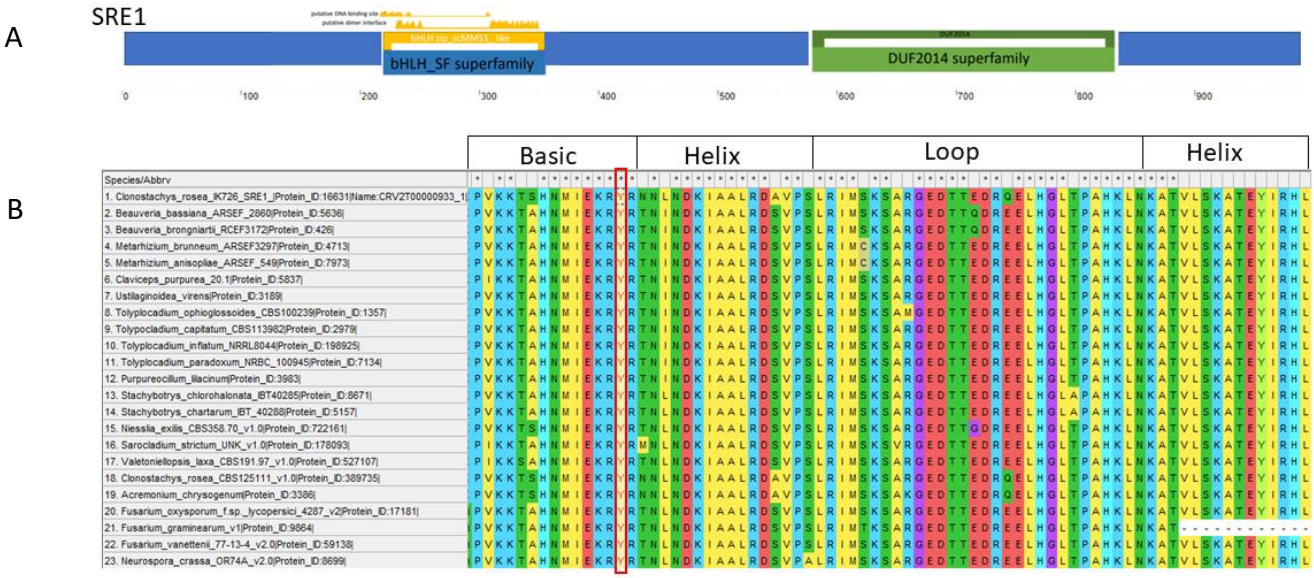

Figure 1: Analysis of the sequences of the SRE1 protein in C. rosea and alignment of SRE1 in Hypocreales. (A) a prediction of the structure of SRE1 in *C. rosea*, including its conserved domains bHLH and DUF2014 based on NCBI conserved domain search and (B) sequence alignment of putative SRE1 homologs using Mega X ver.11 (Tamura et al. 2021) of *C. rosea* and species within Hypocreales, with the unique tyrosine residue highlighted in the basic region.

SRE2 has a shorter protein sequence than SRE1, consisting of 328 residues. It has a predicted basic Helix-Loop-Helix-zipper (bHLHzip) domain on the residue interval 210-316 and similarly to SRE1 the unique tyrosine residue in the basic region (Figure 2). The putative DNA binding site is predicted to be located between the residual interval 210-280 and a putative dimer interface located between residues 220-310 (Figure 2) (Marchler-Bauer & Bryant 2004; Marchler-Bauer et al. 2011, 2015, 2017). The SRE2 protein had a sequence identity close to 60% to the SRE2 protein found in *S. pombe* in a JGI-blastp search (Grigoriev et al. 2014). Compared to SRE1, there is not as high sequence homology of the bHLH region within Hypocreales, especially in the loop region (Figure 2).

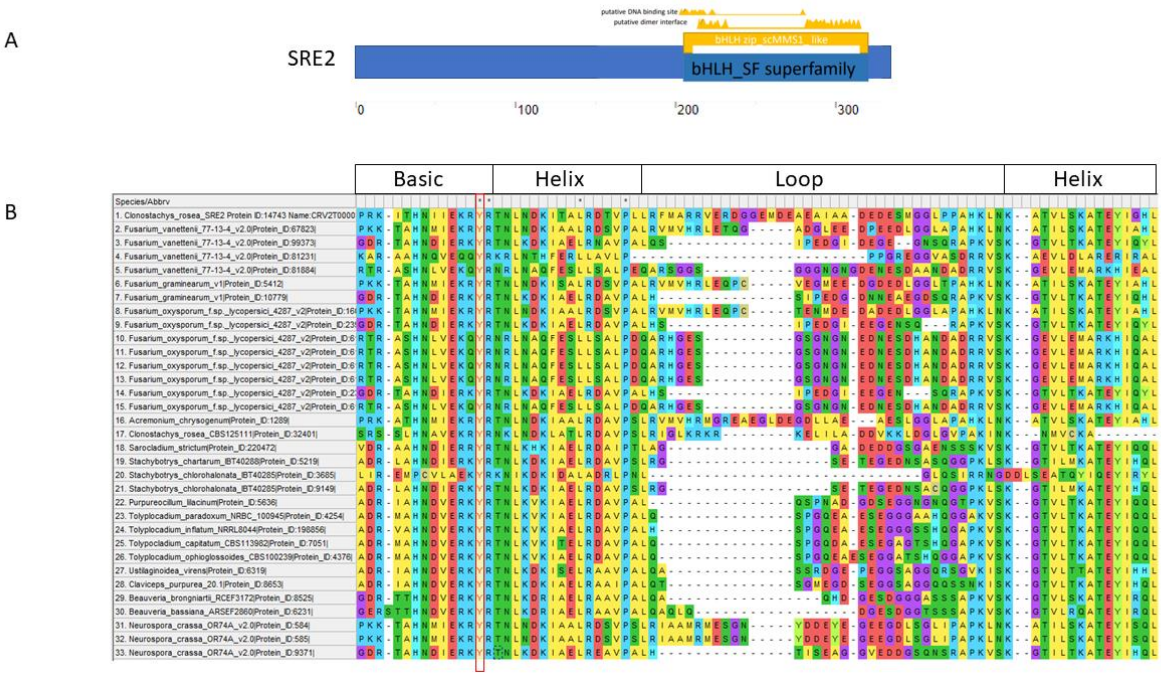

Figure 2: Analysis of the sequences of the SRE2 protein in C. rosea. (A) a prediction of the SRE2 proteins conserved domains bHLH based on NCBI conserved domain and (B) sequence alignment of putative SRE2 homologs using Mega-X ver.11 (Tamura et al. 2021) of *C. rosea* and several species within Hypocreales, with the unique tyrosine residue highlighted in the basic region.

A putative INSIG homolog was found in *C. rosea* using INSIG sequence from *Saccharomyces cerevisiae* (NP_012001.1 Nsg1p) as a query in Blast P search. The putative INSIG homolog (CRV2T00010728_1) in *C. rosea* are predicted to consist of 407 residues and have the INSIG superfamily domain is on the interval of 165-391 residues (Figure 3), and showed 49 percent similarity to *A. fumigatus* (XP_752057.1) (Marchler-Bauer & Bryant 2004; Marchler-Bauer et al. 2011, 2015, 2017).

A 43% sequence identity hit of SCAP in the *C. rosea* IK726 genome was found using the sequence of *S. pombe* Scap1 (NP_596673.1) in a JGI-blastp search (Grigoriev et al. 2014). A NCBI conserved domain search with the putative SCAP protein CRV2T00000306_1 (Protein ID: 16009) predicted that the protein sequence contains the MMPL superfamily domain at the residue interval 318-457 with high statistical evidence. The MMPL superfamily consist of proteins which are putative integral membrane proteins and suggested to be involved in lipid transport. The protein was also predicted to contain the 2A060601 super family at the residue interval 14-457. The 2A060601 superfamily include the Niemann-Pick C1 type protein family and are proteins that are most likely involved in cholesterol transport and cholesterol homeostasis. The putative SCAP sequence was also predicted to contain a domain belonging in the WD40 superfamily at the residue interval 659-730, which is a protein domain found in many eukaryotic proteins that have a

diverse set of functions (Figure 3) (Marchler-Bauer & Bryant 2004; Marchler-Bauer et al. 2011, 2015, 2017).

Similarly to *C. rosea*, the confirmed SCAP homolog in *S. pombe* Scap1 (NP_596673.1) contains the MMPL superfamily, as well as three WD40 repeats (Marchler-Bauer & Bryant 2004; Marchler-Bauer et al. 2011, 2015, 2017), explaining resemblance in sequence found on MycoCosm (Grigoriev et al. 2014).

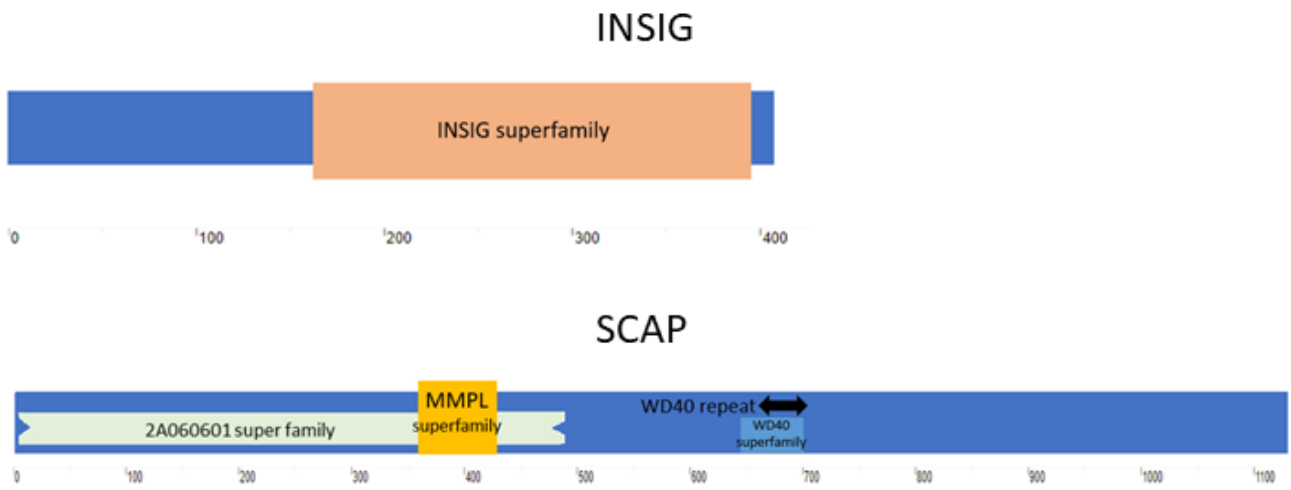

Figure 3: Protein structure of INSIG and SCAP in C. rosea based on prediced conserved domain in NCBI conserved domain search. A) INSIG protein consisting of approximately 400 residues and are predicted to have the INSIG domain between the residue interval 165-391. B) SCAP protein predicted domains MMPL, WD40 and 2A060601.

During the sequence analysis of the putative SCAP protein, an additional SCAP-like protein was found. It will further be referred to as SCAP2.

The SCAP2 protein is 1253 residues and predicted by NCBI CDS to only contain the 2A060601 super family and not the other domains found in the putative SCAP proteins of Pezizomycotina. Instead, the 2A060601 super family is stretching over almost the whole protein (15-1216 residues), having a very high statistical certainty (figure 4). The 2A060601 super family only stretches between roughly 400 residues in SCAP, but the sequence seems to be broken and have a less high statistical certainty CDS search (Marchler-Bauer & Bryant 2004; Marchler-Bauer et al. 2011, 2015, 2017). This could indicate that the region 2A060601 is larger or interrupted with the other domains that is located on the SCAP protein, explaining the lower statistical certainty of the location of the domain.

A SMART (Letunic & Bork 2018; Letunic et al. 2021) sequence search including PFAM domains and signal peptides of SCAP2 gave a more detailed view of the protein but with less statistical certainty. The SMART search located a NPC1 domain located in the N-terminal, which is a domain found in Niemann-Pick C proteins. The protein also contained a transmembrane region, the MMPL domain and Patched domain that is found in Niemann Pick-C proteins (Figure 4).

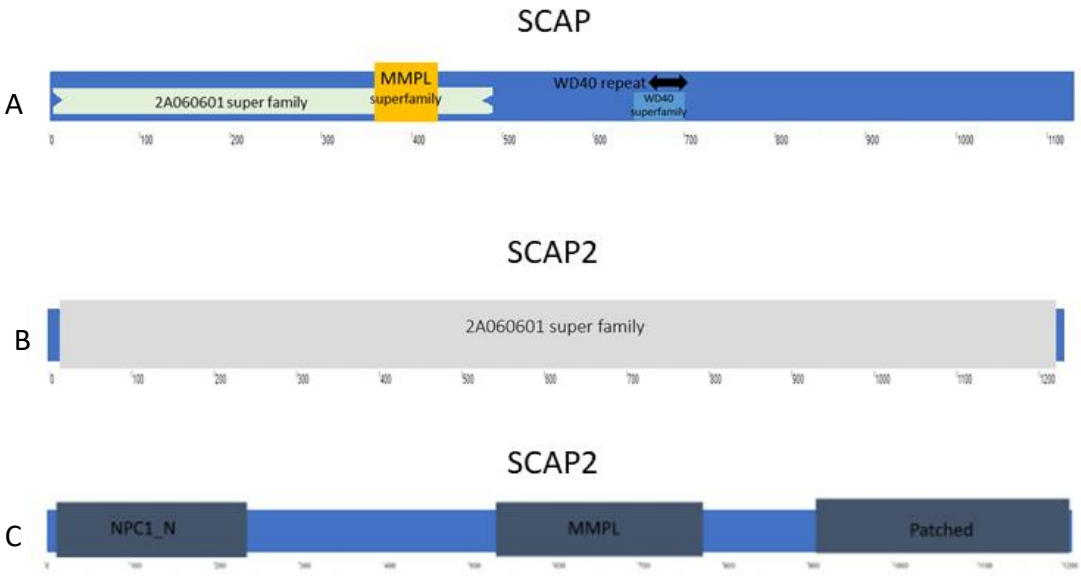

Figure 4: The SCAP and SCAP2 proteins and predicted domains based by NCBI CDS and SMART search. A) SCAP found in *C. rosea*. B) SCAP2 and the predicted domains based by NCBI CDS search and C) SCAP2 and predicted domains based on SMART search.

The SCAP2 proteins had sequence and domain similarity to proteins that are considered putative sphingolipid transporters (Grigoriev et al. 2014). Sphingolipids are necessary for the plasma membranes function, and there are domains in the plasma membrane where sphingolipids are particularly enriched (Santos et al. 2020).

3.2 Phylogenetic analysis on SRE1, SRE2, INSIG, SCAP and SCAP2

All the reference genomes in Hypocreales but *Escovopsis weberi* contained putative SRE1 homologs, figure 5. There were a bHLH protein in *E. weberi*, but it did not contain the unique tyrosine residue and was thus excluded from the analysis. SRE1 might have a common ancestor in Sordariomycetes since *N. crassa* is rooted with the Hypocreales species, but the bootstrap value of 65 is on the verge of being too low for such hypothesis.

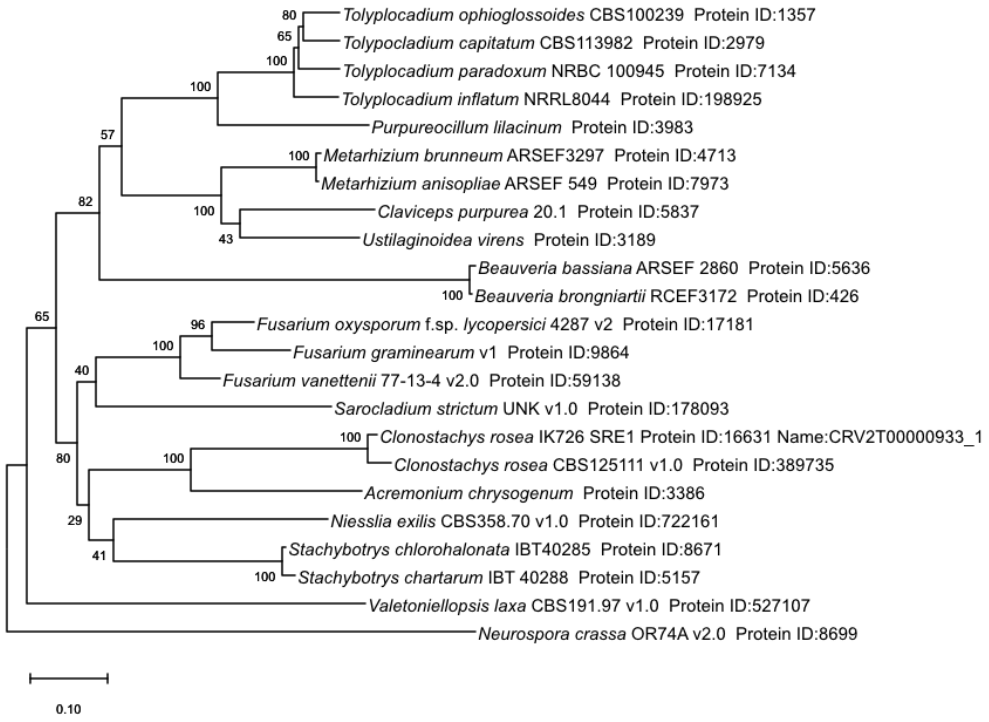

Figure 5:Putative SRE1 homologs in Hypocreales generated with Mega-X ver.11, using the Sordariomycete Neurospora crassa as an outgroup. A common ancestor to SRE1 in Hypocreales is not certain because of low bootstrap value.

A brief location of SRE1 putative homologs was made in JGI-blastp (Grigoriev et al. 2014) with a few species from each order of Pezizomycotina, revealing that putative homologs occurred in Pezizomycetes, Eurotiomycetes, Orbiliomycetes (not in *Drechslerella stenobrocha* 248), Dothiomycetes, Sordariomycetes, Leotiomycetes, Xylonomycetes and Lecanoromycetes (not in *Xanthoria parietina* 46-1-SA22 v1.1). A phylogenetic tree was made with the species found, but the tree had very low support, suggesting that the sequences might be too diverse and that there is low homology between the proteins. The alignment of the sequences showed that there was low homology between the studied proteins in the regions between the bHLH and DUF2014 domains, with some proteins being much longer and some shorter. This can affect the bootstrap values a lot.

The phylogeny of SRE2 in Hypocreales did not follow the phylogeny of species to the same extent as for SRE1. Even though *N. crassa* was the out-group, it clustered and showed that it had the same ancestor as many of the Hypocreales species, which indicate that an ancestor might be located in Sordariomycetes or possibly longer back in Ascomycetes. Gene duplication and horizontal transfer events in *F. oxysporum* f.sp. *lycopersici, F. vanettenii* and *F. graminearum* have

likely occurred at separate occasions, as implicated by the phylogenetic tree, figure 6.

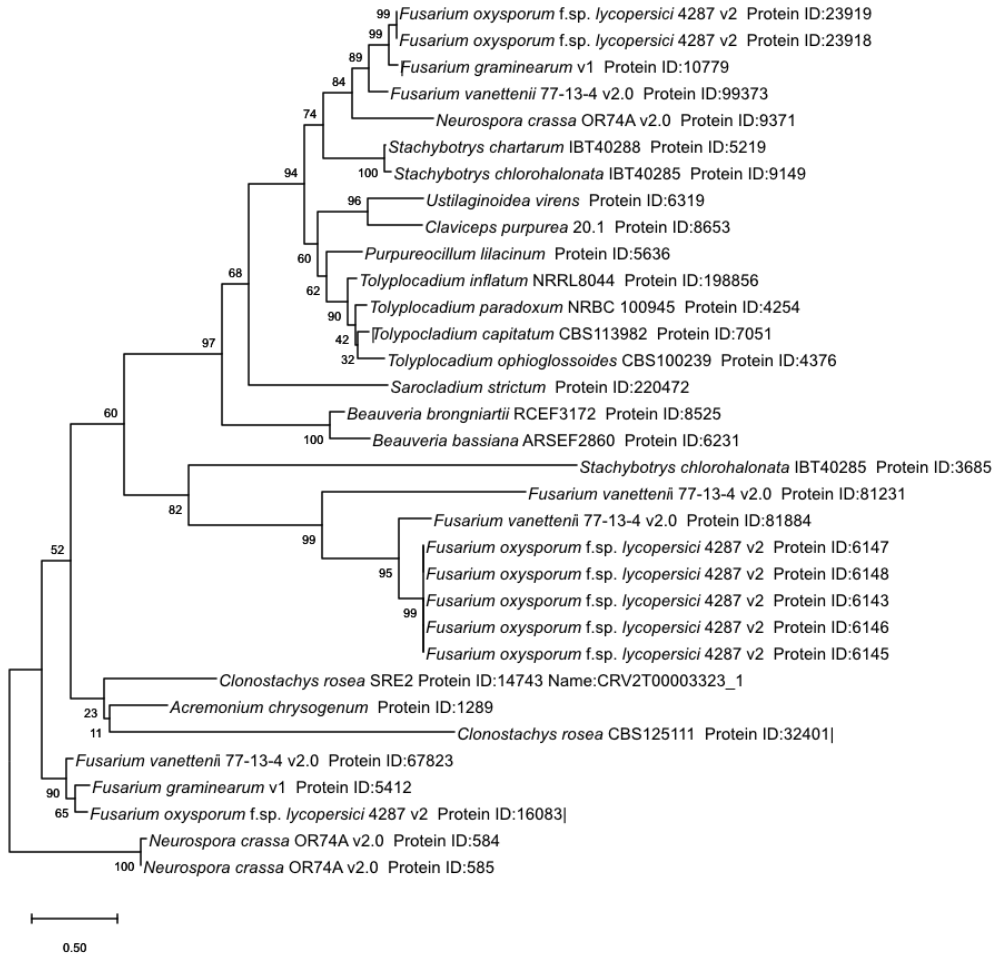

Figure 6: Putative SRE2 homologs in Hypocreales generated with Mega-X ver.11. Several paralogs are located within Hypocreales, and the bootstrap values indicate that *Fusarium* and the outgroup *N. crassa* have attained SRE2 from different ancestors.

A brief investigation of SRE2 proteins in a few species of each order within Pezizomycotina was made. Finding that no SRE2-like protein occurred in Eurotiomycetes, Orbiliomycetes, Pezizomycetes, Xylonomycetes but potentially occurred in Leotiomycetes, Sordariomycetes, and possibly in the Lacanoromycete *Lobaria pulmonaria*, however a SRE2-like protein could not be found in other species within the class.

The phylogeny of INSIG in Hypocreales showed low bootstrap support between branches and a common ancestor was not able to be distinguished, figure 7.

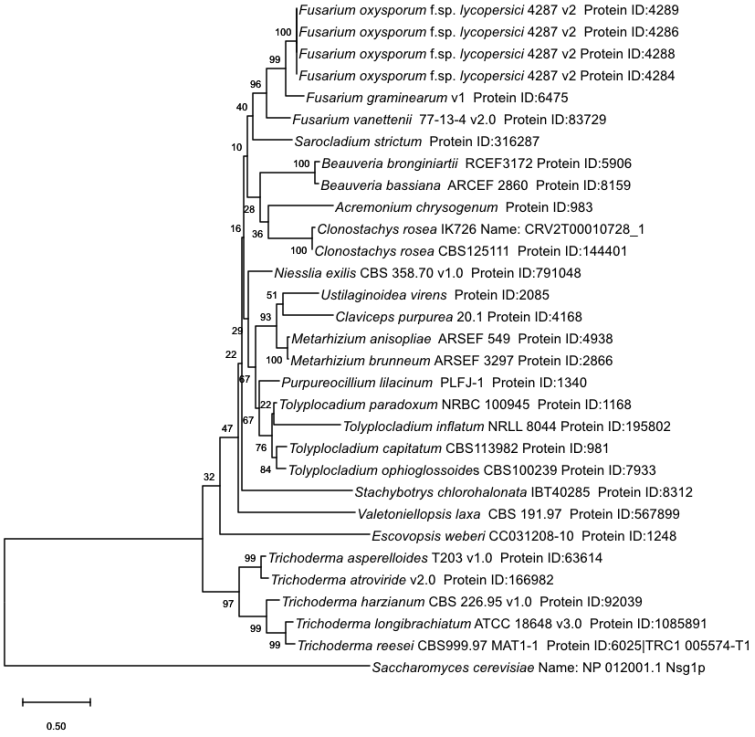

Figure 7: Putative INSIG homologs in Hypocreales generated with Mega-X ver.11, using S. cerevisiae as an outgroup. The low bootstrap support suggests low sequence homology between species.

The low bootstrap support indicates that there is low sequence homology between the species in Hypocreales and that the protein has evolved very different between species.

The amino acid sequences of putative SCAP protein was furthered used in JGI-blastp (Grigoriev et al. 2014) in the Hypocreales reference genomes. SCAP was found in all the reference genomes of Hypocreales, figure 8.

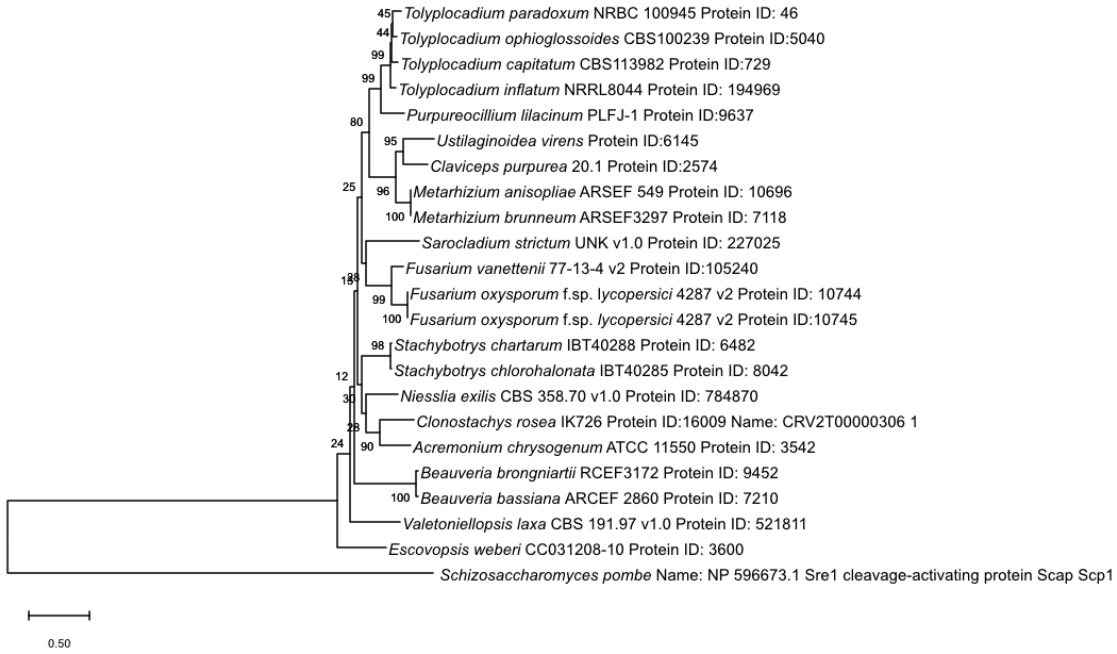

Figure 8: Putative SCAP homologs in Hypocreales generated with Mega-X ver.11, having S. pombe as an outgroup. Low bootstrap support indicates low sequence homology between species.

The phylogenetic tree (Figure 7) followed the species phylogeny to a degree, at least for *Tolypocadium, Purpureocillum, Metarhizium, Claviceps and Ustaliginoidea*. However, *Beauveria* should cluster closer with them if the tree followed the species phylogeny, but the bootstrap values were low (<70) overall and there's a high risk of misinterpretation. The following can be stated with *Valetoniellopsis laxa* and *Sarocladium strictum*.

Using the sequence of *S. pombe* Scap1 (NP_596673.1), there was no blastp hit on *A. fumigatus* (Grigoriev et al. 2014), similar to what had previously been studied by Willger et al. (2008). Implying that SCAP must have been lost in the order of Eurotiales, *A. fumigatus* and possibly other, but not strictly lost in all filamentous fungi. Therefore, a brief phylogenetic analysis using JGI blastp (Grigoriev et al. 2014) with the putative SCAP protein was made, to establish where it was lost in Pezizomycotina. Putative conserved SCAP homologs was found in reference genomes of the Hypocreales, Xylenomycetes, Sordariomycetes, Leotiomycetes, Dothideomycetes and Pezizomycetes, by using the sequence of the putative SCAP homolog in *C. rosea (*Appendix: Figure 1).

The majority of the species within Pezizomycotina had two hits of SCAP on the blastp as previously mentioned in section 3.1. By analysing the protein structure and conserved domains of SCAP2 in NCBI CDS (Marchler-Bauer & Bryant 2004; Marchler-Bauer et al. 2011, 2015, 2017), and SMART (Letunic & Bork 2018; Letunic et al. 2021) as well as studying the phylogeny (Appendix: figure 2) of the

protein with lower sequence identity to SCAP, it was apparent that it was a different protein.

The sequence from SCAP2 in *C. rosea* IK726 was used in JGI-blastp (Grigoriev et al. 2014) and resulted in multiple hits in Pezizomycotina but none in the otherwise used reference genome of *S. pombe*, figure 9.

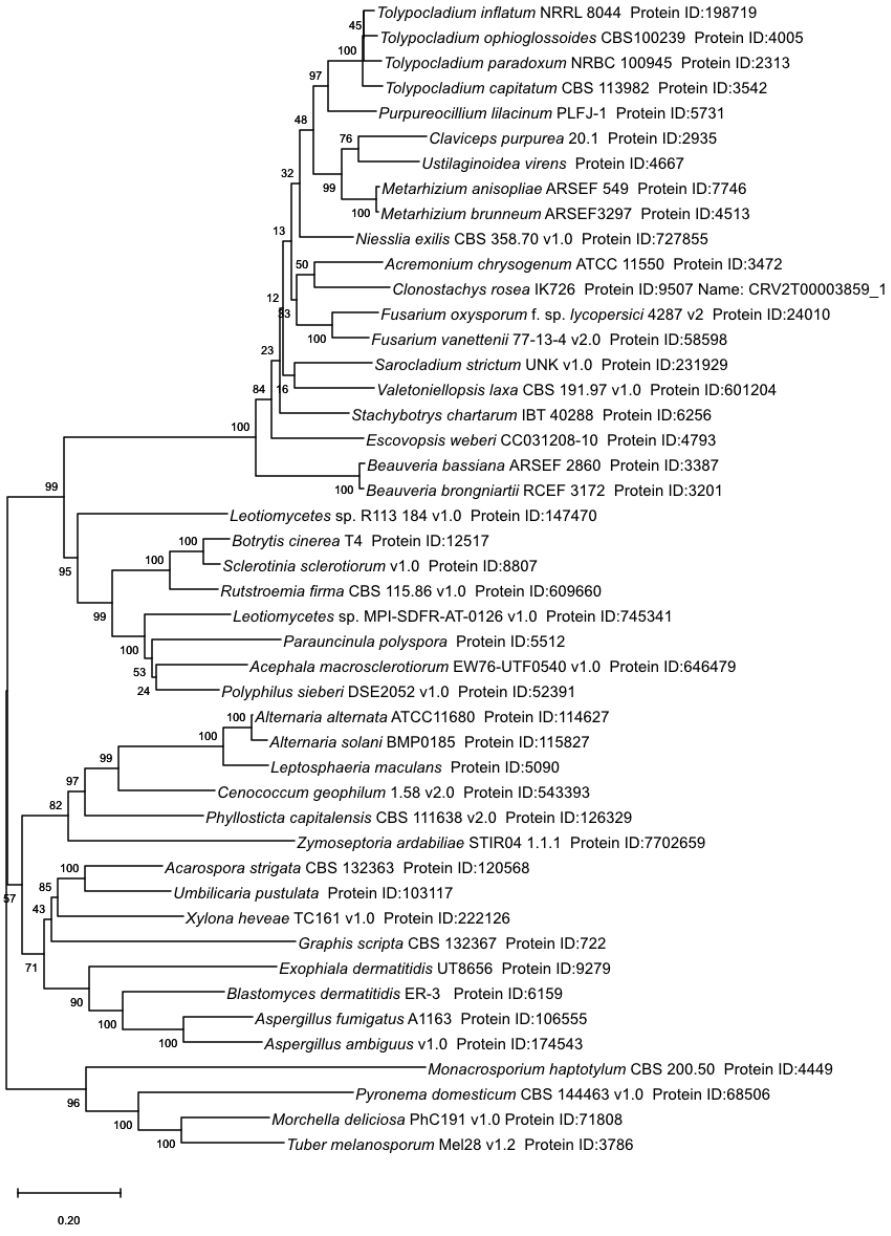

Figure 9: Putative SCAP2 homologs in Pezizomycotina generated with Mega-X ver.11. All species within Hypocreales have a common ancestor according to the 100 bootstrap support, but ancestors between species are not supported. The Leoitomycetes and Sordariomycetes have an ancestor with 99 bootstrap support, but a single ancestor between the branching of species is harder to determine.

Pezizomycotina has a common ancestor both between species and within the class. A single ancestor within Pezizomycotina is not fully supported since bootstrap value is of 57.

The phylogenetic tree (figure 9) shows that there could be a common ancestor to all the different classes within Pezizomycotina, but the support is not that high of 57. The SCAP2 protein seem to be more conserved within Pezizomycotina compared to SCAP since the bootstrap values are higher and sequences are more aligned.

3.3 Generation and validation of *sre2* gene deletion strains

Agrobacterium tumefaciens carrying correct *sre2* gene deletion cassette was validated by restriction analysis of plasmid DNA, which produced expected fragment size (Figure 10).

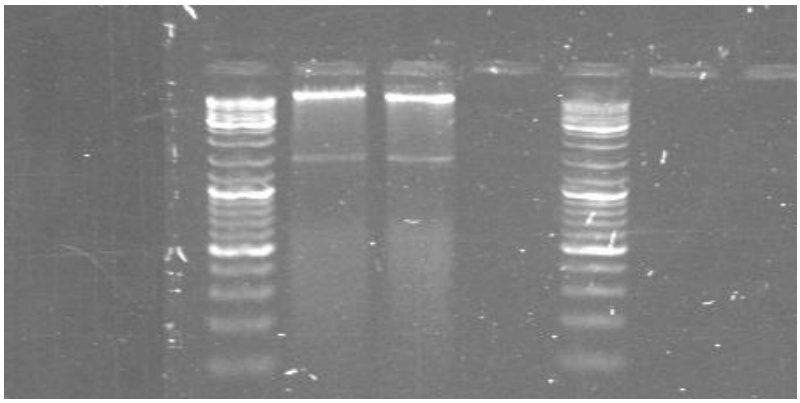

Figure 10:The two plasmids was extracted with the Gene JET plasmid miniprep kit® (Thermo Scientific) from two colonies of AGLI, using enzymatic digestion (Thermo Fisher Scientific, Waltham, MA) and produced fragment of expected size.

A. tumefaciens mediated transformation was used to generate Δ*sre2* strains in *C. rosea*. When *A. tumefaciens* comes in contact with conidia of *C. rosea* and the right conditions are met for infection, *A. tumefaciens* transfers the plasmid containing the gene deletion cassette to the conidia's cell nucleus. Because the sequences flanking our hygromycin resistance gene is homologous to the regions flanking *sre2*, homologous recombination occurs in *C. rosea* and *sre2* is replaced with a hygromycin resistance gene, figure 11.

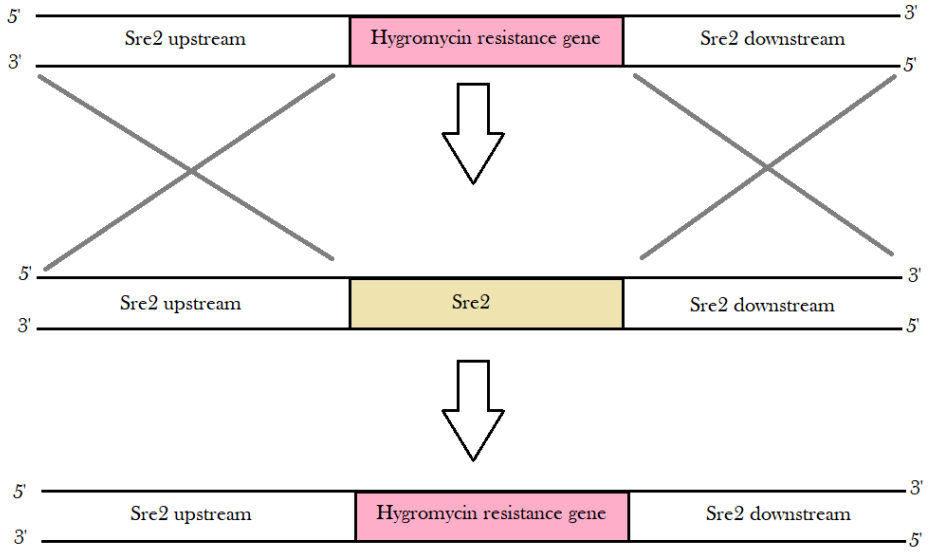

Figure 11: Schematic figure of homologous recombination event where the sre2 gene is replaced with hygromycin resistance gene.

It is possible that the homologous recombination goes wrong and that the sequence is inserted at a random place in the genome, called ectopic insertion. This means that the hygromycin resistance gene will be integrated into the genome, but with *sre2* still being intact and functioning.

PCR amplification of fragments using primers located within the hygB cassette, together with primers located upstream or downstream of the construct, as shown previously in Table 1, generated the expected 2.7 kb size of PCR fragments from gene deletion strains Δ*sre2_14*, Δ*sre2_55* and Δ*sre2_104*, figure 12.

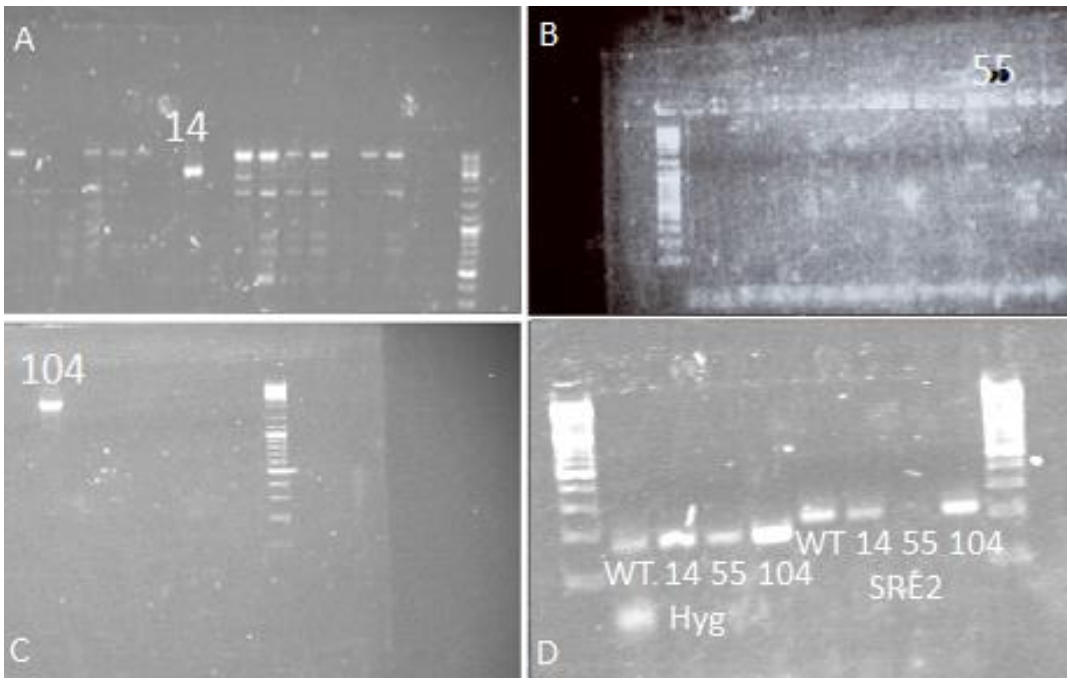

Figure 12: Picture showing the gel electrophoresis results from PCR using sre2-KO-R and Hyg-F primers as well as PCR using the cDNA from RTq-PCR and RNA extraction. (A), (B) and (C) showing the expected size of 2,7kB size of PCR fragments using sre2-KO-R and Hyg-F primers for Δ*sre2*_14, Δ*sre2*_55 and Δ*sre2*_104. (D) Gel results after first RNA extraction.

Reverse transcriptase (RT-) PCR analysis using primers specific to hygB and *sre2* revealed that all gene deletion strains but WT expressed Hygromycin resistance gene and Δ*sre2_14* and Δ*sre2 55* did not express SRE2 but WT and Δ*sre2_ 104* still expressed SRE2, probably containing an ectopic insertion of the cassette, figure 13.

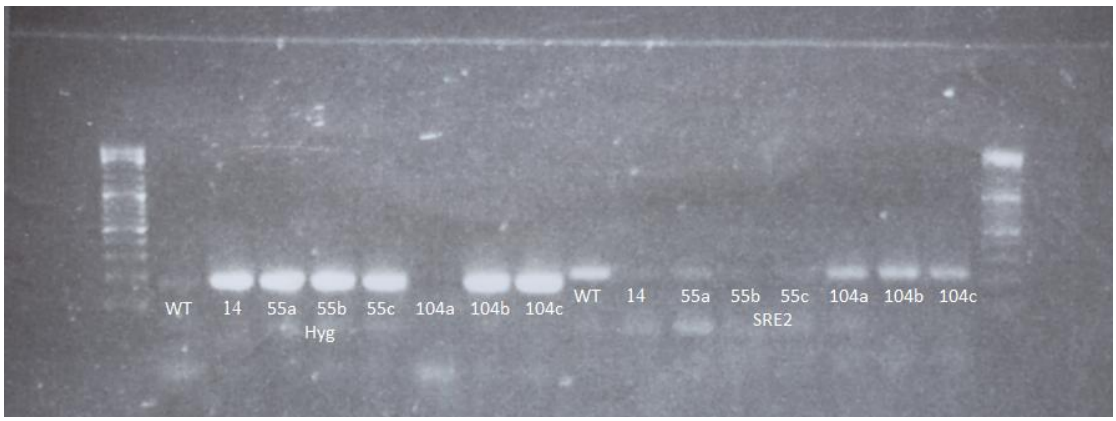

Figure 13: PCR products after RNA extraction for Hyg primers to left and SRE2 primers to the right. WT and 104 expressing SRE2 but no SRE2 expression in 14 and 55 a, b and c colonies. All strains expressed Hyg except for WT and 104 colony a.

Out of the 208 colonies growing on the hygromycin selection plates, two resulted in positive *Δsre2* transformants and was named Δ*sre2_14* and Δ*sre2_55*. The *sre1* gene deletion strains that were generated, validated and provided from the supervisor was named Δ*sre1_1*, Δ*sre1_10* and Δ*sre1_15*.

3.4 Phenotypic characterization of Δ*sre1* and Δ*sre2* gene deletion strains

3.4.1 SRE2 and SRE1 does not influence growth rate

There was a significant difference between the growth rate (mm/day) between Δ*sre1_104* and WT, but this cannot imply that SRE2 have an influence on growth since it only occurred in this specific Δ*sre2* strain. Hence, there was a no significant difference between the colonies mean diameter of WT, Δ*sre1* and the other Δ*sre2* strains on CZ agar media. However, a phenotypical difference in overall mycelia structure could be seen, figure 15.

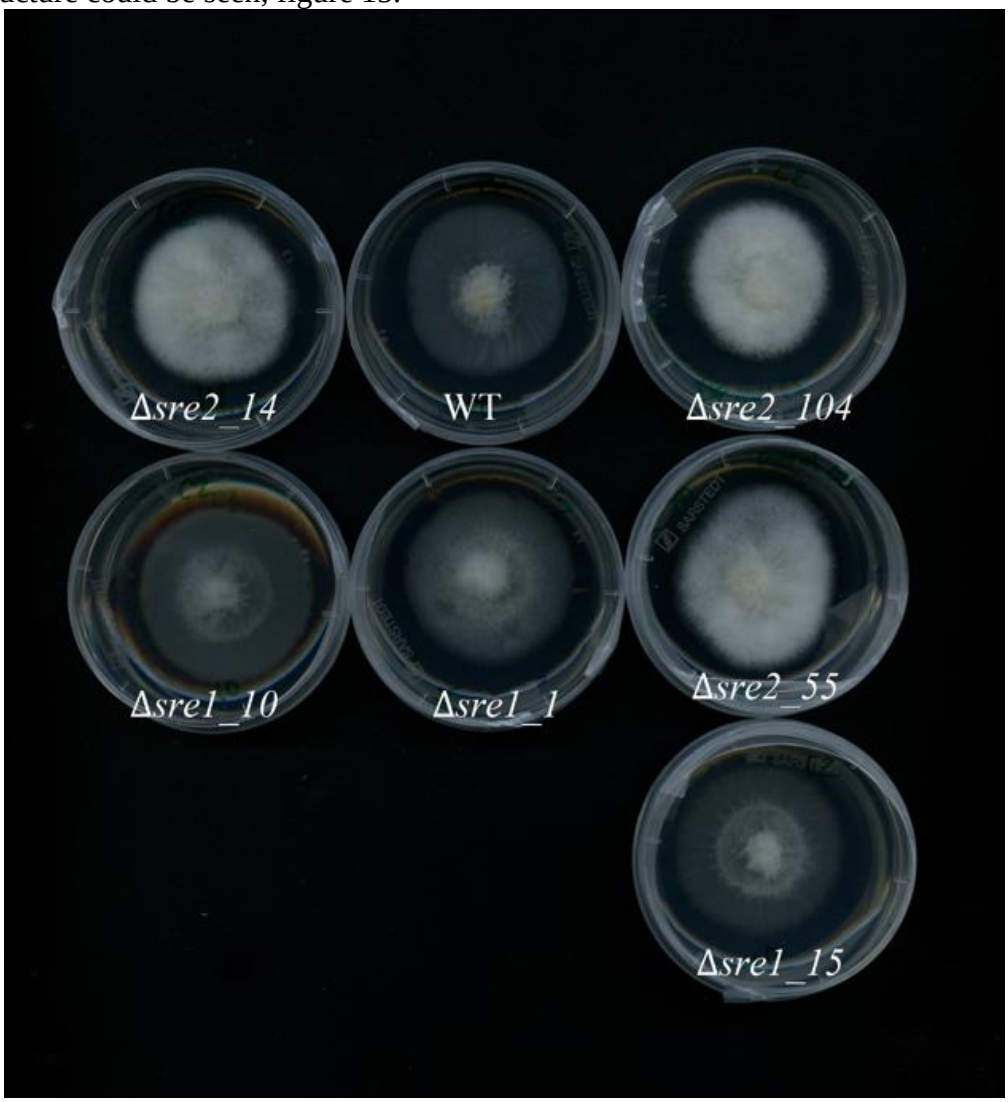

Figure 14: CZ plates with WT and each respective gene deletion strain, 6 days after inoculation.

3.4.2 SRE1 likely contribute to prothioconazole tolerance

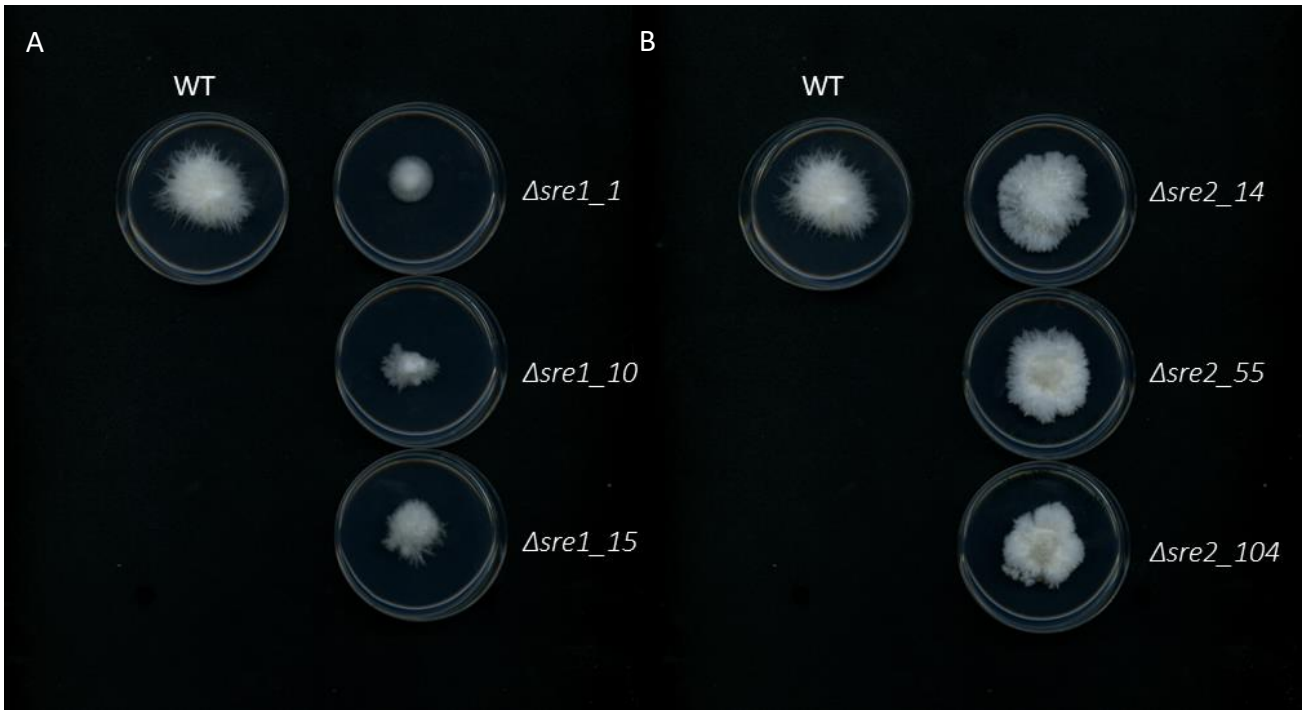

Figure 15: The growth after 14 days of WT and each respective sre1 and sre2 gene deletion strain on CZ plates treated with 1/60 dosage of Proline with the active ingredient prothioconazole.(A) WT to the left and from top to bottom: Δ*sre1_1,* Δ*sre1_10* and Δ*sre1_15 showing a very decreased growth*. (B) WT to the left and from top to bottom*:* Δ*sre2_14,* Δ*sre2_55* and Δ*sre2*_104 having a larger growth than *sre1* gene deletion strains.

There was a significant difference in growth rate averages between the Δ*sre1* strains and WT, on CZ media supplemented with Proline fungicide (figure 16), which also can be visually seen in figure 16. No significant difference in mycelial growth was found between the WT and *sre2* deletion strains 6 dpi, but a significant difference was seen 14 dpi for Δ*sre2_14* and Δ*sre2_55* (figure 16). As Δ*sre2* strains had a similar average growth rate to WT on 4 dpi, there was also a significant difference in growth between the Δ*sre1* and Δ*sre2* strains.

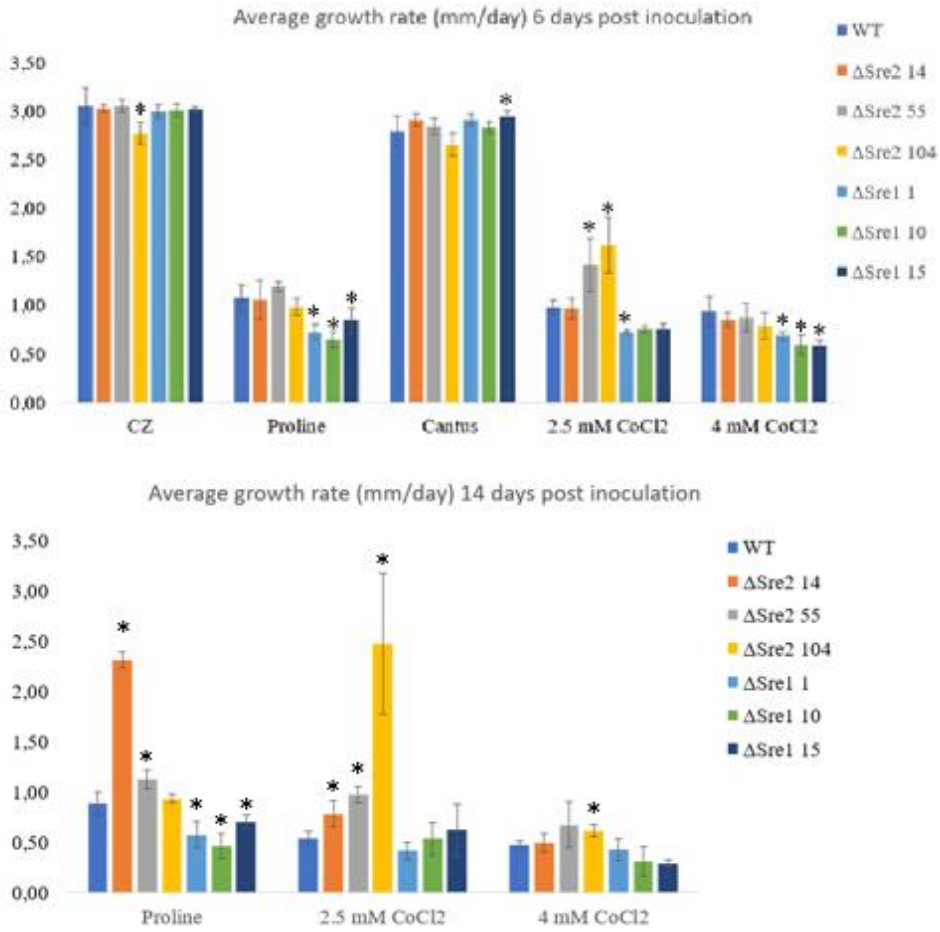

Figure 16: Average growth rate in mm/day for each treatment and each respective strain of C. rosea. A) 6 dpi, significant differences compared to WT are indicated with an asterisk (*). B) 14 dpi, significant values compared to WT is shown in asterisk.

3.4.3 Gene deletion of *sre2* increases hypoxia tolerance

None of the gene deletion strains or WT responded well to the hypoxia inducing $CoCl_2$ treatments. All of the colonies' mycelia grew upwards because of the stress inducing media, and some colonies grew upwards with a force that made the media brake and rise from the plate. However, the Δ*sre2* strains tolerated the hypoxia more than the Δ*sre1* strains, figure 17.

Figure 17: WT, sre1 and sre2 gene deletion strains on CZ plates treated with 2,5 and 4mM CoCl2. (A) WT, Δ*sre1_1*, Δ*sre1_10 and* Δ*sre1_15*, Δ*sre2_14*, Δ*sre2_55 and* Δ*sre2_101* on CZ plates supplemented with 2,5 mM $CoCl_2$. Δ*sre2* strains have the largest growth compared to the other strains. (B) WT, Δ*sre1_1*, Δ*sre1_10 and* Δ*sre1_15*, Δ*sre2_14*, Δ*sre2_55* and Δ*sre2_104* on CZ plates supplemented with 4 mM $CoCl_2$. All colonies showing reduced growth and abnormal mycelia growth but Δ*sre2* strains showing a better tolerance to both $CoCl_2$ compared to Δ*sre1* strains.

The average growth in mm/day was calculated 6 days after inoculation as well as 14 days after inoculation, revealing that the growth rate increased for all colonies after one week of inoculation. There was a significant difference in growth rate averages between the WT and Δ*sre1* strains on 4 mM $CoCl_2$ 4 dpi and a significant difference between WT and Δ*sre2* strains on 2,5 mM $CoCl_2$ 14 dpi. The strains Δ*sre1_10* and Δ*sre1_15* had a *p*-value of 0,05655 and 0,05655 4dpi on 2.5 mM $CoCl_2$,respectively, and could thus not be regarded as significantly different to WT (figure 16).

3.4.4 SRE1 and SRE2 is likely not related to *C. rosea*'s tolerance of Boscalid

The CZ plates supplemented with Boscalid showed that both *C. rosea* WT and gene deletions are highly tolerant of the SDHI fungicide Cantus, containing the active ingredient Boscalid. There was only a significant difference between the growth rate averages of Δ*sre1_15*, but the Δ*sre1* strains had decreased mycelial mass and grew sparser compared to WT and Δ*sre2* strains, figure 18.

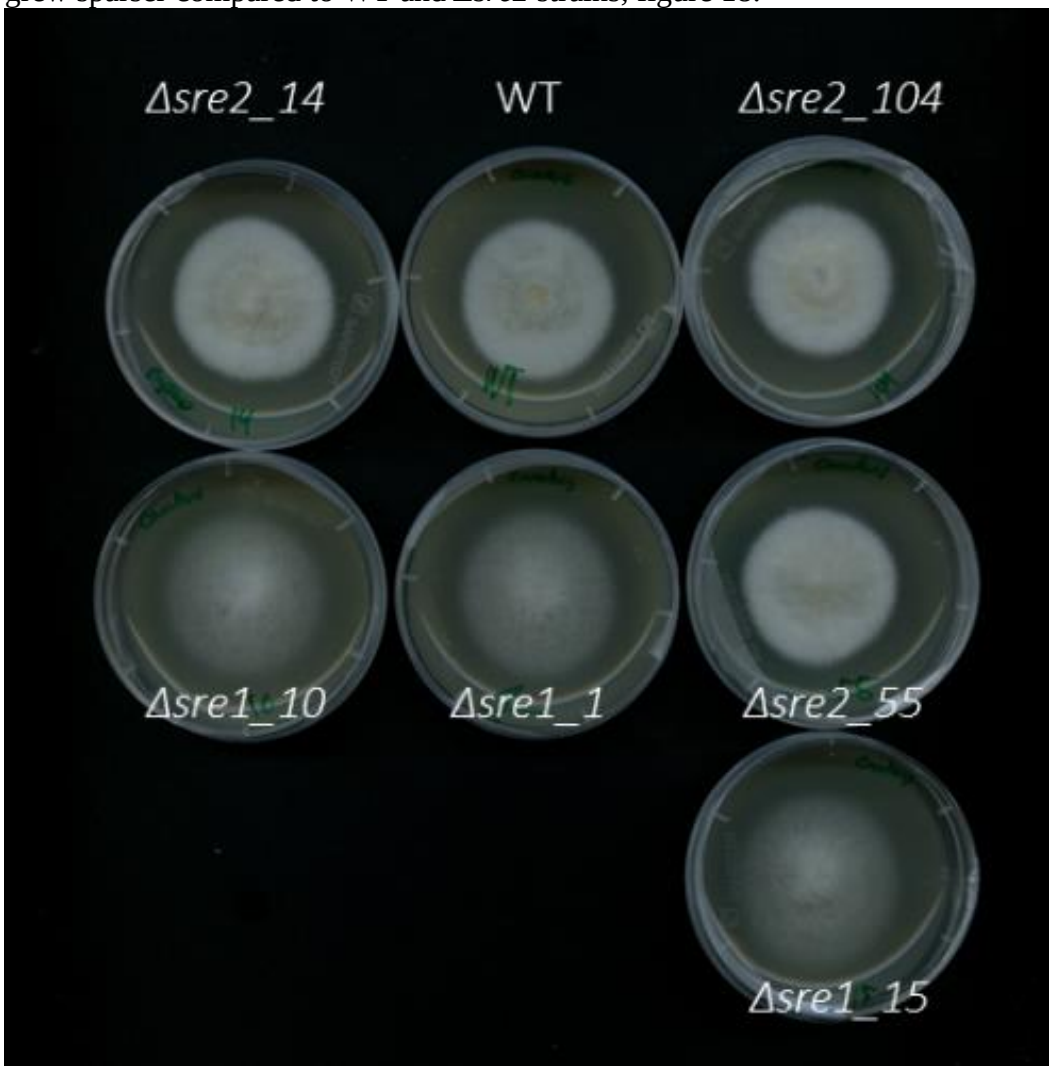

Figure 18: CZ plates treated with the fungicide Cantus® containing the active ingredient Boscalid. Plates 6 days after inoculation, *strain* Δ*sre1_1,* Δ*sre1_10* and Δ*sre1_15* having a sparser and less pigmented mycelia compared to Δ*sre2* strains and WT.

All of the colonies had reached the end of the plate after 14 days, all having similar growth rate on the media. The notable visual difference between the Δ*sre1* and Δ*sre2* strains is that Δ*sre2* strains look more similar to WT, having a more pronounced pigmented mycelia at the inoculation point.

It is apparent that the Boscalid treatment resulted in similar phenotype and growth rate as CZ control, hence the resistance to Boscalid in *C. rosea* IK726 is most likely not related to *sre1* and *sre2*.

3.4.5 Deleting *sre1* and *sre2* does not influence the antagonism of *C. rosea* when interacting with *B. cinerea* and *F. graminearum*

A dual culture confrontation assay was made to measure the antagonism of WT and gene deletion strains. There was no significant difference when comparing the average overgrowth of *C. rosea* WT and gene deletion strains in dual culture with *F. graminearum*. However, the overgrowth by WT on *B. cinerea* was significantly different to Δ*sre2_14*, Δ*sre1_1* and Δ*sre1_15* which all had an increased overgrowth. Δ*Sre1_10* had a p-value of 0,050275 and Δ*sre2_55* a p-value of 0,052481. There was also an increased mycelial growth at the contact point of Δ*sre1* strains during the dual culture with *B. cinerea*, figure 19B.

The two pathogens interacted differently with the gene deletion strains and WT before the contact point (cp). For instance, *F. graminearum* had a slower growth curve in the beginning of the inoculation with the gene deletion strains compared to WT, which then increased when coming closer to the cp. However, there was only a significant difference between WT and Δ*sre1_15*, Δ*sre2_14* and Δ*sre2_104*, figure 19C.

Similarly to the phenotype seen on CZ agar plates, Δ*sre2* strains has a thicker mycelia similar to WT and the mycelia of Δ*sre1* strains is sparser than both WT and Δ*sre2*, figure 19A.

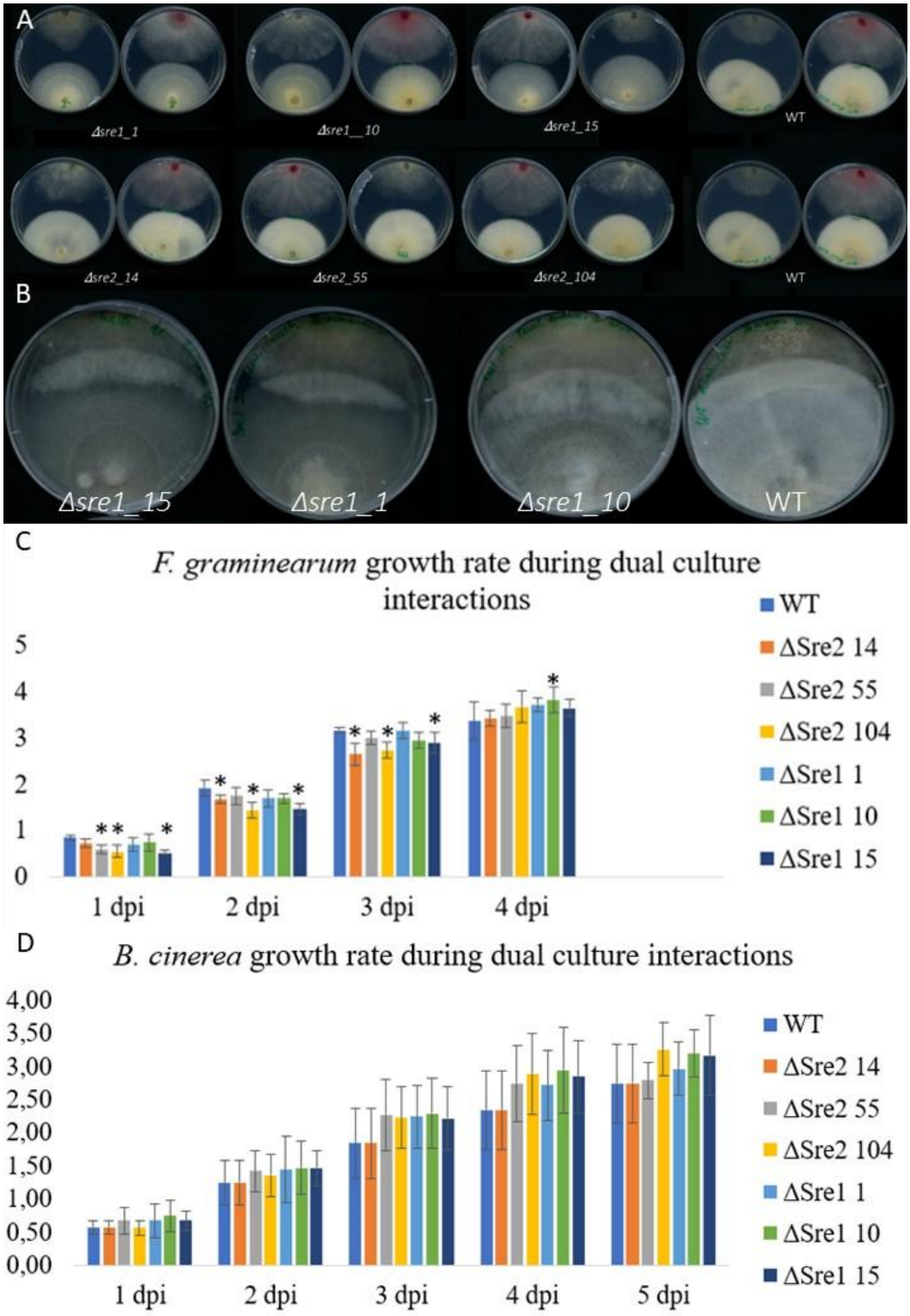

Figure 19: The growth of F. graminearum and B. cinerea when dual cultured with the sre1 and sre2 gene deletion strains and WT. (A) Dual cultivation plates of *F. graminearum* (pink) and *B. cinerea* (grey) with WT, Δ*sre1* and Δ*sre2* strains, picture taken 5 days post inoculation (dpi). (B) Δ*Sre1* strains interacting with *B. cinerea*, having an increased mycelial growth at the contact point. (C) Growth rate in mm/day of *F. graminearum* until contact point with WT, Δ*sre1* and Δ*sre2*. Significant difference to WT shown in asterisk. (D) Growth rate in mm/day of *B. cinerea* until

contact point with WT, Δ*sre1* and Δ*sre2*. There was no significant difference between any of the strains.

Botrytis cinerea seemed to grow faster during the interaction with the gene deletion strains compared to WT which is suggesting that there are different mechanisms in *C. rosea*'s antagonism when interacting with *B. cinerea* or *F. graminearum*. However, no significance difference in growth rate of *B. cinerea* during the interaction with the strains.

B. cinerea has a slower growth rate than *F. graminearum* and the cp occurred one to two days after *C. rosea*'s cp with *F. graminearum.* However, the growth of *B. cinerea* is more linear compared to *F. graminearum* and does not decrease when nearing the cp with *C. rosea* WT. The growth of *F. graminearum* does however decrease when coming closer to WT, suggesting that the antibiosis of *C. rosea* works more efficiently against *F. graminearum* than *B. cinerea* before the cp. SRE1 and SRE2 does also seem to have different roles in the antibiosis of the two pathogens. The loss of SRE1 or SRE2 seem to decrease the antibiosis by *C. rosea* on *B. cinerea* since it grows faster in the dual culture with the gene deletion strains compared to WT. Whereas the loss of SRE2 and SRE1 lead to a decreased growth in *F. graminearum* compared to WT, and a growth that is increases nearing the cp with the gene deletion strains. Suggesting that SRE1 and SRE2 have an influence on the antagonistic ability of *C. rosea* on *F. graminearum*, and that the loss of these proteins lead to a decreased antibiosis close to cp.

C. rosea is overgrowing their fungal pray when in contact, and this feature was not lost in any of the gene deletion strains. However, the growth in mm/day differed between WT and gene deletions, particularly against *B. cinerea*, but the difference is not significant in all gene deletion strains because of the high standard deviation between the WT replicates.

3.5 Deletion of *sre1* and *sre2* does not influence *C. rosea* biocontrol abilities

The gene deletion strains were used in a bioassay with wheat and *F. graminearum* inoculum to determine if SRE1 and SRE2 contribute to *C. rosea*'s biocontrol ability against fusarium foot rot.

The gene deletion strains and WT decreased disease significantly compared to the control that was not inoculated with any *C. rosea*. Applying *C. rosea* increased the germination of seeds, the length of the shoot as well as the fresh- and dry weight of the plants significantly, compared to non-treated control, figure 20. The wheat plants without any inoculum were the healthiest overall, but there was not a significant difference between the germination of seeds coated with *C. rosea* strains compared to the healthy wheat control.

The healthy wheat plants had almost double the fresh weight compared to plants inoculated with *F. graminearum* and *C. rosea*. However, the plants inoculated with *C. rosea* had in average increase in weight by ten-fold compared to unhealthy control. When comparing the dry weight of the strains, the weight was about 6-fold more for strains inoculated with *C. rosea* compared to the control, figure 19F. The dry weight of healthy control was 1,2-1,5 times higher compared to plants inoculated with *C. rosea,* figure 20G.

There was no significant difference in biocontrol ability of WT, Δ*sre1* and Δ*sre2* strains when comparing the disease scores. But there was a significant difference between healthy control, unhealthy control and plants inoculated with *C. rosea*.

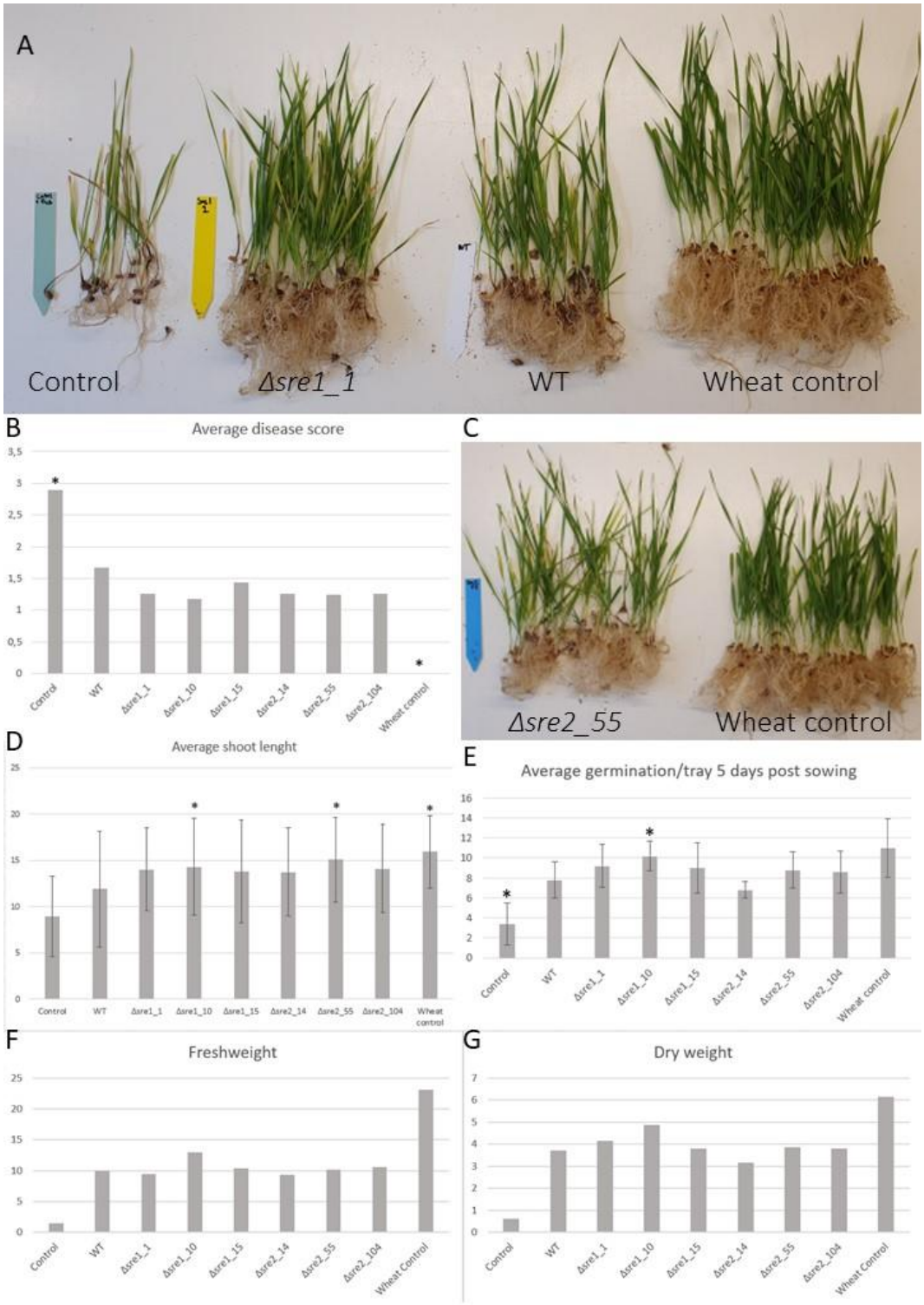

Figure 20: Figures showing the results from the bioassay of wheat plants treated with F. graminearum+respective gene deletion strains. Significant difference to WT shown in asteriks. A) from the left: Control, Δ*sre1_1*, WT and Wheat control. B) Average disease scores for respective strain and controls. Wheat control and control being significant different to WT. C) From the left: Δ*sre2_55* and wheat control. D) average shoot length, Δ*sre1_10*, Δ*sre2*_55 and wheat control having a significant difference to WT. E) Average germination per tray 5 days post sowing. Control

and Δ*sre1_10* being significant different to WT F) fresh weight and G) dry weight for each treatment and applied strain.

One row of WT, Δ*sre1* and Δ*sre2* was used in the wheat control tray without any *F. graminearum* to determine if *C. rosea* had any effect on the wheat plants without any pathogen inoculum, and there was no effect on the germination or growth of these controls, the plants looked healthy and grew similar to the wheat control.

Discussion

4.1 Identification and sequence analysis of SRE1, SRE2, INSIG and SCAP

The identification of putative SCAP and INSIG proteins make it possible to study their interactions with SRE1 and SRE2 in *C. rosea* to develop a greater understanding of their specific function in the sterol regulatory pathway. It is possible that the mammal SREBP pathway is similar to the sterol pathway in Ascomycetes because putative INSIG, SCAP and SREBPs now have been found in species within the division. If their specific function is conserved and follows the same pathway as in mammals needs to be furthered confirmed. Since the studies in *C. neoformas, A. fumigatus* and *S. pombe* have revealed different pathways and ways the SREBPs function with their interacting associating proteins (Bien & Epenshade 2010), it is possible that the SREBP pathway have evolved and function differently in filamentous fungi in the order of Ascomycota as well. The sequence identity in the JGI-blastp of the putative homologs were over 35% to confirmed homologs *in S. pombe* and *A. fumigatus* (Grigoriev et al. 2014), which make it very likely that the proteins are structurally similar (Krissinel 2007). Even though the sequence similarity of *C. rosea* putative SREBP homologs were closer to *A. fumigatus* than to *S. pombe*, SCAP is lacking from *A. fumigatus* which means that the pathway of SREBPs and associated proteins must have been altered. Indicating that the SREBP pathway might have been conserved from yeast to the studied species of Pezizomycotina.

SRE2 is not as common in Pezizomycotina as SRE1, suggesting that SRE2 either is not conserved within the subdivision because it did not have an essential function, or that a potential gene duplication event of SRE1 occurred in an ancestor of Sordariomycetes and Leotiomycetes which was conserved within the orders. The predicted protein structures of SRE1 and SRE2 are similar at the C-terminus region (Marchler-Bauer & Bryant 2004; Marchler-Bauer et al. 2011, 2015, 2017), and could mean that the C-terminus of the SRE1 protein was lost during the duplication event and only N-terminus and the bHLH domain remained. Even if SRE2 is conserved in Hypocreales, bootstrap values are quite low and the sequences are

quite different particularly in N-terminus regions of the protein with mainly the bHLH_zip domain being conserved, probably having the most important function in the protein. But compared to the bHLH region in SRE1, SRE2 show less homology in sequence between the species in Hypocreales after alignment in Mega-X ver.11 (Tamura et al. 2021). A more substantial phylogenetic analysis with SRE1 and SRE2 are necessary to answer where potential ancestors are located and if SRE1 and SRE2 follows the same phylogeny within Pezizomycotina.

Interestingly, *Fusarium* had an abundance of SRE2 paralogs. Which can be related *Fusarium*'s ability to perform horizontal chromosome transfer and horizontal gene transfer (Mehrabi et al. 2011), and the multiple versions of SRE2 in their genome is probably a result from such events. Which is something that can be interpreted through looking at the phylogenetic tree, since the paralogs of SRE2 have different ancestors. Additionally, one of the SRE2 paralogs had the same ancestor as *N. crassa* which is located in another order within Sordariomycetes. To know the specific ancestor in Hypocreales, a more in-depth study of Sordiariomycetes is needed. There is also a potential that some of the proteins with close ID's in *Fusarium* are in fact the same proteins but have been interpreted as separate by the program.

Putative INSIG homologs was also found within Hypocreales, but no phylogeny was made with it in Pezizomycotina because the phylogenetic tree in Hypocreales had low bootstrap values. The INSIG phylogeny tree had low bootstrap support in most of the branches, which make it hard to know specific ancestors, and it's likely that the support would be even lower if the putative INSIG homologs would be compared with other species within Pezizomycotina.

SCAP was conserved within almost the whole Pezizomycotina apart from Lecanoromycetes, Euritiomycetes and Orbiliomycetes. It is difficult to interpret why it was not conserved within these orders since it does not follow the phylogeny of classes and classes related have the putative SCAP protein.

Interestingly, SCAP2 was not initially found in *C. rosea* using the Scp1 sequence from *S. pombe* in the JGI-blastp (Grigoriev et al. 2014). The sequence of SCAP2 was found using the *C. rosea* SCAP sequence while doing JGI-blastp analysis on Pezizomycotina (Grigoriev et al. 2014). JGI-blastp (Grogoriev et al. 2014) using the sequence of *S. pombe* SCP1 did not reveal any hit of SCAP2 in Pezizomycotina, which might be explained by the SCP1 not having any predicted 2A060601 super family domain by NCBI CDS (Marchler-Bauer & Bryant 2004; Marchler-Bauer et al. 2011, 2015, 2017), making SCAP2 almost missed from the phylogenetic analysis. SCAP2 must have had an ancestor in Pezizomycotina, hence why it was not found in *S. pombe*. What SCAP2 does is unknown, and if it has a function similar to SCAP. However, some of the SCAP2 proteins that was found in Pezizomycotina was considered putative sphingolipid transport proteins (Grigoriev et al. 2014). The similarities are that they have predicted domains that are integral

to the membrane that have a sterol sensing domain and provide an important function in lipid transport (Marchler-Bauer & Bryant 2004; Marchler-Bauer et al. 2011, 2015, 2017). The research of SREBPs in *S. pombe* revealed that SRE1 induced genes for sphingolipid biosynthesis (Todd et al. 2006). Could the sphingolipid pathway have developed differently in filamentous fungi, giving rise to an additional protein in the sphingolipid pathway?

However, the similarity of SCAP2 and SCAP could be because of their role in the lipid pathway, but having whole different functions and interactive pathways, and it is possible that SCAP2 do not interact with SREBPs. Considering that SCAP2 was found within the whole Pezizomycotina through the phylogenetic analysis and have clear ancestors to many of the orders, its function is probably necessary, and its specific function could reveal more about the sterol or sphingolipid pathway. Further research of SCAP and SCAP2 in Pezizomycotina is needed to answer their specific function.

4.2 Generation of *sre1* and *sre2* gene deletion strains

The efficiency of the ATMT was particularly low considering only two out of 208 colonies had the gene deletion cassette inserted through HR. This could be the case if the gene is located near the centromeric region or have a difficult chromatin structure (Hooykaas et al. 2018). The efficiency of HR is mainly influenced by the hosts dominant pathway to repair double stranded DNA-breaks, while this mechanism is active in *C. rosea* this is likely not be the source of the error since more successful gene deletion have occurred in *C. rosea*, for instance the *sre1* gene deletion. The G/C content and length of the flanking regions of the gene can also influence the efficiency of HR. The flanking regions bp was over 1000 for the cassette, which is the recommended amount, and should not be the source of inefficient HR. The transcriptional status of the targeted gene can also influence the efficiency (Hooykaas et al. 2018). However, the RNA extraction proved that SRE2 was expressed continuously in WT, and this is likely not the cause for the inefficient HR. The most probable reason that low HR occurred must be that *sre2* is located near the centromeric region or that the chromatin structure around *sre2* make it difficult for HR to take place.

Other methods might prove more efficient to successfully delete *sre2* from the genome. Successful gene deletions can be performed with the CRISPR-cas9 (clustered regularly interspace short palindromic repeats-associated protein-9 nuclease) technology. The first successful CRISPR-cas 9 system to occur in filamentous fungi was made with *Trichoderma reesei* (Liu et al. 2015). A codon-optimized cas9 gene was inserted into the genome first through ATMT with generated a cas9-strain that further could be used successfully in gene engineering with CRISPR (Liu et al. 2015). However, the random integration of cas9 could lead

to unwanted effects and in the case of multinuclear fungi, there is a larger challenge to produce a cas9 strain, but it has proven possible with *Sclerotinia sclerotium* (Li et al. 2018). Since no 'cas9 strain' is made in *C. rosea* IK726 it is not possible to use the CRISPR method and producing a cas9 strain could prove tedious and considering the scope of this study, would not be possible.

4.3 Phenotypic characterization of Δ*sre1* and Δ*sre2* gene deletion strains

The hyphal structure and appearance were affected in the gene deletion strains under induced stress treatments. The *sre1* gene deletion strains grew sparser with less pigmentation compared to the *sre2* gene deletion strains that had an abnormal mycelial growth on the treated plates with prothioconazole and $CoCl_2$. The difference in phenotype between *sre1* and *sre2* deletion strains suggest that SRE1 and SRE2 have different functions in *C. rosea*. Because the Δ*sre1* strains performed worse than Δ*sre2* strains under stress, it is likely that SRE1 have a more essential function in regulation of hypoxia and fungicide tolerance compared to SRE2. Since SRE1 is still active in *sre2* mutants, it is possible that the abnormal and increased growth was caused by SRE1 (over)expression, but the sterol pathway not functioning fully because of the *sre2* gene deletion which gave rise to the specific Δ*sre2* phenotype. In the future, research about Δ*sre1* and Δ*sre2* effect on hyphae development and its role in pigmentation would be interesting.

The bioassay revealed that deletion of *sre1* and *sre2* did not influence *C. rosea*'s biocontrol properties. Even though there were instances where a Δ*sre1* or Δ*sre2* strain performed different to WT, but the difference was not consistent within the other *sre1* or *sre2* gene deletion strain. Hence it is different to conclude if deletion of *sre1* or *sre2* had a different phenotype. Having more replicates could strengthen the experiments validity. Other than that, both gene deletion strains performed well against their fungal prey and decreased disease significantly. Thus, SRE1 and SRE2 does not seem to be essential for the biocontrol abilities of *C. rosea*.

In the dual cultivation assay, *F. graminearum* had a slower growth rate in the beginning of the interaction with the gene deletion strains that increased near the contact point. When interacting with WT, *F. graminearum* grew faster in the beginning of the contact but slowed when nearing the contact point with WT. It might be possible that gene deletion strains had an increased usage of volatile compounds that decreased the growth rate of *F. graminearum* in the early stages, but that SREBPs are necessary in the antibiosis of close interactions which lead to the increased growth of *F. graminearum* near the cp.

Since SREBPs were necessary for pathogenesis in *C. neoformas* and *A. fumigatus*, one of the hypothesis was that the antagonism of *C. rosea* would be negatively affected in the gene deletion strains. The antagonism of *C. rosea* likely follows different mechanism compared to the way these pathogens functions.

Ultimately, a strain with gene deletion of both *sre1* and *sre2* would provide more information about the function of SRE1 and SRE2. However, in consideration to the low efficiency of HR of the *sre2* gene deletion cassette, an additional transformation would not have been possible considering the time restriction of this study.

4.4 Conclusion

C. rosea contain SRE, putative INSIG and SCAP homologs which are associated with the SREBP pathway. *Sre2* gene deletion led to increased growth in hypoxia and on prothioconazole. *Sre1* gene deletion led to a less pigmented mycelia and a decreased tolerance to hypoxia and prothioconazole. The difference in phenotype between *sre1* and *sre2* deletion strains suggest that SRE1 and SRE2 have different functions in *C. rosea*. The gene deletions of *sre1* and *sre2* did not affect *C. rosea*'s biocontrol ability negatively and SRE1 and SRE2 is thus not deemed essential for *C. rosea* IK726 biocontrol abilities against *F. graminearum*.

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Popular science summary

Just like us humans, plants are subjected to numerous diseases caused by different organisms that are referred to as pathogens. There are a big group of fungi that are plant pathogens which causes huge losses of food every year which both translates to economic instability and a threat to our food safety. To limit the impact of fungal pathogens we have developed several strategies. One of them involves the usage of chemical substances called fungicides, which can reduce and kill the fungi that infect our crops. However, many fungal species have developed resistance or tolerance to certain fungicides because of their extensive use, which reduces their efficiency. There is a thus an incentive to use less fungicides and use alternative strategies. Another strategy is to apply a naturally organism to the crop to control the pathogens, just like the saying; the enemy of my enemy is my friend. This method of controlling plant pathogens is called biocontrol. The fungus *Clonostachys rosea* is a natural enemy of several fungal pathogens and can control plant diseases by reducing the growth of fungal pathogens. And even *C. rosea* have developed ability to tolerate relatively higher level of certain fungicides. Azole-fungicides are one of the most commonly used fungicides, and studies in a few fungal species have shown that Sterol Regulatory Element Binding Proteins (SREBPs) are necessary for tolerance to azole-fungicides, pathogenesis and tolerance to environments with low oxygen (hypoxia). Humans and other mammals also have SREBPs and other proteins that interact with SREBPs, for example the proteins SCAP (Sterol Cleavage Activating Protein) and INSIG (INSulin Induced Gene protein). When similar proteins with similar functions and origin occur in different organisms, the proteins are referred to as homologs. One of the aim of this study was to identify SREBP, SCAP and INSIG homologs in the specific order of fungi that *C. rosea* is located in and investigate their evolution by producing a phylogenetic tree, similarly to a family tree. By comparing the sequences and structures of known SCAP and INSIG, putative homologs were found and a phylogenetic tree of them was constructed. To understand if there is a similar mechanism of SREBPs in *C. rosea* as in other fungi and mammals, gene deletions of two SREBPs genes named *sre1* and *sre2* was made in *C. rosea* to study their role in tolerance to fungicide and hypoxia, and biocontrol of fungal plant pathogens on wheat caused by *Fusarium graminearum*. Deleting *sre1* reduced *C. rosea*'s ability to tolerate prothioconazole fungicide and low oxygen. The mycelial structure was also affected in both *sre1* and *sre2* deletions. However, the biocontrol ability of *C. rosea* was not reduced by deleting *sre1* or *sre2*.

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Appendix 1

Phylogenetic tree of SCAP in selected species within Pezizomycotina (Figure 1).

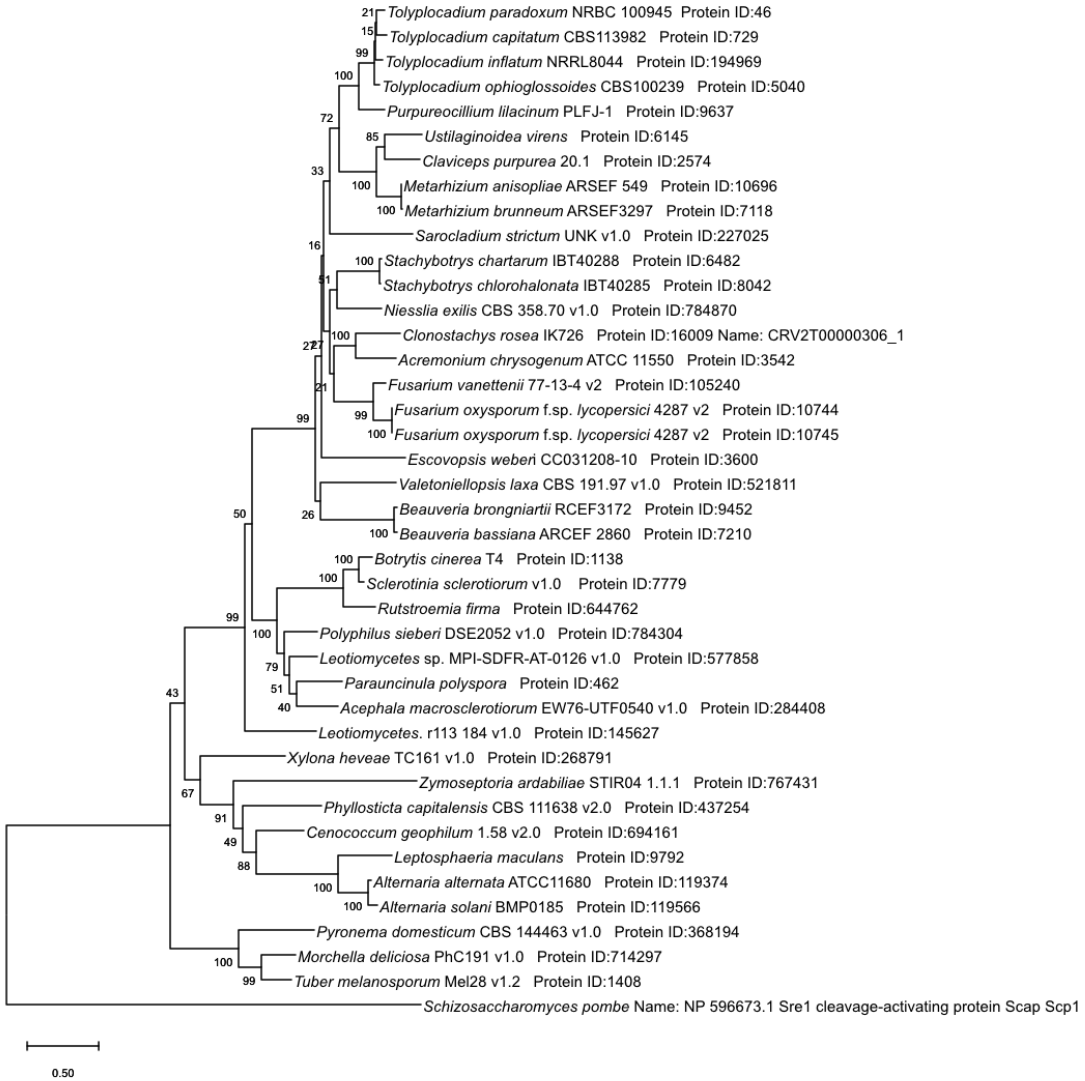

Figure 1: Phylogenetic tree of the putative SCAP protein and potential homologs within Pezizomycotina. S. pombe as an outgroup.

The phylogenetic tree (figure 1) resembled the species phylogeny, but a common ancestor to Pezizomycotina are difficult to establish because of low support of

bootstrap values. However, there seem to be a very certain common ancestor to the Sordariomycetes and Leotiomycetes.

The Pezizomycete *Tuber melanosporum* was one of the species that contained an additional protein that had the very small similarity to SCAP, apart from the protein with the higher resemblance. Both of the protein sequences were analysed for *Tuber melanosporum* Mel28 v1.2 in the phylogenetic tree to see if one of the two proteins was more similar to the small resembling sequences found in Euritiomycetes and Orbiliomycetes. The SCAP-like protein of *Tuber melanosporum* (Protein ID: 3786) clustered with the SCAP-like sequences in Euritiomycetes and Orbiliomycetes revealing that they had a common ancestor (figure 2).

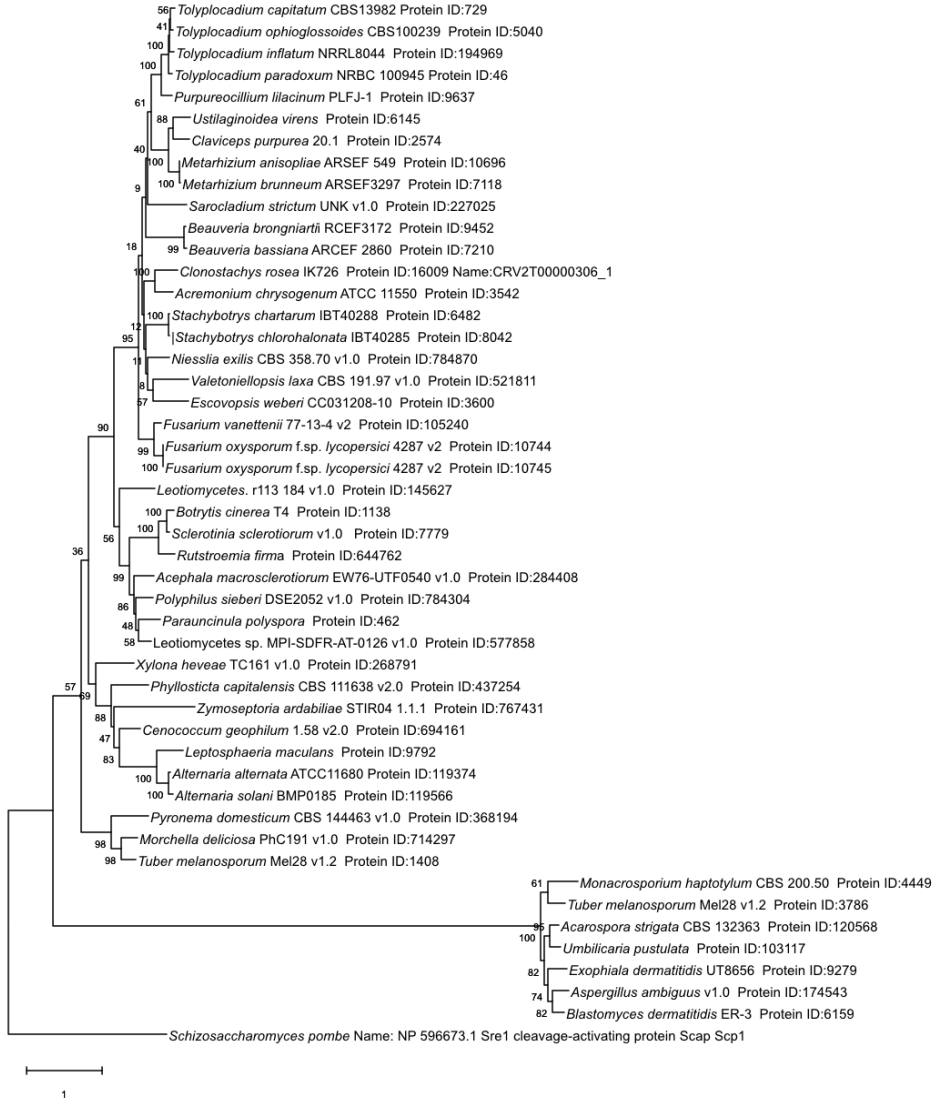

Figure 2. Phylogenetic tree of the putative SCAP protein with potential homologs found in several species of Pezizomycotina. The protein sequences that showed low sequence identity to SCAP clustered together at the bottom of the tree, having a common ancestor.

After making the phylogenetic tree (figure 2). The SCAP-like protein was named SCAP2 and further studied as a separate protein.

No SCAP-like protein occurred was found in the Lecanoromycetes but in *Graphis scripta* CBS 132367. Similarly, no SCAP-like protein was found in *Aspergillus aculeatus* ATCC16872 v1.1, *Aspergillus calidoustus* CBS 113228 v1.0, *Aspergillus niger* ATCC 13496 v2.0, *Penicillium brasilianum* MG11 or *Trichophyton equinum* CBS 127.97 (Grigoriev et al. 2014). No SCAP or SCAP-like protein was found in the JGI-blastp search (Grigoriev et al. 2014) in the Orbiliomycete *Drechslerella stenobrocha* 248 and *Arthrobotrys oligospora* ATCC 24927 genome, but a small resemblance of SCAP was found in *Monacrosporium haptotylum* CBS 200.50.

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