Rhizoctonia solani and sugar beet responses

Genomic and molecular analysis

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Cover: Left *Rhizoctonia solani* mycelium and right Rhizoctonia root rot.
Photo: L. Holmquist

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Abstract

The soil-borne basidiomycete *Rhizoctonia solani* (strain AG2-2) incite root rot disease in sugar beet (*Beta vulgaris*). The overall objective of this thesis work was to enhance the genomic knowledge on this pathogen and induced responses in the host to promote breeding of better performing cultivars. The AG2-2IIIB *R. solani* isolate sequenced in this project had a predicted genome size of 56.02 Mb and encoded 11,897 genes. In comparisons with four other *R. solani* genomes, the AG2-2IIIB genome contained more carbohydrate active enzymes, especially the polysaccharide lyase group represented by the pectate lyase family 1 (PL-1). When predicting for small, cysteine rich and secreted-proteins (effectors) 11 potential candidates were found to be AG2-2IIIB strain specific. In parallel, transcript data was generated from sugar beet breeding lines known to express differential responses to *R. solani* infection. After extensive data mining of the achieved information a handful of genes with potential roles in sugar beet defence were identified. Particularly three Bet v I/Major latex protein (*MLP*) homologous genes caught the interest and were further investigated together with three *R. solani* (Rs) effector candidates selected based on their transcript profiles during infection of sugar beet seedlings. They are: a rare lipoprotein-A like protein (RsRlpA), the chitin-binding lysin motif effector (RsLysM) and a cysteine-rich protein (RsCRP1). The three fungal effectors were induced upon early infection and were heterologously expressed in *Cercospora beticola*, a sugar beet leaf spot fungus, facilitating functional analysis. RsLysM showed perturbation of chitin-triggered plant immunity as expected but did not protect fungal hyphae from degradation. RsRlpA is localized to the plant plasma membrane and has capacity to suppress the hypersensitive response. When monitoring cellular localization of RsCRP1 it was found to target both plant mitochondria and chloroplasts. RsCRP1 was also used in pull-down experiments followed by amino acid sequencing from which a potential interacting protein, a plasma membrane intrinsic protein, BvPIP1;1 was proposed to be a candidate. The studies on the fungal effectors and the potential plant defence candidates involving *BvMLPs* and *BvPIP1;1* are on-going including assays of gene homologs in *Arabidopsis* to promote mechanistic understanding of the sugar beet – *R. solani* interactions together with protein-protein interactions and associated assays. Results to be implemented in resistance breeding.

Keywords: Beta vulgaris, *Cercospora beticola*, effectors, LysM, MLP, resistance, *Rhizoctonia solani*, RNAseq

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Rhizoctonia solani och sockerbeta. Genomiska och molekylära analyser

Sammanfattning

Rhizoctonia solani är en jordburen svamp som tillhör Basidiomycota och orsakar rotröta i sockerbetor (*Beta vulgaris*). Det övergripande målet för denna avhandling var att studera *R. solani* AG2-2IIIB genomet för att identifiera faktorer som har betydelse vid infektionen av sockerbeta samt att få bättre förståelse för hur sockerbetor reagerar vid en infektion. Målet på sikt är att kunna utveckla bättre kontrollstrategier för sockerbeta mot infektion av *R. solani*. *R. solani* AG2-2IIIB hade ett predikterat genom på 56,02 Mb och 11 897 gener. I jämförelse med fyra andra *R. solani* genom var AG2-2IIIB det största och hade fler kolhydrataktiva enzymgrupper, speciellt polysackarid lyaser innehållande pektatlyas-familjen 1 (PL-1), än övriga grupper. Små, cystein-rika och utsöndrade proteiner (effektorer) predikterades i genomet och 11 potentiella kandidater unika för AG2-2IIIB kunder urskiljas. Parallellt genererades transkriptdata från sockerbetslinjer med olika resistensnivåer mot Rhizoctonia infektion. Efter omfattande dataanalyser identifierades en handfull gener som potentiellt har betydelse för sockerbetsförsvaret. I synnerhet tre gener homologa till Bet v I/Major latex proteiner (MLP) urskildes och undersöktes ytterligare. Även tre *R. solani* (Rs) effektorkandidater: ett sällsynt lipoprotein-A (RlpA)-liknande protein, den kitinbindande lysinmotiv (LysM) effektorn och ett cystein-rikt protein (CRP1), utvalda baserat på deras genexpression vid infektion av sockerbetsplantor studerades i detalj. De tre svamp-effektorgenerna inducerades vid tidig infektion och för att kunna göra funktionella analyser transformerades de in i *Cercospora beticola*, en bladfläcksorsakande sockerbetspatogen. *RsLysM* visade som förväntat en störning av kitin-utlöst växtimmunitet men skyddar inte svamphyferna från nedbrytning orsakad av kitinaser. RsRlpA lokaliseras till växtplasmamembranet och kan undertrycka hyperkänslig respons. Den cellulära lokaliseringen av RsCRP1 fanns både i växt-mitokondrier och kloroplaster. RsCRP1 användes också i proteininteraktionsstudier där neddragningsförsök följt av aminosyrasekvensering visade att ett plasma-membran protein, BvPIP1;1 potentiellt interagerar med RsCRP1. Studier av svamp-effektorerna och de potentiella resistensgenerna i sockerbeta som innefattar *BvMLP* gener och *BvPIP1;1* pågår och inkluderar proteininteraktioner och analyser av genhomologer i *Arabidopsis* för att öka förståelsen av händelser mellan sockerbeta och *R. solani*. Resultaten av dessa studier är tänkta att användas vid resistensförädling.

Nyckelord: Beta vulgaris, *Cercospora beticola*, effektorer, LysM, MLP, resistens, *Rhizoctonia solani*, RNA sekvensering, sockerbeta

Dedication

To Mattias, Alfred, and Elsa,

It is not in the stars to hold our destiny but in ourselves.
William Shakespeare

Contents

List of publications

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

I Wibberg D[§], **Andersson L**[§], Tzelepis G, Rupp O, Blom J, Jelonek L, Pühler A, Fogelqvist J, Varrelmann M, Schlüter A, Dixelius C. 2016. Genome analysis of the sugar beet pathogen *Rhizoctonia solani* AG2-2IIIB revealed high numbers in secreted proteins and cell wall degrading enzymes. *BMC Genomics*. 17:245

II **Holmquist L**, Fogelqvist J, Cohn J, Dölfors F, Varrelmann M, Kraft T, and Dixelius C. Identification of genes contributing to *Rhizoctonia solani* defense responses in sugar beet. In manuscript

III Dölfors F, **Holmquist L**, Moschou P, Dixelius C, Tzelepis G. 2018. The The *Rhizoctonia solani* RsLysM and RsRlpA effector proteins contribute to virulence by suppressing chitin-triggered immunity and hypersensitive response. *BioRxiv*. doi.org/10.1101/395582

IV Tzelepis G, **Holmquist L**, Dölfors F, Dixelius C. The *Rhizoctonia solani* RsCRP1 effector promotes virulence and impacts the sugar beet BvPIP1;1 membrane protein. In manuscript

§ = The authors contributed equally.

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Additional publications

Wibberg D[§], **Andersson L**[§], Rupp O, Goesmannd A, Pühler A, Varrelmann M, Dixelius C, Andreas Schlüter A. 2016. Draft genome sequence of the sugar beet pathogen *Rhizoctonia solani* AG2-2IIIB strain BBA69670. *J Biotechnol.* 222:11-12

Dölfors F, **Holmquist L**, Tzelepis G, Dixelius C. *Rhizoctonia solani* infection assay of young sugar beet and *Arabidopsis* plants. In manuscript

§ = The authors contributed equally.

The contribution of Louise Holmquist to the papers included in this thesis was as follows:

I Participated in research planning, provided materials, extracted DNA and RNA, performed part of data analysis and participated in manuscript writing.

II Participated in research planning, provided materials, performed parts of the research, bioinformatics and additional data analysis. Participated in manuscript writing.

III Provided materials, participated in statistical analysis and in manuscript writing.

IV Participated in research planning, provided materials, made phylogenetic and statistical analysis, took part in manuscript writing.

1 Introduction

Sugar is a common molecule that makes our food taste better and act as a preservative. Sugar beet is one of two main sources of sugar and contributes about 20% of the world's sugar production; the rest mainly derives from sugar cane (International Sugar organization, 2017). The end product extracted from the plants, white table sugar, is composed of pure sucrose and is the same product regardless of which of the two plant species it is extracted from. There are other sources of sugar in nature, for example maple syrup, agave nectar, honey and dates, but sucrose is most concentrated in sugar beets and sugar cane (Phillips *et al*., 2009). Another sweetener, 100-300 times sweeter than sucrose and with no calories, is stevioside extracted from *Stevia rebaudiana* (Goyal *et al*., 2010). Besides the white table sugar, sugar beets are also used for animal feed and bioethanol production. Beside humans and animals, many microorganisms are attracted to tissue enriched in sugar, where they pose a threat to crop production if large-scale multiplication occurs. Pathogenic organisms need to be taken care of in one or another way to ensure sugar beets of good quality. One strategy is to control pests and pathogens with chemical applications. This is costly, not always effective and most of all not beneficial for the environment. Another (and more environment-friendly) alternative is to grow resistant varieties. In many cases there is a negative correlation between disease resistance and high sugar yield and it is a difficult task for the breeders to combine the two characters.

In this project next generation sequencing technologies have been used in an attempt to better understand the interaction between sugar beet and *Rhizoctonia solani*. Results are envisioned to be implemented in on-going breeding work.

1.1 Sugar beet

1.1.1 The history of sugar beet breeding

It was as late as 1747 that a scientist succeeded to extract sugar from a sugar beet for the first time. This progress was made by Andreas Sigismund Marggraf and in 1801 his student Franz Carl Achard built the first pilot factory in France (Cooke and Scott, 1993). During the Napoleonic period at the beginning of the 19th century a lot of factories were built all over Europe as a result of the high prices of imported cane sugar. This time is considered as the start for sugar beet breeding. Achard discovered that roots from different species, and even from seeds from the same plant, differed a lot in sugar content and he started to breed for high sugar content. In the 1870s breeding diverged into two beet types, high sugar content or high root yield. The challenge was to combine the two polygenetic characters to obtain a big root with high sugar content.

Early on it was understood that the beet cyst nematodes were a problem if beets were repeatedly grown in the same field (Cooke and Scott, 1993). By the end of the 19th century farmers had learned to handle the nematode infestations by widening the crop rotation schemes. Other diseases were now noticed, like the fungal disease Cercospora leaf spot and the viral disease curly top.

Early sugar beet cultivation was associated with labour-intensive work eliminating weeds from the fields and plant thinning. The seeds were multigerm, meaning that three or more shoots emerged from each seed (Fig. 1). The rows had to be thinned leaving only one plantlet to grow, and this was hard work.

Figure 1. Germinating sugar beet seeds. Left multigerm seeds and right monogerm seeds. Photo: L. Holmquist

A great success was the discovery of a monogerm plant in the 1930s. The first monogerm variety was produced and marketed in the United States (1957) and in Western Europe from the mid-1960s (Draycott, 2006). The first herbicide, propham, was available to growers in the USA in the 1950s after an extensive

research programme and together with the monogerm seed these developments drastically decreased the workload for the farmers. Another important step forward in sugar beet breeding was the detection of cytoplasmic male sterility (CMS), which is used today in the breeding of hybrid varieties (Owen, 1945). More information on this can be found in section 1.1.3.

The first factory in Sweden was built in 1854 in Landskrona and during the 1880s the sugar production in Sweden increased 10-fold with eight new sugar factories built in the southern parts of the country (Bosemark, 1997). The first breeding activities in Sweden started in Landskrona in 1907 and by 1928 only seeds from Hilleshög, the Swedish breeding company, were planted in Sweden.

An important step forward for the sugar beet research came in 1989 when molecular markers were implemented in the breeding programmes.

1.1.2 Sugar beet production

4.5 million hectares of sugar beets were harvested in the world in 2016 of which 75% were grown in Europe (www.fao.org/faostat/en/#data/QC, 20180807). Sugar beets are grown commercially throughout the world in cooler and temperate climates (Fig. 2). The main producing regions are the European Union, the United States, the Russian Federation, Turkey, Ukraine, Iran, Japan and China. Sugar beets are a good complement to sugar cane in terms of growth requirements. Sugar cane grows in tropical regions, has a 12 months growth period and needs more water than sugar beet that grows in temperate regions and with a 6-month growth period.

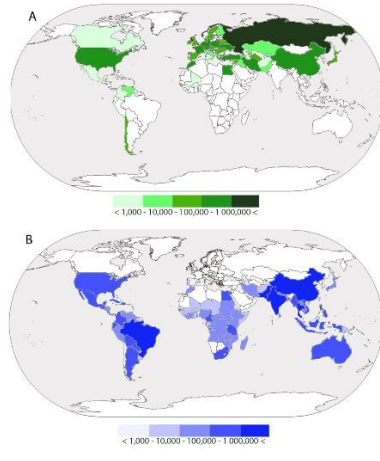

Figure 2. Countries where A, sugar beets (green) and B, sugar cane (blue) were grown and harvested in 2016. Colour indicates hectares of harvested sugar beets/sugar cane per country. Information collected from www.faostat.com

The seeds are drilled in the spring and the roots are harvested in the autumn ahead of frost. The highest sugar concentration is in the lower part of the root and gradually decreases towards the crown (Fig. 3). Commercial sugar beet yields are between 50 and 100 metric tons of clean beet/ha, with a sugar concentration of 17-18% of fresh weight, yielding 8-18 tons of sugar/ha (Draycott, 2006). Besides pure sugar several useful by-products are produced in the sugar refinery, for example pulp and molasses for feed supplements for livestock (Elferink *et al*., 2008) and waste lime as a soil amendment to increase soil pH levels. Sugar beets are also used as raw material for the ethanol component in biofuel production. From one ton of fresh sugar beet roots 100-120 litres of ethanol can be produced (Panella, 2010). This makes it one of the most efficient crops for ethanol production per hectare. New potential areas of application are as bioplastics (Liu *et al*., 2011) or as a blood supplement through the extraction of haemoglobin (Leiva-Eriksson *et al*., 2014).

Figure 3. Sugar beet. A indicates the crown of the plant. Photo: MariboHilleshög Research

The production of sugar within the European Union has been regulated since 1968 when support was introduced for growers as part of the Common Agricultural Policy to improve food self-sufficiency within EU (Bureau *et al.*, 1997). This quota system was abolished in 2017 and the market is now deregulated. It is speculated that this change will lead to an increase in sugar beet production due to the free choice of growing as much as a farmer wants and the refiners are free to export sugar outside of EU. Whether higher access to sugar will lead to a decrease in prices within EU is still unclear.

1.1.3 Sugar beet breeding

Sugar beet, *Beta vulgaris* ssp. *vulgaris*, belongs to the subfamily Betoideae in Amaranthaceae (Schwichtenberg *et al*., 2016). It is a diploid plant with nine pairs of chromosomes and it originates from the Mediterranean region. The sea beet *Beta vulgaris* subsp. *maritima* is a wild relative often used in breeding. Sea beet is resistant to many pathogens and insects, tolerant to drought, heat and salinity and it can easily be crossed with sugar beet (Biancardi *et al*., 2012). Introgression of traits from *Beta vulgaris* subsp. *maritima* is the main source for broadening the narrow gene pool of sugar beet. All cultivated beets are biennial and require a cold period, vernalization, to change from vegetative to reproductive stage. The reproductive stage is used in breeding and seed production to develop new varieties whereas the vegetative stage is used in farmer production. To generate as high yield as possible the growing season needs to be as long as possible and one way of prolonging the season is to plant early in spring (Draycott, 2006). This increases the risk for seed stalk development, bolting, that can be induced by low spring temperatures and therefore there is a need for bolting tolerance. Flower induction is influenced by day length as well as temperature, and manipulation of these factors can be used to shorten the breeding cycle. Many of the wild Mediterranean forms of Beta species are annuals, a trait regulated by the dominant *B* gene. Plants carrying this gene bolt extremely quickly if light and temperature are favourable, which could be used to speed up breeding. On the other hand, the presence of the dominant *B* gene in commercially cultivated beets is strongly negative since it will result in beets that will bolt and flower in the fields. This makes it very difficult to use the *B* gene in breeding. The *B* gene, or *BvBTC1* as it is also called, interacts with two other genes to control flowering, *BvFT1* and *BvFT2*. It is suggested that the biennial-growth habit of the cultivated beet emerged from a selection of partial loss-of-function in the *BvBTC1* allele (Pin, 2012).

Commercial sugar beets are 3-way hybrids. To be able to produce these hybrids a male sterility system is used. There are two types of male sterility, a combination of nuclear and cytoplasmic sterility and only nuclear (genetic) sterility (Biancardi *et al*., 2005). The first type of male sterility provides a complete control of pollination while the second type is used for cross-pollinations. The genetic-cytoplasmic male sterility (CMS) is maternally transmitted. In hybrid production, CMS plants are pollinated by maintainer plants (O-types), which carry the same sterility genes as the male sterile plants but in normal cytoplasm (Draycott, 2006). The offspring, which is referred to as an F_1MS line, is also male sterile and is used as a mother plant in a second cross with a third line that is referred to as a pollinator. The new seed is now what we call the hybrid seed produced for the market.

1.1.4 Sugar beet pests and diseases

Sugar beet attracts a lot of different pathogens causing a number of diseases. More or less all soils where sugar beets are grown around the world are infested with the plasmodiophorid *Polymyxa betae* which transmits the beet necrotic yellow vein virus causing Rhizomania disease (Tamada and Asher, 2016). The risk of Rhizomania disease is thus high and the only way to handle the disease is by growing resistant cultivars. Beet cyst nematodes (*Heterodera schachtii*) (Bohlmann and Sobczak, 2014) are also a worldwide problem and using nematode tolerant or resistant varieties are important. In Scandinavia, the most prevalent diseases are Aphanomyces damping off and root rot (*Aphanomyces cochlioides*) and Ramularia leaf spot (*Ramularia beticola*) (Windels, 2000; Videira *et al*., 2016). Both diseases are caused by pathogens which prefer a cooler and humid climate. In warmer climates like southern Europe, Rhizoctonia root rot (*Rhizoctonia solani*) and Cercospora leaf spot (*Cercospora beticola*) are the most common fungal diseases (Sneh *et al*., 1996; Weiland and Koch, 2004). In the United States all of the diseases mentioned above are more prevalent and more severe than in Europe. More information on resistance genes can be found in section 1.5.1.

Rhizoctonia root and crown rot

Rhizoctonia root and crown rot of sugar beet is caused by the widespread soil-borne fungus *Rhizoctonia solani*. The disease was first reported in 1915 in the United States by Howard Austin Edson (Mukhopadhyay, 1987). Rhizoctonia root and crown rot is primarily a disease causing symptoms on the root. It affects sugar beets in all growing areas but is more severe in hot climates and in heavy, poorly drained and wet fields (Cooke and Scott, 1993; Harveson *et al*., 2009; Bolton *et al*., 2010). The disease is estimated to affect 24% of the acreage in the United States and 5-10% in Europe (Harveson *et al*., 2009). In recent years an increase of the disease has been seen both in the United States as well as in Europe (Ithurrart *et al*., 2004; Bolton *et al*., 2010). Commercial varieties with a strong resistance to the disease are available but the drawbacks are a lower yield potential in the absence of the disease and lack of resistance to other important diseases (Jacobsen *et al*., 2004; Strausbaugh *et al*., 2013). Farmers in the United States rely on fungicides instead of highly resistant cultivars and the risk for fungicide resistance is threatening. Many different fungicides with different active ingredients are available for the control of the disease (Arabiat and Khan, 2016). Timing of application is difficult and critical since it needs to be done early, prior to initial infection to prevent disease establishment (Bolton *et al*., 2010). In Europe there are no registered fungicides available and the only way

to handle the disease is by agronomical strategies like crop rotation, plant residue management and soil tillage practices and most importantly availability of resistant varieties (Buhre *et al*., 2009).

Figure 4. Sugar beet seedlings infected with *Rhizoctonia solani*. Photo: F. Dölfors

The disease can appear in different forms and with different symptoms; root rot and crown rot, damping off and foliar blight. The fungus can cause a pre-emergence damping-off (Mukhopadhyay, 1987). The dead sprouts are difficult to observe in the soil because of their relatively small size and farmers often think they have a poor stand due to poor quality of seeds rather than to pre-emergence damping off. A more common symptom is damping-off of emerged seedlings (Fig. 4). It starts with a dark brown to black lesion on the hypocotyl just at the soil surface (Cooke and Scott, 1993; Harveson *et al*., 2009). The fungus continues to advance along the hypocotyl and a sharp line between diseased and healthy tissue can be seen (Fig. 4). The collar (crown) of an infected seedling breaks easily at or near the soil line, but the roots generally remain healthy until the plant dies (Mukhopadhyay, 1987). When the hypocotyl is heavily colonized the plants rapidly collapse.

Figure 5. Rhizoctonia root rot infection of sugar beets in field. Photo: L. Holmquist

The first sign of root and crown rot is a sudden wilting and chlorosis of the leaves and with dark brown to black lesions at the base of the petioles (Cooke and Scott, 1993; Harveson *et al*., 2009). The leaves then collapse, fall to the ground and die, but remain attached to the crown (Fig. 5). Soil infection is often patchy and symptoms are not always seen above ground even though roots are heavily infected. Crown rot starts in the crown of the root and extends down the taproot (Fig. 6). The disease development is often associated with soil being deposited on the crown during cultivation (Harveson *et al*., 2009).

Figure 6. Rhizoctonia crown rot on sugar beet root. Photo: MariboHilleshög Research

Root rot on the other hand often starts in the tip of the root and progresses upwards on the taproot. Roots show varied degrees of dark brown to black rot (Cooke and Scott, 1993). Deep cracks or holes can sometimes emerge that deform the root (Harveson *et al*., 2009). Inside the root there is generally a sharp line between diseased and healthy tissue (Fig. 7). The infected tissue is often located in the periderm of the root and is not spread into the root until the disease is severe (Harveson *et al*., 2009).

Figure 7. Sugar beet root with severe root rot symptoms caused by *Rhizoctonia solani*. Photo: L. Holmquist

Dry rot canker is another form of the disease which is less common. The symptoms are dark brown, circular lesions on the surface of the root, about 1.5-

25 mm in diameter (Cooke and Scott, 1993; Harveson *et al*., 2009). Beneath the lesions deep cankers filled with fungal mycelium can be seen. Under warm, humid conditions there are certain strains of *R. solani* that can induce foliar blight (Cooke and Scott, 1993). Cotyledons are diseased and lesions appear on older leaves. Foliar blight is favoured by heavy rain that splashes infested mud onto the foliage (Mukhopadhyay, 1987).

1.2 Genome sequencing

The first complete protein-coding gene sequence, the coat protein of bacteriophage MS2, was elucidated in 1972 using the 2-D fractionation method (Min-Jou *et al*., 1972). This method was replaced by Sanger's 'plus and minus' system in 1975 and at the same time Maxam and Gilbert developed a method using radiolabelled DNA (Heather and Chain, 2016). The Maxam and Gilbert method was the first technique to be widely adopted, and thus might be considered the 'first-generation' DNA sequencing method. However the major breakthrough came with the introduction of the Sanger sequencing method (Sanger *et al*., 1977). This method is also called the chain termination method because of the dye-labelled chain-terminating dideoxy-nucleotides used. The advantages with this method were high-quality and relatively long DNA sequences.

Pyrosequencing was licensed by 454 Life Sciences and seen as the first next generation sequencing (NGS) technique (Ronaghi *et al*., 1998). In 1998 Balasubramanian and Klenerman founded the Solexa company where they developed a new method called sequencing-by-synthesis (Balasubramanian, 1999). Solexa and its technology was acquired by Illumina in 2007 and this technology is by far the most common today. Platforms other than Illumina available today are Ion Torrent and Pacific Biosciences (Quail *et al*., 2012). In the last few years the development has gone quickly and longer sequences and pair-end data with higher accuracy can now be generated. Next generation sequencing approaches have also been a revolution for speed and costs of sequencing. Today a human genome can be sequenced in a single day compared to when the first draft human genome was sequenced using Sanger sequencing and took a whole decade. The cost of sequencing has fallen dramatically and a whole human genome is now down to less than US$1,000 (Goodwin *et al*., 2016). The techniques are constantly being improved and the sequence information gets more reliable. Today the problem is not the lack of data but rather the limited time available to analyse data as well as advanced bioinformatics tools to understand the data generated.

1.2.1 Genome sequencing of plants

Arabidopsis thaliana was the first plant genome to be sequenced (The Arabidopsis genome initiative, 2000) followed by rice and black cottonwood, *Populus trichocarpa* (Goff *et al*., 2002; Tuskan *et al*., 2006). Today hundreds of plant genomes, many of them important crops (Table 1), are sequenced and publicly available and of great use for plant science.

Table 1. *Genome size and number of predicted gene models for some important crops*

Crop	Latin name	Genome size	No. protein coding genes	Reference
Wheat	*Triticum aestivum*	17 Gb	104,091	Clavijo *et al*., 2017
Corn	*Zea mays*	2.3 Gb	32,000	Schnable *et al*., 2009
Sugar beet	*Beta vulgaris*	567 Mb	27,421	Dohm *et al*., 2014
Soybean	*Glycine max*	1.1 Gb	46,430	Schmutz *et al*., 2010
Rice	*Oryza sativa*	389 Mb	37,544	International rice genome sequencing project, 2005
Barley	*Hordeum vulgare*	4.79 Gb	39,031	Mascher *et al*., 2017
Potato	*Solanum tuberosum*	844 Mb	39,031	Xu *et al*., 2011

1.2.2 Genome sequencing of fungi

The ascomycete *Saccharomyces cerevisiae*, was the first fungus to have its genome sequenced (Goffeau *et al*., 1996) and the crust forming fungus, *Phanerochaete chrysosporium*, the first basidiomycete (Martinez *et al*., 2004). By 2016 over 1,000 fungal species genomes had been sequenced and available to the public and this has rapidly increased since then (Aylward *et al*., 2017). Of the 1,090 genome sequences available in 2016, the largest category (35.5%) comprised of pathogenic species of which plant pathogens form the majority. Of the 191 plant pathogenic fungal species with available genomes, 61.3 % cause diseases on food crops. The genomes of plant pathogens are slightly larger than those of other fungal species sequenced to date and they contain fewer predicted coding sequences in relation to their genome size (Table 2).

Table 2. *Genome size and number of protein coding genes for some important plant pathogens*

Plant pathogen	Disease	Genome size	No. protein coding genes	Reference
Rhizoctonia solani AG2-2IIIB	Rhizoctonia root rot	56 Mb	11,897	Wibberg *et al*., 2016a,b
Magnaporthe grisea	Rice blast	40 Mb	11,109	Dean *et al*., 2005
Ustilago maydis	Corn smut	20 Mb	6,902	Kämper *et al*., 2006
Blumeria graminis	Powdery mildew	82 Mb	6,540	Wicker *et al*., 2013
Fusarium graminearum	Head blight	36 Mb	11,640	Cuomo *et al*., 2007
Leptosphaeria maculans	Blackleg disease	45 Mb	12,469	Rouxel *et al*., 2011
Puccinia graminis f. sp. tritici	Stem rust	89 Mb	17,773	Duplessis *et al*., 2011
Phytophthora infestans	Potato blight	240 Mb	17,797	Haas *et al*., 2009

1.3 *Rhizoctonia solani*

Rhizoctonia solani Kühn (teleomorph: *Thanatephorus cucumeris*) is a soil-borne basidiomycete and a pathogen on a wide range of crops and plant species (Harveson *et al*., 2009). The asexual stage of *R. solani* is often seen as its predominant stage, whereas the sexual stage can rarely be found in agricultural fields (Cubeta and Vilgalys, 1997). *Thanatephorus* basidiospores are very difficult to germinate and if single basidiospore isolates have been successfully produced they are usually less virulent and have more limited saprophytic capabilities (Cubeta and Vilgalys, 1997). Basidiospores can be wind spread and serve as inoculum for foliar diseases but in general basidiospores are not the primary inoculum for disease. Most *R. solani* infections are initiated by sclerotia or mycelia from debris which can survive in the soil for many years (Cubeta and Vilgalys, 1997).

Hyphae are characteristic, coarse, pale- to dark brown and they branch near the distal septum of the hyphal cell, usually at right angles in young vegetative hyphae and are constricted at the point of origin. Individual cells are multinucleate with 4 to 14 nuclei per cell. Isolates of *R. solani* vary greatly in their cultural appearance, in their growth characteristics and in their pathogenicity towards plants, both in terms of host-plant specialization and in terms of virulence (Sneh *et al*., 1996).

The most common method to categorize *R. solani* is based on hyphal cell wall fusion between different isolates. The resulting fusion products form the division

into anastomosis groups (AGs). AGs are widely used since they reflect diversity between isolates. Some AGs have very specific hosts while others have a broad host range (Table 3). Some AGs are further divided into subgroups based on host range, colony morphology, pathogenicity, zymogram patterns and other characteristics (Sneh *et al*., 1996). Among molecular methods used for distinguishing between AGs and different subgroups, sequencing of the ribosomal region's internal transcribed spacer (ITS) is the most widely used strategy (Sharon *et al*., 2006). Phylogenetic analysis based on ITS sequences can in many cases cluster similar AGs and subgroups together. AG and subgroup specific PCR primers are available in many cases and can be used as a diagnostic tool (Bounou *et al*., 1999; Salazar *et al*., 2000, Grosch *et al*., 2007).

Table 3. *Estimated genome size, number of predicted protein coding genes, host range and genome sequencing reference of Rhizoctonia solani anastomosis groups and subgroups*

Anastomosis group & subgroup	Genome size (Mb)	No. protein coding genes	Host range	Genome reference
AG1-1A	36.9	10,489	Rice	Zheng *et al*., 2013, Nadarajah *et al*., 2017
AG1-1B	42.8	12,616	Bean, rice, soybean, figs, hydrangea, cabbage, lettuce	Wibberg *et al*., 2013
AG2-1			Cauliflower, canola, oilseed rape, cabbage	
AG2-2IIIB	56.02	11,897	Sugar beet, soybean, maize	Wibberg *et al*., 2016a,b
AG2-2IV			Sugar beet	
AG3	51.0	12,720	Potato, tomato, cotton, tobacco, maize	Wibberg *et al*., 2017; Cubeta *et al*., 2014
AG4			Canola, tomato, potato, soybean, cotton, oilseed rape, cabbage, sugar beet	
AG5			Potato, soybean	
AG8	39.8	13,420	Wheat, barley, canola, legumes	Hane *et al*., 2014
AG9			Potatoes, canola	
AG10			Canola	
AG11			Wheat	

R. solani lives in the soil as a primitive organism with modest nutrient requirements (Mukhopadhyay, 1987). As a saprophyte it can utilize many organic compounds as an energy source and thus can live on dead or

decomposing plant debris for months. *R. solani* survives as thickened hyphae, sclerotia, bulbils or basidiospores in crop residues and soil (Harveson *et al*., 2009) and is generally spread by rain, irrigation, or flood-water, or with tools or anything else that carries contaminated soil (Sneh *et al*., 1996).

AG2-2 is the main isolate type attacking sugar beets. When temperatures reach 12°C the overwintered propagules of *R. solani* germinate and infect the beet seedlings. The fungus is active between 12-35°C with optimal activity at 25-33°C when the soil is wet. Sclerotia are the primary survival structures and therefore an important source of inoculum. The sclerotia germinate under humid conditions by producing new mycelia threads (Mukhopadhyay, 1987). Exudates from germinating seedlings and actively growing roots stimulate sclerotia to form hyphae which initiate colonization upon reaching the plant root (Mukhopadhyay, 1987; Sneh *et al*., 1996).

Based on current knowledge, the different AGs have slightly variable ways of infecting the plant host but a generalized procedure is as follows. The hyphae have first a round shape and grow over the plant surface without being attached to the plant. They then become flat and firmly attached to the plant, forming side branches at right angles (T-shaped branches). At this stage the infection process can continue in two ways. Either the branches give rise to short swollen hyphae, which is most common for isolates infecting foliage; alternatively multiple T-shaped branches are formed resulting in dome-shaped infection cushions, often the way for stem and root infecting isolates. The hyphae in the infection cushion also have swollen tips that adhere tightly to the host surface. Several of the swollen tips simultaneously form infection pegs that penetrate the surface. The penetration is probably a combination of mechanical pressure and enzyme activity but this is not confirmed. The invading hyphae rapidly ramify through the host tissue, causing it to turn brown and collapse.

1.4 Plant defence mechanisms

The understanding of plant defence and related mechanisms has grown extensively the last 15 years and is described in many review articles (Gohre and Robatzek, 2008; Spoel and Dong, 2012; Mengiste, 2012; Muthamilarasan and Prasad, 2013; Newman *et al*., 2013; Wang *et al*., 2014; Bigeard *et al*., 2015; Presti *et al*., 2015). Since plants cannot move and escape a threatening invader they have different ways to protect themselves. Plant pathogens are commonly divided into three groups based on life-style: biotrophs that require living host cells for growth, necrotrophs that kill and thrive on dead host cells, or hemibiotrophs that have an initial period of biotrophy followed by necrotrophy. Basal resistance or innate immunity is the first level of defence that protects

plants against a broad category of pathogens. General elicitors, or microbe-associated molecular patterns (MAMPs), are conserved structures of microbes that are sensed by a broad spectrum of plants. MAMPs are recognized by pattern-recognition receptors (PRRs) that trigger immediate defence responses leading to basal or non-host resistance. MAMPs represent a broad category of compounds and all are essential for microbial life including pathogens, hence named pathogen-associated molecular patterns (PAMPs). The activation of PRR signalling results in rapid responses that include the accumulation of reactive oxygen intermediates, activation of ion channels, activation of specific defence-related mitogen-activated protein kinase (MAPK) cascades, and extensive transcriptional reprogramming of the host (Boller and Felix, 2009). Collectively this leads to an accumulation of antimicrobial compounds including proteinases, chitinases and glucanases that damage pathogen structures. Also enzyme inhibitors directed toward molecules produced by the pathogen are formed, as well as other non-proteinaceous antimicrobial molecules. Specialized pathogens are able to overcome basal host immunity by either avoiding the detection of PAMPs or interfering with pathogen-triggered immunity (PTI) by delaying, suppressing or reprogramming host responses.

A general definition of effectors is pathogen-produced molecules that have a specific effect on one or more genotypes of a host or non-host plant (Vleeshouwers and Oliver, 2014). The recognition of pathogen effectors by plant resistance (R) proteins may generate a hypersensitive response (HR) and local cell death, an event often leading to effector-triggered immunity (ETI) (Wang *et al*., 2014). For biotrophs this leads to failure to survive and infect. For necrotrophs this leads to effector-triggered susceptibility (ETS) and the pathogen can continue the colonization of the host. The majority of known *R* genes in plants encode nucleotide-binding leucin-rich repeat (NB-LRR) proteins (Dangl and Jones, 2001). Effector perception by NB-LRRs is highly specific and can be either direct (with the receptor binding the effector) or indirect (involving accessory proteins) (Dodd and Rathjen, 2010). Accessory proteins can be pathogen virulence targets or structural imitators of such targets. PTI and ETI responses are similar but often differ in their strength to protect the plant from disease development (Jones and Dangl, 2006; Presti *et al*., 2015).

1.5 Marker-assisted breeding

Marker-assisted breeding uses molecular markers to indirectly select for traits of interest. A DNA marker is a variation in the DNA, i.e. point mutation, insertion, deletion or error in replication of tandem repeated DNA (Collard and Mackill, 2008). Quantitative trait loci (QTLs) are phenotypically defined chromosomal

regions that contribute to allelic variation for a biological trait. Many agronomically important traits like yield, quality, abiotic stress and some disease resistance are inherited quantitatively, meaning many genes control these complex traits and each gene has a small and cumulative effect on the target trait. Linkage maps are used for the identification of quantitative traits using QTL analysis.

Marker-assisted selection (MAS) uses molecular markers known to be associated with a trait of interest to facilitate selection of a desirable allele influencing the target trait (Bhat *et al*., 2016). The MAS application is most effective for traits that are controlled by fewer numbers of QTL having major effect on trait expression. Genomic selection is however more efficient for traits controlled by many QTL regions. Genomic selection estimates the genetic value of each individual, based on a large set of markers distributed across the whole genome, and is not based on few markers as in MAS (Bhat *et al*., 2016). In genomic selection a prediction model based on genotypic and phenotypic data of a training population is used to derive genomic estimated breeding values for all the individuals of a breeding population from their genomic profile (Meuwissen *et al*., 2001). All molecular markers available for a candidate trait are used to predict the breeding value and the outcome is used to predict individuals that will perform better and are suitable to select as parents of the next generation.

Genome wide association mapping or GWAS, finds single nucleotide polymorphisms (SNPs) within the whole genome that are associated with a trait of interest. GWAS can be performed on the same population as genomic selection (Zhang *et al.*, 2014). The genetic architecture revealed by association mapping can be used to inform the genomic selection models, for example if highly significant SNPs are revealed by a genome wide association study, these SNPs could be fitted as fixed effects in a genomic selection model (Begum *et al*., 2015).

1.5.1 Resistance genes, QTLs and molecular markers in sugar beet

Disease resistance in a crop can either be due to one major gene or regulated quantitatively by several genes. In the sugar beet reference genome 715 resistance gene analogs have been predicted (Dohm *et al*., 2014). The predicted domain distribution is: 518 with similarity to the serine (threonine) protein kinase domain, 80 have nucleotide-binding site (NBS) and leucin rich repeat (LRR) domains, 57 have a single NBS domain and 60 have only a LRR domain. Examples of resistances encoded by a major gene are the *Rz1* and *Hs1* genes for resistance to the Rhizomania virus disease and the nematode (*Heterodera*

schachtii) resistance (Cai *et al*., 1997; De Biaggi *et al*., 2010). Presently, four QTLs located on four different chromosomes are known to promote resistance to Rhizoctonia root and crown rot in sugar beet (Lein *et al*., 2008; Kraft personal communication). The QTL regions are wide (in total covering 10-15% of the genome) and include several negative traits as well. It is important to make the mapping more precise to be able to identify recombinants and thereby remove some of the yield drag associated with Rhizoctonia resistance.

Different types of DNA-based markers have been used for genetic analyses in sugar beet over time (Barzen *et al*., 1992; Uphoff and Wricke, 1995; Schondelmaier *et al*., 1996; Laurent *et al*., 2007; Schneider *et al*., 2007; Smulders *et al*., 2010; Izzatullayeva *et al*., 2014; Stevanato *et al*., 2014). For a long time simple sequence repeat (SSR) markers were preferred in plant breeding, due to their high reproducibility, hypervariability, multiallelism, codominant inheritance, extensive genome coverage, chromosome-specific location and easy automated detection by PCR (Taški-Ajduković *et al*., 2017). In sugar beet, a few hundred SSR markers have been developed for various purposes (Rae *et al*., 2000; Arnaud *et al*., 2003; Richards *et al*., 2004; Viard *et al*., 2004; Laurent *et al*., 2007; McGrath *et al*., 2007; Fénart *et al*., 2008; Arnaud *et al*., 2009). Today, SNP markers are almost exclusively used in sugar beet breeding. Even though they are bi-allelic and therefore less informative, the screening is much easier to automate and therefore many more markers can be used as a compensation for less information. Different methods are available for the detection of SNPs; hybridization, enzymatic cleavage, ligation and primer extension (Kim and Misra, 2007). One of the first methods was restriction fragment length polymorphism (RFLP) that uses allele-specific restriction enzymes to cleave DNA at a certain base (Botstein *et al*., 1980). A widely used method today is the TaqMan system (De La Vega *et al*., 2005) which combines hybridization and nuclease activity using fluorescently-tagged, allele-specific probes detected with PCR. For high-throughput analysis different chip technologies, like microarrays, are often used (Thomson, 2014). These multiplex solutions is suitable for largescale studies requiring genotypic data for individual samples with thousands of SNPs. For crop improvement when only low to medium number of markers are needed but for a large number of samples it is more suitable to use uniplex systems like TaqMan or KASP (Kompetitive allele specific PCR) (Semagn *et al*., 2014).

2 Aims of the study

Soils infested with *Rhizoctonia solani* are increasing. The situation has led to a demand for resistant hybrid cultivars. In Europe there are no registered chemical treatments against the pathogen that is causing root rot of sugar beets. In the United States chemical treatments are available but the timing of application is difficult and a combination with resistant varieties is necessary.

The emphasis of this work was to study the plant pathogen *Rhizoctonia solani* and its interaction with the host *Beta vulgaris*.

Specific objectives were to:

- Sequence the genome of a *Rhizoctonia solani* AG2-2IIIB, a highly pathogenic, disease-inciting pathogen to sugar beet
- Run comparative genomics to study host specificity, pathogenicity factors and especially effectors potentially responsible for host infection.
- Analyse sugar beet transcriptomes, comparing partially resistant and susceptible genotypes, with the aim of finding genes involved in the defence response.

3 Results and discussion

3.1 *Rhizoctonia solani* comparative genomics

Rhizoctonia solani is an important disease on many crops and other plants around the world (Harveson *et al*., 2009). Different crops are infected by different anastomosis groups of *R. solani*. The host specificity and infection mechanisms are areas of low knowledge and understanding.

Sugar beet is mainly infected by the AG2-2 strain, which has a relatively wide host range (while AG3, for example, predominantly attacks potato). In an attempt to understand more about the *R. solani* - sugar beet pathosystem, the genome of a highly pathogenic *R. solani* AG2-2IIIB isolate was sequenced (**I**).

Genomes of plant pathogenic fungi differ a lot in size (Table 2) for example *Ustilago maydis* has an estimated genome size of only 20 Mb, while many Pucciniomycete (rust) species have genomes larger than 100 Mb (Aylward *et al*., 2017). The average genome size of all sequenced basidiomycetes is 57 Mb compared to ascomycetes with an average size of 39 Mb. *Rhizoctonia solani* AG2-2IIIB has an estimated genome size of 56 Mb (**I**) and 11,897 predicted protein coding genes, placing it between *Ustilago maydis* with 6,902 and *Puccinia graminis* f. sp. *tritici* (89 Mb) with 17,773 predicted protein coding genes (Table 2). Explanations for the small number of genes and small genome size in *Ustilago maydis* are the absence of expansions of gene families and small or no introns (Kämper *et al*., 2006).

Compared to other sequenced *R. solani* isolates the AG2-2IIIB isolate has the largest genome size (Table 3) but the number of predicted protein-coding genes is about the same for all AGs (**I**). The core genome of all five isolates analysed consists of only 2,704 predicted genes representing 19-25% of all genes (**I**). However, 4,908 genes are specific for the AG2-2IIIB isolate (**I**). This shows that

there is a huge variation between the different anastomosis groups and there is a need to improve the old fashion division of groups within this species.

Plant pathogenic fungi secrete metabolites during their interaction with other organisms or with biological matter in the environment. A secretory metabolite can be a hormone, enzyme, toxin and antimicrobial peptide. We predicted 1,142 secreted proteins to be encoded in the AG2-2IIIB genome (**I**). Compared to the other *R. solani* isolates AG2-2IIIB has the highest number of secreted proteins. 473 secreted proteins of the AG2-2IIIB isolate are unique compared to the other AGs.

We chose to look more closely into two groups of genes that we believe are of importance for the fungus in the interaction with its host; cell wall degrading enzymes and additional effector proteins.

3.1.1 Cell wall degrading enzymes

The first barrier that a fungus needs to overcome to be able to infect its host is the cell wall. It is well known that plant cell wall degrading carbohydrate active enzymes (CAZymes) play important roles during fungal infection (Kubicek *et al*., 2014). A large number of genes encoding fungal cell wall degrading enzymes are present in phytopathogenic fungal genomes. However, the expression patterns and the exact roles of these enzymes during fungal infection and host colonization are not fully understood (Lyu *et al*., 2015). We assume that different sets of CAZymes in the different *R. solani* strains could be involved in host specificity.

CAZymes are responsible for the breakdown, synthesis or modification of glycoconjugates and complex carbohydrates (Cantarel *et al.*, 2008). A large group of the fungal secreted proteins are CAZymes. They are currently divided into five main CAZyme classes: glycosyltransferases (GTs), glycoside hydrolases (GHs), polysaccharide lyases (PLs), carbohydrate esterases (CEs) and auxiliary activities (AAs) as well as one associated class: carbohydrate-binding modules (CBMs) (Levasseur *et al*., 2013).

We identified 1,097 predicted CAZymes in *R. solani* AG2-2IIIB (**I**) which is a high number compared to other *R. solani* AGs and also compared to other basidiomycetes, ascomycetes and oomycetes (Table 4). All CAZy groups are expanded in *R. solani* AG2-2IIIB except in the comparison with the necrotrophic fungus *Fusarium oxysporum*. The PL group is largely expanded in *R. solani* AG2-2IIIB also in the comparison with *F. oxysporum*. Polysaccharide lyases are a group of enzymes that cleave uronic acid-containing polysaccharide chains via a β-elimination mechanism to generate an unsaturated hexenuronic acid residue and a new non-reducing end of the product (Yip and Withers, 2006). The most

abundant PL classes in *R. solani* AG2-2IIIB are PL1 and PL3 both representing pectate lyases (**I**). Pectate lyases are secreted from bacteria to cause soft rot in their hosts (Wegener, 2002).

Table 4. *Putative fungal genes for Carbohydrate-active enzymes (CAZymes) in the R. solani AG2-2IIIB genome as compared to other fungal species. Total number of genes in the genome (Σ), Glycoside hydrolase (GH), glycosyltransferase (GT), carbohydrate esterase (CE), auxiliary activity (AA), carbohydrate-binding module (CBM), polysaccharide lyase (PL), total number of CAZymes (ΣCAZy). Information gathered from dbCAN 2014-09-01*

Basidiomycota	**Σ**	**GH**	**GT**	**CE**	**AA**	**CBM**	**PL**	**ΣCAZy**
R. solani AG2-2IIIB	14250	399	112	176	171	136	103	1097
R. solani AG1-IB	12713	347	100	141	132	130	92	942
R. solani AG1-IA	10516	183	71	60	53	47	31	445
R. solani AG3	12726	321	94	132	119	109	71	846
R. solani AG8	13952	220	78	93	81	80	48	600
Coprinopsis cinerea	13393	171	84	96	85	86	15	537
Phanerochaete chrysosporium	13602	176	75	62	80	53	7	453
Ustilago maydis	6666	103	63	60	28	10	3	267
Postia placenta	9083	134	32	61	27	22	1	277
Schizophyllum commune	16319	232	85	99	72	43	17	548
Cryptococcus neoformans	6552	90	68	29	15	16	5	223
Serpula lacrymans	12917	179	70	85	0	44	7	385
Laccaria bicolor	23132	163	87	60	38	31	7	386
Puccia graminis	15979	322	204	148	48	32	14	768
Ascomycota	**Σ**	**GH**	**GT**	**CE**	**AA**	**CBM**	**PL**	**ΣCAZy**
Aspergillus nidulans	9520	253	91	105	75	61	20	606
Saccharomyces cerevisiae	4904	206	92	69	56	45	5	473
Neurospora crassa	10785	185	86	66	57	52	4	450
Trichoderma reesei	9115	46	53	13	6	10	0	128
Fusarium oxysporum	26719	495	200	256	157	165	27	1300
Verticillium dahliae	10535	277	101	123	102	92	37	732
Oomycota	**Σ**	**GH**	**GT**	**CE**	**AA**	**CBM**	**PL**	**ΣCAZy**
Pythium ultimum	14096	113	98	37	13	38	17	318

Sugar beet roots have a high pectin content, up to 40–50% of the cell wall dry matter (Guillemin *et al.*, 2005), and this may be a reason for the expanded PL groups. Maize on the other hand, which also is a host of *R. solani* AG2-2IIIB, is

a monocot and has a low content of pectin in the cell walls (Abedon *et al*., 2006). In general primary cell walls of dicotyledonous plants contain 35% pectin while grasses contain 2–10% pectin (Voragen et al, 2009). The expanded set of CAZymes in *R. solani* AG2-2IIIB may be an adjustment to be able to infect different hosts.

3.1.2 Effectors important in host - pathogen interaction

Effector proteins can function as toxins to directly induce plant cell death, but they can also suppress or evade plant defence responses and thereby favour an early pathogen colonization of the host. Many fungal effectors are known to be small, secreted and cysteine-rich proteins. We predicted 126 proteins with a signal peptide at the N-terminal and high cysteine content (Rafiqi *et al*., 2013). After removing proteins with longer sequences than 400 amino acids, 61 predicted effectors remained (**I**). Among these 11 were unique to *R. solani* AG2-2IIIB in comparison with the other AGs and their gene expression levels in a compatible system have been tested with qRT-PCR (**I**). Three of them; a cysteine-rich protein (*RsCRP1*), a rare lipoprotein-A-like protein (*RsRlpA*) and a CHAT domain protein showed a significantly higher expression level at an early time-point after host infection as compared to mycelia from culture (**III, IV**).

Necrosis-inducing proteins were first discovered in *Fusarium oxysporum* where the necrosis and ethylene inducing protein, Nep1, was purified and shown to be capable of triggering plant cell death (Bailey, 1995). Since that time many other Nep1-like proteins (NLPs) have been discovered in a variety of organisms including fungi, oomycetes and bacteria. NLPs are proposed to perform dual functions in the plant−pathogen interactions, acting both as triggers of immune responses and as toxin-like virulence factors known to promote leaf necrosis (Zaparoli *et al*., 2011). *R. solani* is a necrosis inducing pathogen and even though we expected to find NLPs, none were predicted for any of the *R. solani* AGs sequenced so far (**I**).

LysM is another class of conserved fungal effectors that carry no recognizable protein domains other than lysin motifs (LysMs) (Garvey *et al*., 1986; Béliveau *et al*., 1991). LysM effectors occur in both pathogenic and non-pathogenic fungi. Effectors with a LysM domain can mask fungal chitin so that the pathogen can escape detection by the plant and in some cases they also affects appressorial function (Kombrink and Thomma, 2013; Takahara *et al*., 2016). In *R. solani* AG2-2IIIB we have identified one protein containing two LysM domains (RsLysM) (**I**). The *RsLysM* gene was highly induced upon sugar beet infection (**III**).

3.1.3 Three effector proteins with potential roles in host infection

There are protocols describing transformation of *R. solani* (Robinson and Deacon, 2001; Wu and O´Brien, 2009; Liu *et al.*, 2010; Ying-qing *et al.*, 2011) but to my knowledge no one has succeeded to repeat them to set up a stable transformation system for *R. solani* AG2-2IIIB to evaluate gene functionality. Instead we used the leaf spot inducing fungus *Cercospora beticola* and transformed with the three effector-protein coding genes *RsLysM, RsRlpA* and *RsCRP1* sequences, driven by the *PgdpA* constitutively expressed promoter (**III**;**IV**). *C. beticola* overexpressing *RsLysM* (*RsLysM+*) or *RsCRP1* (*RsCRP1+*) showed an increase in necrotic lesion size compared to wildtype (Ty1) when inoculated on sugar beet leaves (**III**;**IV**). This was not seen for the *RsRlpA* overexpressing strain (*RsRlpA+*) (**III**). Fungal biomass in inoculated sugar beet leaves was higher for *RsLysM+* and *RsRlpA+* strains compared to wild type and empty vector (**III**;**IV**). The data also shows that *RsRlpA* suppresses the hypersensitive response in *N. benthamiana* leaves; this may be the explanation for the absence of increased necrotic lesion size in inoculated sugar beet leaves. *RsLysM* was expressed in *Pichia pastoris* and the protein was purified and used in a chitin-binding assay were it bound to all tested forms of chitin (**III**). The LysM in *R. solani* is probably masking chitin in the same way as LysM from ascomycetes. All together the data indicate a role of the three effectors in virulence to sugar beet. In addition RsCRP1 was seen to target both mitochondria and chloroplasts when Agro-infiltrated in *N. benthamiana* leaves (**IV**). To target such diverse plant organelles can be a good strategy for a fungus with a relatively broad host range.

3.1.4 Pathogen effector putatively interacts with a membrane protein in sugar beet plant cells

The predicted effector gene *RsCRP1* was highly induced as early as 4 days after inoculation to sugar beet (**IV**) and is therefore an interesting candidate effector gene. We were interested to know how this protein interacts with the host sugar beet. Pull-down analysis followed by MALDI MS/MS analysis showed a potential interaction between RsCRP1 and a plasma membrane intrinsic protein, PIP1;1 in sugar beet (**IV**). According to the RNAseq analysis, the gene coding for *BvPIP1;1* was differentially expressed between the different genotypes at 5dpi. Further studies to evaluate the importance of this transmembrane protein for the interaction between *R. solani* and sugar beet are in progress.

3.2 Sugar beet response to fungal invasion

Known resistance to *R. solani* in sugar beet is quantitative and the QTL regions are large. Within these regions there are, in addition to the resistance genes, unwanted traits that give rise to an undesirable yield drag. If we can identify genes associated with the disease resistance trait, new markers can be developed and used so that the selection can be made on smaller regions. In this way the unwanted traits can be reduced and the performance of the resistant varieties can be improved.

We studied gene expression differences between partially resistant and susceptible genotypes. In an early response to the pathogen, 217 genes were up-regulated in partially resistant genotypes and gene ontology (GO) analysis showed that 11 of these genes had functions related to biotic stress (**II**). Four of them were peroxidase homologs and three were annotated as NBS-LRR disease resistance genes and these are potentially involved in an early host response to *R. solani*.

In a comparison between partially resistant and susceptible genotypes at different time-points after inoculation 660 genes were significantly differentially expressed (**II**). EuKaryotic Orthologous Group (KOG) analysis of these genes revealed four genes associated to known defence mechanisms and nine genes annotated to the cell wall category. Of the genes differentially expressed at the later time-point, three genes were in the response to biotic stimulus GO group, all three annotated as Major latex protein (MLP)-like protein 43 encoding genes.

Other genes that were differentially expressed in the dataset were genes with AP2/ERF domains, cytochrome P450 genes, xyloglucan endotransglucosylases, WRKY transcription factors, an ethylene response factor, a cysteine-rich receptor-like protein kinase, a COBRA-like protein (cell wall structure), and a pectinesterase inhibitor (**II**). Gene expression for some of these genes was confirmed with qRT-PCR.

We chose to investigate the *MLP* genes and their effect on resistance in more detailed studies.

3.2.1 MLPs might be important for resistance

Major latex protein (MLP) was first identified in the opium poppy latex (Nessler *et al*., 1990; Nessler and Burnett, 1992). Homologs called MLP-like proteins (MLPs) have been found in many other plants (Aggelis *et al*., 1997; Wu *et al*., 2008; Yang *et al*., 2015; Gai *et al*., 2018; Zhang *et al*., 2018). In cotton, a gene called *GhMLP28* is involved in resistance against the pathogen *Verticillium dahliae* (Yang *et al*., 2015) and in mulberry a *MLP* gene is involved in disease tolerance against phytoplasma (Gai *et al*., 2018). Gene expression differences of

the three *BvMLP* genes described earlier were confirmed with qRT-PCR and the partially resistant genotypes showed an increased expression at the later time-point for two of these genes (**II**). In an attempt to show the effect of the *BvMLP* genes, they were transformed into *Arabidopsis thaliana* (**II**). *A. thaliana* is susceptible to *R. solani* AG2-2IIIB, and we could test the effect of the overexpression lines as well as of knock-out mutants by inoculation with the fungus (**II**). Results were ambiguous: only one overexpressed line showed a decrease in fungal colonization. For the knock-out mutants we could not detect a significant effect (**II**). We believe that the *MLP* genes in sugar beet are recessive and we speculate that we could get a clearer result from *Arabidopsis* double mutants.

3.2.2 Genes with similar expression patterns

Weighted gene co-expression network analysis (WGCNA) can be used for investigating how genes jointly affect complex diseases. All sugar beet genes expressed in the RNAseq experiment were divided into modules depending on their expression profiles (**II**). We looked for biotic stress related genes as well as cell wall related genes and found nine modules containing such genes. Only one of those had differentially expressed genes including the same three *MLP* genes as found earlier. Other differentially expressed genes in the same module were a MYB46 transcription factor, a plant disease resistance response protein (DRR206) and a flavonoid *O*-methyltransferase protein. MYB46 is involved in the regulation of secondary wall formation by the biosynthesis of cellulose, hemicellulose and lignin components. DRR206 is involved in *R. solani* resistance in pea and is therefore also an interesting candidate in sugar beet.

3.3 Rhizoctonia root rot phenotyping - a difficult task

R. solani disease symptoms in sugar beet fields are often patchy and it is difficult to perform uniform disease trials. Artificial inoculation is often used to reduce variation in the field experiments and the fungus is then often proliferated on barley kernels or millet seeds (Scholten *et al*., 2001; Bolton *et al*., 2010). Despite this it is difficult to get reproducible results from year to year, mainly due to environmental factors. To evaluate resistance levels in sugar beet varieties, greenhouse trials can be used as a complement to field trials. However it can also be difficult in the greenhouse to get a good correlation between fungal mycelium and disease severity. Since *R. solani* rarely produces any spores it is difficult to inoculate with the same amount of the pathogen each time. Not even a given amount of fungal biomass in the plant does ensure a given level of

infection. To be able to make reproducible inoculation studies on sugar beet seedlings as well as *Arabidopsis* plants we developed an inoculation method based on maize flour and perlite (Dölfors *et al*., additional manuscript). Nevertheless, it is still difficult to correlate fungal biomass in soil as well as biomass in roots and whole plants with disease development and this may interfere with our and others' results. In our experiments we used *Arabidopsis* as a model system for functional studies of sugar beet genes potentially involved in resistance. *Arabidopsis* is a dicot species with a different type of root system compared to sugar beet. The ambiguous results from our functional studies may be influenced by the difference in root structure between *Arabidopsis* and sugar beet. One strategy to improve the reproducibility would be to test hydroponic procedures as has been done in interaction studies of the soil-borne fungi *Verticillium dahliae* and *V. longisporum* (Fradin *et al*., 2011; Roos *et al*., 2015).

3.4 Remaining work

Experimentally several analyses remain to be performed to clarify the importance and function of the effector and defence candidates highlighted in this thesis. The work includes: various analyses on protein levels, microscopy, reactive oxygen species (ROS) detection, a range of validation experiments, optimization of *Arabidopsis* screens and scoring of additional single and double mutants, and last not least, new marker information to be evaluated in breeding materials.

4 Conclusions

Main conclusions from this thesis work are:

- *R. solani* AG2-2IIIB, causing severe root rot disease of sugar beet, has the largest genome compared with other sequenced *R. solani* AGs isolates.

- Pectate lyases are an expanded group of cell wall degrading enzymes in *R. solani* AG2-2IIIB and may be needed for the breakdown of the high amount of pectin in sugar beet roots.

- Three *R. solani* effector candidates have been identified as potentially involved in the infection of sugar beet.

- Three *MLP* genes in sugar beet are potentially involved in the resistance to *R. solani* AG2-2IIIB.

5 Future perspectives

For many years farmers have relied on fungicides to produce highly profitable crops. Today there are strong movements to change such procedures. For example, *C. beticola,* causing leaf spot disease on sugar beets, has been controlled with frequent applications of fungicides belonging to benzimidazoles, triazoles, organotin derivatives and strobilurins (Weiland and Koch, 2004). Such high chemical pressure has led to the development of fungicide resistant fungal strains in many sugar beet growing areas, which is a great threat to the sugar production. Today there is a strong need for new control methods in combination with high yielding and resistant sugar beet hybrids.

The disease pressure incited by *R. solani* is presently increasing in Europe, and in US they are starting to see a loss of function of the applied fungicides (Arabiat and Khan, 2016; Khan per. comm.). Pyraclostrobin is no longer effective and only combinations of different active ingredients give an efficient control. This is serious and leads to an increasing demand for Rhizoctonia resistant varieties with a good performance in USA as well as in Europe. The development of new control strategies are an interesting future perspective where results from this study can give some clues on how these treatments needs to be designed.

A new chemical protection strategy against Rhizoctonia would be welcomed, but most likely any such treatment should be combined with (or replaced by) a strong disease resistance in sugar beet. To be competitive with other profitable crops it is also necessary to combine resistance with high sugar yield. If we can validate that some of the genes identified in this project are indeed responsible for the resistance to Rhizoctonia, then this can contribute to a more efficient development of varieties that combine a good resistance level with high yield.

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Popular science summary

Sugar beets account for about 20% of the world's sugar production. The sugar is produced in the taproot and accumulates throughout the growing season of five to eight months. In the soil where the beets are grown there are many different fungi and other pathogens that could cause disease on the sugar beet roots. *Rhizoctonia solani* is one fungus that is widespread in fields where sugar beets are grown. The fungus causes root rot disease on the beets, which suffer and either die or at best survive with a much lower sugar content. The process by which *R. solani* infects the host is not well understood and in this project we wanted to find out more about this. We therefore sequenced the genome of *R. solani* AG2-2IIIB and looked for genes potentially involved in the infection process. We identified three genes coding for proteins secreted by the fungus and showed in functionally studies that these proteins are involved in causing disease. For the functional studies we used the leaf spot disease causing fungus *Cercospora beticola* and transformed it with the three genes of interest, a lysin motif (*LysM*) effector, a rare lipoprotein-A (*RlpA*) like protein and a cysteine-rich protein (*CRP1*). LysM and CRP1 caused increased lesion size on sugar beet leaves and LysM and RlpA gave higher fungal DNA content in the leaves. These results indicate a role of these genes in the infection process and can be used when new control strategies are being developed against *R. solani*. We also wanted to know more about resistance mechanisms in sugar beet against *R. solani* and we identified genes with different gene expression in partially resistant and susceptible genotypes when infected with the fungus. Especially three Major latex protein (*MLP*) genes were identified and further studied in *Arabidopsis* transgenic plants. We could see, for at least one of these genes overexpressed in *Arabidopsis*, an indication of a lower infection. *MLP* genes can perhaps be used in breeding to select more precisely for new genotypes with resistance to the disease. The interaction between the fungus *R. solani* and the host sugar beet remain complicated, with many unknown factors in this interplay, but the results from this project is a step towards more knowledge.

Populärvetenskaplig sammanfattning

Sockerbetor står för cirka 20 procent av världens sockerproduktion. Sockret produceras i roten och ackumuleras under hela växtsäsongen från vår till sen höst. I jorden där betorna växer finns det många olika svampar och andra patogener som kan orsaka sjukdomar på sockerbetor. *Rhizoctonia solani* är en sådan svamp och den kan förekomma i de flesta odlingsområden men föredrar varmt och fuktigt klimat. Svampen orsakar rotröta på betorna vilket gör att växterna antingen dör eller i bästa fall överlever men med en betydligt lägre sockerhalt vid skörd. Miljöförändringar, så som ett varmare klimat, samt ändrade odlingstekniker och grödor i växtföljden har lett till en ökning av denna svamp i jorden. Man vet inte så mycket om hur svampen infekterar sockerbetor och ett av målen med denna avhandling var att studera gener i svampen som är involverade i infektionsprocessen. Svampgenomet sekvenserades och studerades i detalj och framför allt tre intressanta gener identifierades och de kodar för; en lysin-motiv innehållande effektor (LysM), ett sällsynt lipoprotein-A (RlpA) likt protein och ett cysteinrikt protein (CRP1). För att studera dessa närmare transformerades de in i *Cercospora beticola* och dessa överuttryckta isolat användes för att infektera sockerbetor. Vi kunde då se att LysM och CRP1 orsakade större bladfläckar jämfört med vildtypen och att LysM och RlpA gav högre svamp DNA i bladen. Dessa resultat indikerar att generna är involverade i infektionsprocessen och de kan i framtiden användas när nya kontrollstrategier utvecklas. Ett annat mål med avhandlingen var att studera resistens mekanismer i sockerbeta mot *R. solani*. Genuttryck i sockerbetor med olika resistensnivåer studerades med hjälp av sekvensering av RNA och vi identifierade framför allt ett par ”Major latex protein” (*MLP*) gener av intresse. Dessa studerades i modellväxten *Arabidopsis thaliana* genom att överuttrycka samt slå ut dem. Resultaten var inte entydiga men vi såg en indikation på att en eller flera *MLP* gener kan vara av betydelse för resistensen och förhoppningsvis kan dessa användas i framtiden i sockerbetsförädlingen för att mer exakt kunna välja genotyper med resistens mot sjukdomen.

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