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Evaluation of sugar beet genes involved in *Rhizoctonia solani* resistance

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Utvärdering av sockerbetsgener involverade i *Rhizoctonia solani* resistens

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Popular science summary

Food security and plant protection will always be two interconnected issues that are of concern for farmers and breeders, but also consumers. As the amount of arable land is decreasing in many parts of the world because of changing climate, and with an increasing global population, securing crop yields will become more and more important. As more food has to be produced with fewer available resources, it is going to be important to produce stable yields with as little variation as possible. Variation in yields can be a result of various outside factors where climate factors like temperature, precipitation and sunlight are common factors that cause yield variations in most crop systems. In addition to climate factors crops can be affected by pests and pathogens, which is another common factor that can cause yield variations.

One common pathogen that can affect sugar beet yields is *Rhizoctonia solani*, which is a soil-borne pathogenic fungus that has a wide host range and can cause root damage and yield losses. *R. solani* can cause the diseases root-rot, crown-rot and damping-off, and affect approximately 25% of the cultivated sugar beet area in the United States and approximately 10 % of the cultivated sugar beet area in Europe. As sugar beet is an economically important crop efforts are being made to develop varieties that better can withstand pathogens like *R. solani*. This can reduce yield losses when sugar beets are cultivated in fields that are infested with *R. solani*, and result in more stable yields being produced.

This thesis focuses on evaluating sugar beet genes involved in resistance against *R. solani*, and the work done in this thesis could potentially be used to facilitate the breeding of more tolerant sugar beet varieties. The evaluation of genes was done by verifying results from a previous RNA-sequencing study where resistance genes against *R. solani* were identified, as well as by analysing genetic differences between tolerant and susceptible sugar beet lines. The RNA-sequencing study was verified through RT-qPCR, a method that can be used to study gene expression. The RT-qPCR experiment did, however, not produce the same results as the RNA sequencing study, possibly due to genetic differences between genotypes or variation in different aspects of the experimental setup.

Genetic variation between tolerant and susceptible sugar beet lines in a larger population was also investigated for one gene, and showed a potential pattern for the gene tested between the tolerant and susceptible sugar beet lines. This pattern could potentially be used to develop molecular markers, which would facilitate the breeding of sugar beets with increased *R. solani* resistance. However, as the tested population consisted mainly of tolerant and medium tolerant individuals, further research is needed on susceptible individuals before it is determined whether or not a pattern exists.

Abstract

Sugar beet (*Beta vulgaris ssp. vulgaris*) is one of the most cultivated crops in Sweden and contributes to approximately 14 % of the sugar crops grown in the world, the remaining 86 % being sugar cane (OECD-FAO 2019). As with any commercially produced crop, sugar beets can be exposed to pests and pathogens, which can cause yield losses. One common pathogen in sugar beet production is *Rhizoctonia solani*, which is a soil-borne pathogenic fungus, estimated to affect approximately 25 % of the cultivated sugar beet area in the United States and approximately 10 % of the cultivated sugar beet area in Europe (Harveson et al. 2009). It causes three diseases in sugar beets: crown-rot, root-rot and damping-off, which result in root damage but differ at point of infection. As sugar beet is an economically important crop efforts are being made by breeding companies to develop sugar beet varieties with increased tolerance to pathogens like *R. solani*.

In this thesis, genes that are believed to be involved in *R. solani* resistance are evaluated in sugar beet material provided by MariboHilleshög. The expression patterns of five genes, previously discovered through RNA sequencing, were tested through RT-qPCR in two tolerant and two susceptible sugar beet genotypes to determine if the RNA sequencing results could be replicated. The RT-qPCR showed that the RNA sequencing results could not be fully replicated as only some genes followed the expected expression pattern between tolerant and susceptible genotypes. This was possibly a result of genetic differences between genotypes, or because of uneven *R. solani* infection in the analysed material.

The correlation between allele distribution and *R. solani* tolerance was also examined for one gene in a larger sugar beet population consisting of 95 lines of varying *R. solani* resistance, and showed that there possibly could be a correlation between allele distribution and *R. solani* tolerance for the gene tested. However, as the population consisted mainly of tolerant and medium tolerant individuals, further research is needed on susceptible individuals before it is determined whether or not a pattern exists.

Introduction

MariboHilleshög

Already in the 1870s the sugar production industry in Sweden was well-established but relied mostly on extracting sugar from imported sugar cane (Bosemark 1997). During the 1890s nine sugar beet factories were built in southern Sweden resulting in a ten-fold increase in sugar beet production and an increased demand for sugar beet seed. During this time, most sugar beet seed had been imported from Germany, but, as the German climate differs from that of Sweden, the German sugar beet seeds were not well-adapted to Swedish growth conditions. In the early 1900s, breeding trials were started to create sugar beet seeds better adapted to Swedish climate, and in 1907 the sugar beet breeding company Hilleshög was founded.

In 2017 Hilleshög was acquired by the Danish seed production company DLF and was merged with the Danish sugar beet breeding company Maribo, also owned by DLF, to create MariboHilleshög (MariboHilleshög 2020). DLF is ranked among the top 10 largest seed companies in the world measured by revenue and has roughly 1200 employees in 20 countries with headquarters in Denmark. They specialize in forage and turf seeds, seed and ware potatoes and sugar and fodder beet seeds, and MariboHilleshög in Landskrona is the base of the R&D operation for sugar beet breeding (DLF 2018). MariboHilleshög has more than 300 employees in both Europe and the United States, with approximately 80 employees at the location in Landskrona. In Europe seed production takes place mainly in Italy and France, and in the United States seed production takes place in Oregon, while seed processing and R&D operations take place in Colorado (MariboHilleshög 2020).

History of beet cultivation

Beets have been cultivated for thousands of years, and the earliest written records of beet cultivation can be dated back to ancient Greece (Mabey 1996). The ancestors of the beets we know today originated from the Mediterranean, and during the ancient times beets were cultivated in both the Greek and Roman civilizations but were then grown mainly for their leaves and not for their roots. References to beets can be found in both Greek and Roman literature, and the name *Beta* is believed to be derived from Sicily (Biancardi et al. 2010). During the Middle Ages beets spread from the Mediterranean to Spain and France, and written records issued by Charles the Great in 812 state that beets were specifically registered as a plant that should be cultivated in the gardens of the imperial estates (Francis 2006). By the end of the 1400th century, beets were likely grown all over Europe and in 1538 the first detailed description of different beet varieties were given by Caesalpinus in his book *De plantis*. From Europe beets continued to spread to the rest of the world, and in 1880 sugar beet cultivation was introduced to Japan as the first sugar beet factory was raised in Hokkaido (Winner 1993). At about the same time, sugar beets were also introduced to North America as the first commercial sugar beet factory was constructed in California, USA, in 1879, and only a few years later, sugar beets were introduced to Canada as Canadian sugar beet production started in approximately 1881 (ASGA 2019).

It was not until fairly recently that we began extracting sugar from beets, as beets have been grown for their leaves and for animal feed throughout most of their cultivated history. It was believed that the low sugar content of beets could never result in profitable sugar extraction compared to the amount of sugar that could be extracted from sugar cane, but this changed in 1801 when Franz Carl Achard got approval from the Prussian king Frederick William III to build the first ever beet sugar factory in Cunern (prior to 1945 in Germany, but currently in Poland) (Winner 1993). The work leading up to the construction of the world's first beet sugar factory was started by the German chemist Andreas Sigismund Marggraf, who investigated sugar extraction from beets and to whom Franz Carl Achard was a student. By crystallizing beet juice, Marggraf was in 1747 the first to prove that the white sugar crystals, which made sugar beets sweet, were the same white sugar crystals that were extracted from sugar cane. Marggraf's work was published in the *Prussian Academy of Science* in 1749, and, although he had successfully extracted sugar, a very exclusive commodity, from sugar beets, the public did not pay much attention to his findings and he later gave up his work on trying to extract sugar from beets on a larger scale (Francis 2006).

Marggraf's work was, however, not done in vain as his student Franz Carl Achard saw the potential in being able to extract sugar from common beets and began working on producing beets with higher sugar content (Francis 2006). Achard bred fodder beets, commonly grown for animal feed, and realized that the beets with white skin, white flesh and conical roots contained the most sugar. By breeding these beets with each other, he was able to increase the sugar content from 1.6 % to 4 % of the root's fresh weight, and in 1799 he published his results and asked for support from the Prussian king Frederick William III. The king appointed a committee to investigate Achard's work, and, as the committee was positive, the king approved the construction of the first ever sugar beet factory in Cunern in 1801 (Biancardi et al. 2010).

Figure 1. Sugar production building at the site of the first sugar beet factory in Cunern, photographed in 1912 (Francis 2006).

Sugar beet biology and production

Beets constitute multiple varieties that belong to the species *Beta vulgaris ssp. vulgaris* and are part of the *Amaranthaceae* family (formerly *Chenopodiaceae)* (Biancardi et al. 2010). *Beta vulgaris ssp. vulgaris* includes varieties like red beet, leaf beet and fodder beet, as well as sugar beet. Sugar beet is diploid with a chromosome number of 18 and has a biennial life cycle where it produces a thick root, which is harvested for sugar extraction, the first year and flower and produce seeds the second year (Klotz 2005). A period of vernalisation is required to induce flowering, and sugar beets therefore only transition into their reproductive phase the second year after being exposed to winter temperatures between 4–8°C for approximately 10–14 weeks (Pathak et al. 2011). As colder climate is required for the production of sugar beet seeds, sugar beet cultivation is limited in countries that rely on self-sustained seed production but do not experience colder temperatures during the winter months. Sugar beets are therefore mostly grown in the temperate climates of Europe and North America, while sugar cane make up the majority of the sugar crops grown in the tropical and subtropical climates of Asia and South America (Cooke & Scott 1993).

After establishment the sugar beet seedling starts producing leaves, and after six weeks the plant has between 8–10 leaves in a rosette-formation around the crown (Klotz 2005). The leaves are dark-green and glossy and have an ovate to cordate shape. During early establishment root growth is slow, but as the growing season progresses the root will continue to make up an increasing portion of the plant's total dry weight, see *Figure 2* (Elliott & Weston 1993). The plant will stay in its vegetative phase throughout the first season during which leaf production continues and the root accumulates sucrose. During the second season, after a period of vernalisation and with increasing day length, the plant will transition into its generative phase, during which it produces bolters (inflorescence stalks) which can grow 1.2–1.8m high (Pathak et al. 2011). Indeterminate racemes form at the end of the bolters on which pedicellate (having short floral stalks), green-yellow flowers are produced that occur as single flowers or in clusters of 2–7 (Elliott & Weston 1993). The flowers lack petals and instead consist of a tricarpellate pistil surrounded by five stamens and five sepals supported by a bract. Sugar beets start flowering approximately 5–6 weeks after the generative phase has been initiated and the flowers are mostly cross-pollinated since beets are highly self-sterile (Klotz 2005). The pollen is easily spread with the wind but is sensitive to moisture and can lose its viability within 24 h under dry conditions.

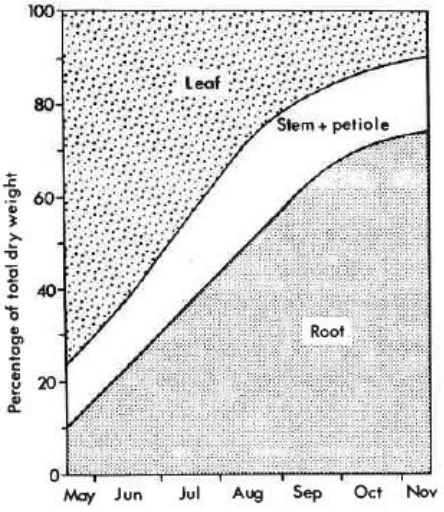

Figure 2. Ratio of root and leaf dry weight throughout the growing season (Elliott & Weston 1993).

The storage organ from which sugar is extracted is made up mostly of the true root but also consists of the swollen hypocotyl, the neck, and the compressed stem, the crown (Elliott & Weston 1993). The crown makes up about 5–15 % of the fresh weight of the harvested root and is the top most part of the beet on which the leaves are attached to the plant (Klotz 2005). The neck is located between the crown and the root and is often the thickest part of the harvested storage organ. As the growing season progresses the root increases both its primary and secondary growth and becomes both longer and thicker. The secondary growth of the root is a result of generation and expansion of cambial cells located between the secondary xylem

and the secondary phloem (Hosford et al. 1984). The cambial cells form rings around the core of the root and give rise to both vascular tissue and sugar-storing parenchymal cells. Throughout the growing season the plant generates between 12–15 cambial rings and the simultaneous growth and proliferation of these cambial rings is what increases the root diameter of the beet (Zamski & Azenkot 1981).

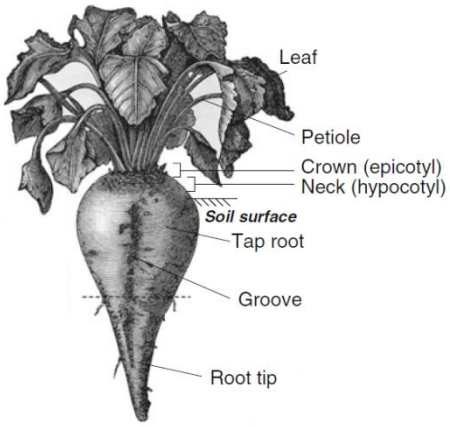

Figure 3. Schematic drawing showing asugar beet plant with the different parts of the plant marked out (Biancardi et al. 2010).

Sugar beet is one of the most commonly grown crops in Sweden and is one of two plants from which white table sugar is produced, the other being sugar cane (*Saccharum officinarum*) (Elliott & Weston 1993). They can be sown in all soil types and prefer a neutral pH but might have reduced growth on very wet soils (Draycott 2006). Sugar beets are most often sown in early spring and are harvested in the autumn after approximately 5-9 months (Biancardi et al. 2010). Seeds are sown in rows 45–55 cm apart and the seeds typically germinate after 3–5 days where factors like temperature and water availability impact the rate of germination (Milford 2006). Optimal germination occurs at temperatures between 22–25 ℃ and is significantly reduced at temperatures above 35 ℃ or below 3 ℃. An adequate supply of water is also important for high germination rates as the seed's water content increases from 10 % in the dry seed to 90 % in the germinating seedling (Biancardi et al. 2010). Before sowing, the seeds are often primed, which induce germination responses in the seeds and result in quicker and more uniform germination. The seeds are also often pelleted, which facilitates sowing by creating a smooth, round seed that is easy to plant with a sowing machine. The pellet also contains pesticides, fungicides and germination promoting compounds, which protect the seedling and improve germination rates (Chomontowski et al. 2019).

When the sugar beet is harvested, the top (crown and leaves) is removed (due to low sugar content and high amount of impurities) and the beet is either transported directly for sugar extraction or left in storage piles (Biancardi et al. 2010). The harvested beets are washed to remove soil and stones and are then cut into thin slices, so called *cossettes* (Dutton & Huijbregts 2006). The sliced cossettes are put into hot water, which extracts the sugar through diffusion, creating a liquid called "raw juice". The raw juice is purified through lime and carbon dioxide treatments to create "thin juice", which then is concentrated through evaporation to make "thick juice". The thick juice is then crystallized under high temperature and vacuum to create white crystallized sugar, which often is purified multiple times before being packaged and sold.

Statistics of sugar beet production

Sugar beets are grown mostly in Europe, Asia and North America and contribute to approximately 14 % of the sugar crops grown in the world; the remaining 86 % being sugar cane (OECD-FAO 2019). In 2018 the majority of sugar beets grown in the world were produced in Europe (67.2 %), which was followed by Asia (15.4 %), North and South America (12 %), and Africa (5.5 %), and in total 275 million tonnes of sugar beets were produced. Over the last 10 years the biggest producers in the world has been Russia (15 %), France (14 %), the United States of America (11 %), Germany (10 %) and Turkey (7 %), and in 2018 these countries produced 57 % of all the sugar beets in the world (FAO 2020). During the same time period, Sweden produced on average 2 million tonnes sugar beets per year, equivalent of approximately 0.5 % of the total world production (SCB 2020). Although Sweden is a fairly small country, sugar beets are one of the most cultivated agricultural crops, and together with cereals like wheat and barley they make up a majority of the crops produced. In 2018 approximately 6 million tonnes of produce was harvested in Sweden (excluding pasture) whereof 1.7 million tonnes (28 %) were sugar beets, 1.6 million tonnes (27 %) were wheat and 1.4 million tonnes (23 %) were barley (SCB 2020).

Sugar beet cultivation has become more efficient over the last 60 years, which is reflected in both the amount of sugar beets produced each year, but also in the amount of land used for sugar beet cultivation. Between 1961 and 1976, sugar beet production saw a steady increase as the total amount of sugar beets produced in the world increased from 160 million tonnes in 1961 to 296 million tonnes in 1976, see *Figure 4* (FAO 2020). The harvested area also increased during this time period as sugar beets were cultivated on 6.9 million ha in 1961 compared to 9.4 million ha in 1976. After 1976 the amount of sugar beets produced worldwide has remained fairly unchanged as approximately 300 million tonnes sugar beets are produced each year, however, the land area used for cultivation of sugar beets has been declining. Since 1976 the harvested area has shown a steady decline and is today lower than in 1961 as 6.9 million ha were harvested in 1961 compared to only 4.8 million ha harvested in 2018. Despite the continued decline of area used for sugar beet cultivation, the amount of produced sugar beets has remains fairly stable indicating that sugar beet cultivation is becoming more and more efficient.

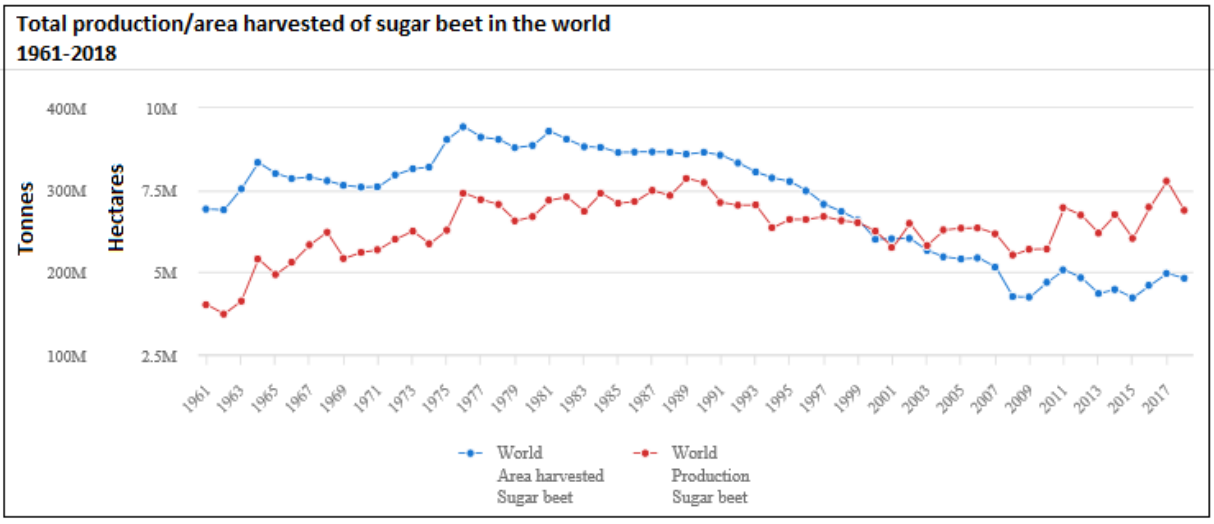

Figure 4. Total world production and total area harvested of sugar beets between 1961–2018. The total world production (tonnes produced) is shown as a red-dotted line, and the total area harvested (hectares) is shown as a blue-dotted line (FAO 2020).

Sugar beet breeding

Beets have been bred for higher sugar content since Franz Carl Achard started working on sugar extraction in beets in the late 1700s (Francis 2006). The most important objective of sugar beet breeding is to increase the sugar yield. Through selective breeding and improved agricultural practices, the sugar content of beet roots have been increased to approximately 18 % of the fresh weight (and 75 % of the dry weight) (Draycott 2006; Biancardi & Skaracis 2005). The sugar yield of a beet is affected by growth conditions and pests and diseases, which have a high impact on the development of the beet. Traits that improve plant vigour or generate resistance are therefore common targets for breeders as these traits may directly or indirectly increase the sugar yield (Biancardi et al. 2010). Breeders may also focus on traits that do not directly increase the sugar yield but in other ways are of economic interest. One example of such a trait is monogerm seed character, which has a significant impact on the economic efficiency of sugar beet production. The discovery of monogerm seeds, made by the Russian sugar beet geneticist V. F. Savitsky in 1948, has been described as one of the greatest achievements in sugar beet breeding, and today the majority of all commercial sugar beet seeds are of monogerm seed character (Bosemark 1993; Biancardi et al. 2010). A monogerm seed only contains one true seed, whereas a multigerm seed consists of 2–5 fused true seeds. When a monogerm seed germinates, it only generates one seedling, while a multigerm seed can generate multiple seedlings. As competition between too tightly spaced seedlings reduce the overall sugar yield, excess seedlings have to be thinned out, which, before monogerm seeds were discovered, required approximately 100 man-hours per hectare (Savitsky 1950). The discovery of the monogerm seed character therefore significantly reduced labour costs and made sugar beet production more efficient.

Most commercial seeds produced today are three-way hybrids, being a cross between three parental lines (Märländer et al. 2011). The three-way hybrid seeds consist of a crossing between a monogerm male-sterile (MS) mother plant, a monogerm maintainer line (O-type) and a multigerm pollinator line, see figure 5. This type of breeding was made possible after F. V. Owen discovered maternally transmitted cytoplasmic male-sterility (CMS) in 1945, which allowed for controlled crossings between lines without the risk of self-pollination (Duvick 1959). Although beets are naturally highly self-incompatible, many lines used in sugar beet breeding today are self-fertile and can therefore easily self-pollinate. As the objective of the breeder is to create superior lines by crossing strong lines with each other, having self-fertile lines is inefficient as self-pollinated seeds cannot be used and have to be removed. Using lines with CMS is therefore a tool commonly used by sugar beet breeders, which eliminates the risk of self-fertilization and promotes out-crossing (Märländer et al. 2011). To maintain the male-sterility in the CMS-line it has to be inherited in the progeny, and this is made possible by crossing the CMS-line with a so called maintainer line, or O-type line (Sneep & Hendriksen 1979). The progeny between the CMS-line and O-type line is then crossed with a multigerm line to create the three-way hybrid commercial seed. Seeds can also be made as two-way hybrids in which the CMS-line is directly crossed with the multigerm line to produce the commercial seed. This is, however, only done if the CMS-line is vigorous on its own, as the high level of inbreeding often results in inefficient seed production when the O-type line is left out. Highly homozygous lines often produce smaller and less vigorous plants, which affect both the amount of seeds produced and the quality of the produced seeds. Heterosis, or hybrid vigour, is the effect in which you can see an improved vigour in a hybrid-line compared to its parents and this is utilized when crossing the CMS-line with the O-type line (Baranwal et al. 2012). By breaking up the genetic homogeneity the progeny between the CMS-line and the O-type line is often more vigorous compared to its parents which results in

more cost-efficient seed production. It is, however, more difficult to produce the desired genetic composition in the commercial seed when three parents are crossed instead of two (as genes in three genomes have to be aligned instead of two), and although the inclusion of the O-type line might generate higher profit margins by increasing cost-efficiency, a two-way hybrid is easier to produce.

To determine the genotype of a seedling and to easily identify allelic variation breeders often utilize genetic markers, which significantly speed up the breeding process, as breeders do not have to depend on phenotyping a fully developed plant (Märländer et al. 2011). A genetic marker is a sequence of DNA at a specific location in a genome that contains slight variations, which can be used to identify and distinguish individuals in a population. Genetic markers have been used since the 1980s and a variety of genetic markers exists with some common examples being RFLP (restriction fragment length polymorphism), AFLP (amplified fragment length polymorphism), SSR (simple sequence repeats) and SNP (single nucleotide polymorphism) (Mammadov et al. 2012). SNPs (often pronounced "*snips*") are a fairly new method for identifying genomic variation, as SNP-markers only have been used since the early 2000s (Khlestkina & Salina 2005). SNPs are the most abundant form of genetic variation and can be used to identify individuals within a population through single base-pair substitution. A SNP is a base-pair at a specific position in a genome that is not universally the same for all individuals within that population. If the base-pair at a specific position for example is a "G" in some individuals and a "C" in others, it can be used to distinguish between the two different groups of individuals. This variation can occur millions of times in a genome and exists within individuals of all species (Mammadov et al. 2012). Breeders utilize this genomic variation when developing SNP-markers, which allows for quick determination of the genotype of an individual.

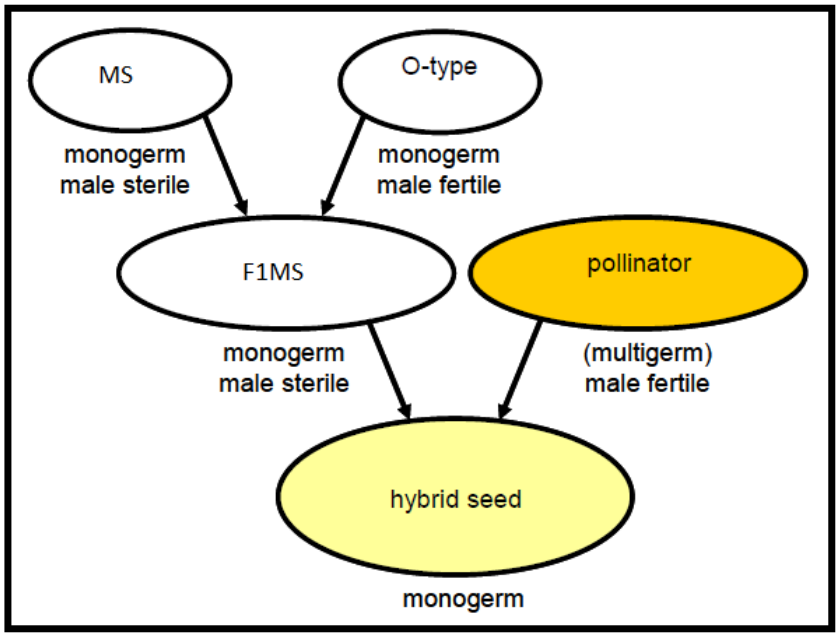

Figure 5. Schematic drawing showing the breeding process in which the three-way commercial hybrid seed is produced. To promote stronger progeny the male-sterile (MS) mother line is crossed with an O-type maintainer line. The progeny (F1MS) is then crossed with a multigerm pollinator to produce the three-way hybrid seed (Märländer et al. 2011, has been edited).

Rhizoctonia solani

The *Rhizoctonia* genus was first described by A. P. De Candolle in 1815 and includes multiple plant pathogens, the most important being *Rhizoctonia solani* (Parmeter & Whitney 1970; Ogoshi 1996). *R. solani* is a fungal pathogen that affects approximately 25 % of the cultivated sugar beet area in the United States and approximately 10 % of the cultivated sugar beet area in Europe (Harveson et al. 2009). It has a wide host range and can infect crops like soybean and maize, as well as various weeds and can cause severe yield losses under high infection pressure. *R. solani* can cause yield losses up to 50 % in sugar beet cultivation and up to 75% in table beet cultivation, and infections are often more severe when the crop rotation is shortened (Panella 2005). *R. solani* is a soil borne pathogen that survives as hyphae, bulbils or sclerotia in the soil or on plant debris and is divided into anastomosis groups (AG) depending on their ability to anastomose (fusion of hyphae) with each other (Asher & Hanson 2006). Thirteen AGs have been identified, and most pathogenic sugar beet isolates belong to AG2-2 and AG4 with some pathogenic isolates being found in AG1, 4 and 5. Isolates belonging to AG4 more commonly infect seedlings, while isolates belonging to AG2-2 more often infect the developed plant. However, since sugar beet seeds often are pelleted with fungicides, isolates from AG4 usually constitute less of a problem compared to isolates from AG2-2 (Kucharska et al. 2017).

Although *R. solani* can propagate sexually, sexual reproduction is uncommon, and the fungus therefore rarely produces spores and instead is spread vegetatively (Asher & Hanson 2006). *R. solani* causes crown-rot and root-rot, which both result in root damage but differ at point of infection. Crown-rot is the result of a *R. solani* infection starting above soil in the crown and is often associated with soil being deposited on the crown during early cultivation, while root-rot instead is initiated below the soil and progresses up the root (Harveson et al. 2009). The infection is initiated when hyphae has come in contact with the root and has started growing on the root surface (Keijer 1996). After 10–12h the hyphae are firmly attached to the root, and after 15h side branches start to form. The side branches typically form at perpendicular angles to each other, creating T-shaped crossings, which is a characteristic trait for *R. solani* hyphae (*Figure 6*). The T-shaped side branches give rise to appressorial structures that form infection pegs, which the fungus uses to penetrate the epidermis and infect the root. The first symptoms of *R. solani* infection are wilting plants and chlorosis followed by brown or black lesions at the base of the petioles (Asher & Hanson 2006). As the infection progresses dark, circular lesions develop on the surface of the root and spread to cover a larger surface area. When the lesions grow bigger, they create cavities in the root and if the root is cut open a clear line can often be seen between infected and healthy tissue, see *Figure 6*. *R. solani* can also cause damping-off in seedlings if the hypocotyl is infected, which causes the seedling to die. A clear line can often be seen between infected and healthy tissue in the infected seedling hypocotyl, and when the infection has encircled the hypocotyl the seedling collapses and dies. *R. solani* show optimal growth at temperatures between 25–33 °C but can cause infections at temperatures between 12–35 °C. It thrives in wet soil and under humid conditions and can occur in all soil types but is more severe in heavy, poorly drained soils (Harveson et al. 2009).

R. solani is most commonly managed through a combination of fungicide application, agricultural practices and cultivation of tolerant varieties (Kucharska et al. 2017). As the pathogen has a wide host range and can survive in the soil and on plant debris, removing crop residue and weeds as well as having a well-planned crop rotation are good preventative measures for reducing *R. solani* infection. In infected fields it is recommended not to grow sugar beets more often than every 3 years and host crops like soybean and maize should be

avoided in the crop rotation (Harveson et al. 2009). At severe infection pressure, fungicides can be used. In the United States, fungicides like azoxystrobin are approved for the control of both crown-rot and root-rot, but in the EU no fungicides are approved for either disease (Panella 2005). Application is also tricky as the timing of the application is critical, and management strategies therefore rely on the cultivation of tolerant varieties (Brent & Hollomon 2007). Although the term resistance might be relevant when discussing sugar beet cultivars that show less severe symptoms when grown in fields infested with *R. solani*, tolerance is a more correct term as no sugar beet lines show complete resistance against *R. solani*. Even though no sugar beet cultivars show complete resistance to *R. solani*, many varieties have a high tolerance, and cultivation of these varieties can reduce yield losses under high infection pressure (Panella 2005). Breeding sugar beet for *R. solani* resistance began in the late 1950s when John Gaskill started a crown- and root-rot breeding program at the U.S. Department of Agriculture (USDA) research station in Fort Collins, Colorado. Breeding for disease resistance have been considered one of the most important improvements in sugar beet production over the last 50 years and continue to be a major target for breeders as improved disease resistance reduce the dependence on fungicides (Biancardi et al. 2010).

Resistance to many pathogens are often not simple traits (traits controlled by only one gene) but are instead controlled by multiple genes that form complex networks (Bosemark 2006). Traits that are controlled by multiple genes are so called quantitative traits, and the position on a chromosome under which the genes coding for a quantitative trait is located is called a quantitative trait locus (QTL) (Collard & Mackill 2008). Genes that belong to a QTL can be located through genome-wide association studies (GWAS), which analyses the correlation between genomic variation and the frequency of specific phenotypes (Mills & Rahal 2019). QTL regions can then be selected for by linking a marker to the QTL region and screening for the related marker. However, for effective screening of a QTL region, the QTL region and the marker need to be tightly linked. A marker is tightly linked if the distance between the marker and the QTL region is short, reducing the risk of recombination (crossing-over between chromosomes) occurring between the marker and the QTL region (Collard et al. 2005). This is important for effective screening of quantitative traits, as a loosely linked marker would not always be inherited together with the genes of interest. A loosely linked marker would therefore not effectively be able to separate individuals containing the QTL region from the rest of the population resulting in inefficient breeding.

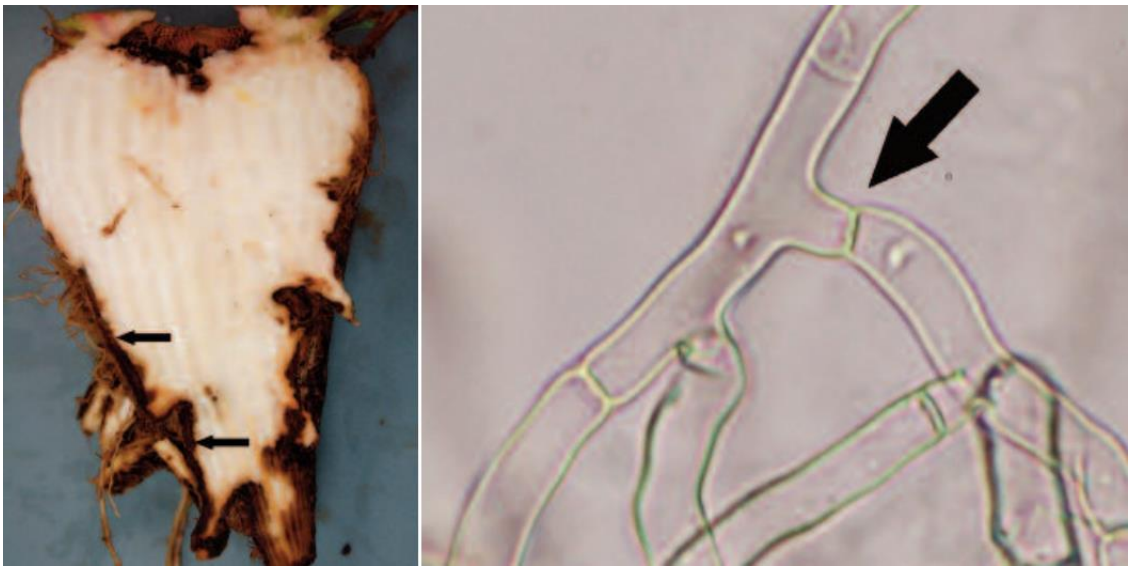

Figure 6. Left: Intersection of sugar beet root infected with R. solani where a clear line can be seen between infected and healthy tissue, marked with arrows. Right: Characteristic T-crossing of a branching R. solani hyphae growing in a perpendicular angle, marked with an arrow (Neher & Gallian 2011, has been edited).

Polymerase chain reaction

Polymerase chain reaction (PCR) was invented by the American biochemist Kary Mullis in 1983 for which he was awarded the Nobel Prize in 1993 (Mullis & Faloona 1987; Mak & Saunders 2006). PCR is a diverse research tool, which today is used in labs all over the world and relies on exponential amplification of DNA. By incubating template DNA with DNA-synthesizing enzymes under varying temperatures, a high level of DNA amplification can be achieved. The template DNA is amplified in cycles and is in theory doubled each PCR cycle resulting in an exponential amplification (Goni et al. 2009). A sample containing 500 copies of template DNA would therefore after 40 cycles contain 5E14 copies, making PCR an efficient method for increasing the DNA concentration in a sample. Increasing the DNA concentration in a sample facilitates further down-stream analyses and makes it possible to visualise the DNA when analysed by gel electrophoresis. If DNA fragments are amplified and should be separated on a gel through gel electrophoresis, a higher DNA concentration in the samples improves the visibility on the gel. DNA amplification has a wide field of application and is commonly utilized in medical laboratories and criminal forensics as well as in research settings (Mak & Saunders 2006).

To amplify template DNA, genomic DNA first has to be extracted from sample tissues, which often is done with the help of DNA extraction kits. To synthesize new copies of the template DNA nucleotide bases (dNTPs), heat resistant polymerase and primers are needed, as well as buffer in which the PCR reactants can be suspended in (Mullis & Faloona 1987). Heat resistant polymerase synthesizes new DNA strands by binding to single-stranded DNA and creating a copy of the opposing strand. PCR primers are short sequences of DNA, which are designed to match a specific location in the template DNA (i.e. the target gene) and are required for the polymerase to bind to the DNA strand (Garibyan & Avashia 2013). To amplify the template DNA in a sample the sample is first heated to a high temperature (around 95 ℃) which causes the DNA to denature, resulting in a separation of the double-stranded DNA into two separate single strands (Mullis & Faloona 1987). The temperature is then lowered (to the temperature that facilitates the primer binding to the DNA), which causes the primers to bind to the separated DNA strands. The temperature is then raised again (to 72 ℃), which activates the polymerase resulting in amplification of the DNA strands, creating a new copy of template DNA for each existing DNA strand. This cycle is repeated up to 40 times and the template DNA is doubled each cycle.

PCR can be used to study gene expression by analysing mRNA instead of DNA, as the synthesis of mRNA is a result of gene expression. Since RNA is more easily degraded than DNA, it cannot be used directly in a PCR and therefore first has to be converted into so called complementary DNA (cDNA) (Soni & Abdin 2017). cDNA is double-stranded DNA without introns and is made by reverse transcribing RNA with reverse transcriptase (RT) enzymes. The cDNA can then be used to run a RT-PCR, specifying that RNA is being amplified instead of DNA, which otherwise function the same way as a normal PCR (Goni et al. 2009). Quantitative PCR (qPCR) is a PCR in which the amplification can be followed in real time by adding a fluorescent dye to the PCR reaction (Soni & Abdin 2017). The fluorescence can be generated with various reactants, but *SYBR Green* is one commonly used qPCR reactant that generate fluorescence by binding to newly formed double-stranded DNA complexes during DNA extension (Ponchel et al. 2003). RT-PCR and qPCR can be combined (RT-qPCR) and can be used to analyse gene expression (Schmittgen & Livak 2008). As the strength of the fluorescent signal is relative to the amount of starting DNA (or cDNA) a sample contains,

RT-qPCR can be used to analyse gene expression by measuring the intensity of the generated fluorescence over time.

When gene expression is analysed through RT-qPCR the fluorescence from the gene of interest is always measured in relation to the fluorescence of a reference gene (Goni et al. 2009). The reference gene can be any gene that is stably expressed in the samples from which the RNA is extracted and can be used to normalize the fluorescence from different samples expressing the same gene of interest. It is, however, important that the expression of the reference gene is even in all tissues and that it is not affected by outside factors such as age, stress or pathogen infection, as that would affect the reference gene's ability to normalize samples. When running an RT-qPCR, a sigmoid curve is generated as DNA is amplified exponentially, resulting in a stronger increase in fluorescence during early cycles and a plateau in the generated fluorescence towards the later cycles, see *Figure 7* (Rutledge 2004). Generally, the fluorescence is not measured at the end of an RT-qPCR run, but is instead measured when a threshold value is reached, the C_t-value (cycle threshold value, sometimes also called *CP*) (Schmittgen & Livak 2008). The C_t-value is the number of cycles required for the generated fluorescence to reach the threshold and is relative to the amount of cDNA in a sample. A sample containing a lot of cDNA (for the gene of interest), as a result of higher gene expression, will reach the fluorescence threshold quicker and will generate a lower C_t-value compared to a sample containing less cDNA for the same gene.

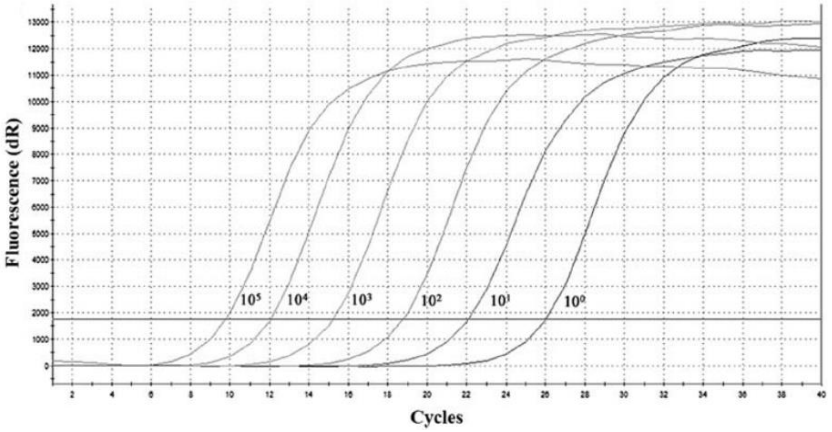

Figure 7. Example of a qPCR amplification curve with six different samples each represented by a different curve. The earlier the fluorescence from a sample reaches the fluorescence threshold (C_t) the more starting DNA that sample contains. The left most curve therefore contain the highest amount of starting DNA and the right most curve contain the lowest amount of starting DNA (Liu et al. 2016).

In general, an RT-qPCR experiment used to analyse gene expression is set up to compare the C_t-values of four different data groups: the treated group for the gene of interest, the treated group for the reference gene, the untreated group for the gene of interest, and the untreated group for the reference gene (Goni et al. 2009). The generated C_t-value for each group can be used to calculate differences in gene expression between treated and untreated samples with the use of the *delta-delta C_t* method. The delta-delta C_t method normalizes the samples containing the gene of interest to the samples containing the reference gene (first delta), and then compares the treated group to the untreated group (second delta) (Schmittgen & Livak 2008). This generates a fold-change value (positive fold-change equals up regulation and negative fold-change equals down regulation), but the delta-delta C_t method has a disadvantage in that it is not taking the primer efficiency into account (Goni et al. 2009). In theory the DNA in a sample is doubled each PCR cycle, but this is only true if the primers used are always able to bind to the DNA. If this is not the case then differences in C_t-values might not be a result of differences in gene expression but might instead be a result of differences in primer efficiency. A more precise method for calculating fold-change is therefore to use the *Pfaffl* method, which takes the primer efficiency of both the gene of interest and the reference gene into account, see *Figure 8* (Pfaffl 2004). Primer efficiency can

be determined by making a dilution series of cDNA and then plotting the C_t-values from the different dilutions against each other. The difference in primer efficiency between the gene of interest and the reference gene should not differ more than 10 % and should therefore be in the range of 90–110 %. Primer efficiency over 100 % can be a sign of pipetting errors, or it can be a sign of amplification of non-specific products like primer-dimers. Primer efficiency over 100 % can also be a sign of the presence of inhibitors as the dilutions that contain higher concentrations of DNA likely also contain a higher concentration of inhibitors, which delay amplification and result in higher Ct-values (Pfaffl 2004). A primer efficiency that is lower than 90 % can be a sign of poor primer design or suboptimal reaction conditions and if the primer efficiency is very low, designing new primers should be considered.

$$\text{ratio} = \frac{(E_{target})^{\Delta CP_{target}\,(control - sample)}}{(E_{ref})^{\Delta CP_{ref}\,(control - sample)}}$$

Figur 8. Pfaffl's modified delta-delta C_t equation which takes the primer efficiency into account, where E_{target} is the efficiency of the target gene primer and E_{ref} is the efficiency of the reference gene primer (Pfaffl 2004).

Purpose of thesis

In 2018 Louise Holmquist, Trait Introgression lead at MariboHilleshög, finished her PhD thesis, *Rhizoctonia solani and sugar beet responses*, which investigated the pathogenic fungus *Rhizoctonia solani* and the genetic differences between tolerant and susceptible sugar beet lines. The PhD project consisted of analyses of root samples taken from tolerant and susceptible lines inoculated with *R. solani* from which RNA was extracted and sequenced. The gene expression profiling was compared between lines and multiple potential resistance genes were identified. The identified resistance genes were expressed at significantly different levels in tolerant and susceptible lines post inoculation and were therefore assumed to, in some way, be involved in the sugar beet's response to *R. solani* infection.

When screening for *R. solani* resistance using molecular markers, MariboHilleshög look at four different quantitative trait loci (QTL). These QTL regions have a positive effect on resistance but have a negative effect on yield. The QTLs are wide, including up to 25 % of the genome, which cause them to also include unwanted genes that have a negative effect on yield. With more specific markers the QTL regions could be narrowed down, which could reduce the amount of yield-reducing genes. This could however at the same time result in the loss of important genes involved in the resistance, creating a sugar beet line with higher sugar yield but reduced *R. solani* resistance.

This master thesis follows up on the work done by Louise Holmquist and continues investigating the potential resistance genes discovered during the PhD project. To develop a better understanding of the sugar beet genes involved in the response to *R. solani*, genes that were discovered in the experiments done by Louise Holmquist were further analysed to confirm that they were in fact differentially expressed in tolerant and susceptible lines. As RNA sequencing is costly this was instead done through RT-qPCR based methods using the same lines as in the PhD project. Secondly the pattern of allelic variation of the discovered resistance genes in tolerant and susceptible lines was investigated. The frequency of alleles for the potential resistance genes was compared in a larger population (with lines of varying tolerance) to determine if there was any correlation between resistance and allelic distribution. If a correlation between allelic distribution and *R. solani* resistance were discovered it could have been used to develop more precise QTL markers for screening of *R. solani* resistance.

In this master thesis we try to answer the following two questions:

- *Can the results from the experiment that identified the potential resistance genes be replicated in new material from the same lines?*

- *Is there a pattern of allelic distribution for the potential resistance genes in tolerant and susceptible lines?*

Material & method

Sowing seeds of genotype 1S, 2S, 3T and 4T

To determine whether the results from Louise Holmquist's PhD project, which identified multiple potential genes involved in resistance through RNA sequencing, could be replicated, an RT-qPCR based experimental setup was arranged. Seeds from the same lines were sown, which included two lines susceptible to *R. solani* infection (genotype 1S and 2S), and two lines tolerant to *R. solani* infection (genotype 3T and 4T), see *Table 1*. The line's *R. solani* tolerance level had previously been determined, and 20 seeds from each line were sown in sterile (steamed) soil in a greenhouse at MariboHilleshög, Landskrona.

Table 1. Resistance to R. solani for the four investigated sugar beet genotypes.

Genotype	Resistance
1S	*Susceptible*
2S	*Susceptible*
3T	*Tolerant*
4T	*Tolerant*

Preparation of inoculum and inoculation

R. solani AG2-2IIIB was grown on agar plates for 1 week and was then cut into 1 cm^2 pieces and transferred to a plastic bag containing 1 kg autoclaved barley kernels that functioned as the substrate for fungal amplification. Four *R. solani* plates were transferred to the bag, which was then sealed and incubated in room temperature for 4 weeks. The barley kernels were then dried in a drying cabinet for 3 days before being used for inoculation.

8-week-old plants from genotype 1S, 2S, 3T, and 4T were inoculated with the barley kernels carrying *R. solani*. Normally during inoculation trials with *R. solani*, barley kernels are ground up and spread out on top of the soil in the pots. However, to ensure that the plants would be exposed to root-rot and not crown-rot, whole barley kernels were instead pushed down into the soil. Each plant was inoculated with 4 barley kernels placed around the root approximately 1 cm from the root and approximately 1 cm deep.

Sampling

After inoculation, root tissues were sampled from each plant at four different time points. Three biological replicates for each genotype and time point were taken at 1 DPI (days post inoculation), 2 DPI, 3 DPI and 5 DPI. Plants were also sampled before inoculation (at DPI 0) to set a base-line for the gene expression of each genotype. Plants were removed from the pot and washed quickly under running tap water to remove soil. The top (crown and leaves) as well as the tip of the root were removed for easier handling, and a small slice (approximately 0.5 cm thick and 2 cm in diameter) from the middle of the root was removed with a scalpel. The root sample was taken at approximately the same depth as the barley kernels were placed, and four technical replicates were taken for each biological replicate. The root samples were put in 1.5 ml eppendorf tubes and were immediately placed in liquid nitrogen to ensure that the mRNA in the tissue was kept intact.

To confirm the viability of the *R. solani* isolate, inoculated barley kernels were put on agar plates. After 2 weeks the hyphae growth was assessed visually, and the structure of the hyphae was determined with an *Olympus BX53* light microscope. To confirm that plants of each genotype could become infected through inoculation with *R. solani* on barley kernels, and to assess the tolerance of each genotype, 5 plants from each genotype were left inoculated

in a greenhouse for 6 weeks. After 6 weeks the plants were scored for disease symptoms on a scale from 1 to 9 where 1 is completely dead, 3 is severely infected, 5 is quite infected, 7 is lightly infected, and 9 is uninfected.

RNA extraction and cDNA synthesis

RNA was extracted from the root following the *Invitrogen RNAqueous RNA isolation kit* protocol. The elution step was done with 2x 40 µl elution buffer (instead of 2x 40 µl + 2x 30 µl elution buffer) and the first elution was run through the filter twice. The concentration of the extracted RNA was determined with an *Ultrospec 2100 pro* spectrophotometer, and all samples were diluted with distilled water to the same concentration (90 ng/µl). The diluted RNA was converted into cDNA following the *Bio-Rad iScript cDNA Synthesis kit* protocol and was then used for RT-qPCR.

Primer design for RT-qPCR

In *Table 2* the genes analysed with RT-qPCR and their corresponding primers and primer efficiencies are listed. For all RT-qPCR runs *ICDH* was used as a reference gene. Primers for each gene (except *ICDH* for which primers already existed) were designed with the web-based primer designing tool *Primer3Plus* (http://www.bioinformatics.nl/cgi-bin/primer3plus/primer3plus.cgi), specifying *qPCR* and with a modified GC interval of 40–60 % (instead of the preset GC interval of 20–80 %). Primers were chosen based on the following criteria: 18–24 base pairs, 40–60 % GC-ratio, maximum 3 G or C in the last 5 base pairs of the 3' end, melting temperature (Tm) 58–62 °C. To determine the possibility of formation of primer-dimers (self-binding of forward and reverse primers) all primer pairs were analysed with ThermoFisher's web-based primer-dimer tool *Multiple Primer Analyzer* (https://www.thermofisher.com/se/en/home/brands/thermo-scientific/molecular-biology/molecular-biology-learning-center/molecular-biology-resource-library/thermo-scientific-web-tools/multiple-primer-analyzer.html). Primer efficiency was determined with a 5-fold dilution series with 5 samples containing pooled cDNA from three biological replicates (i.e. three individual plants of the same genotype) following the method used by (*Pfaffl* 2004).

Table 2. List of the genes analysed with RT-qPCR as well as the primer sequence, primer length, Tm, GC-ratio and primer efficiency, as determined by the Primer3Plus primer designing tool.

Gene	Primer sequence	Length	Tm	GC %	Efficiency
18537	F: TGAAGAAGTGTAAAATGGCGAAC	23 bp	60.5 °C	39.1 %	99.78 %
	R: TCCCCGCTCTCAACAACTT	19 bp	60.8 °C	52.6 %	
1973.1	F: TGGTTTATGTCTTTTGCCTCCT	22 bp	60 °C	40.9 %	109.28 %
	R: TTTGGCAGTCCATGTTCTTTC	21 bp	60.1 °C	42.9 %	
3674.1	F: CTGGGAACCAGCTGACTGAG	20 bp	61 °C	60 %	112.12 %
	R: GGCTTTGTAACAGCCCGTAA	20 bp	60.1 °C	50 %	
18538.1	F: GCTTCTCAATCCTCATTTGGAG	22 bp	60.2 °C	45.5 %	91.39 %
	R: TCAGCTTCCCTTTCCAAAAA	20 bp	59.8 °C	40 %	
036930	F: GGTCGGTCTTTTTGCTATGC	20 bp	59.7 °C	50 %	102.47 %
	R: CTCCCGGAAACGTAAAAACC	20 bp	60.7 °C	50 %	
ICDH	F: CACACCAGATGAAGGCCGT	19 bp	60 °C	57.9 %	98.92 %
	R: CCCTGAAGACCGTGCCAT	18 bp	60 °C	61.1 %	

RT-qPCR

The cDNA from each of the biological replicates for each time point and genotype was pooled for each RT-qPCR run (except one control run for gene 036930, which was run with both pooled and unpooled cDNA). Although statistical variance between biological replicates is lost by pooling the cDNA, the pooled cDNA promotes a higher level of through-put and

reduces RT-qPCR costs by increasing the number of genotypes that can be represented per RT-qPCR plate. As the expected results for each gene tested was a relatively large difference in expression between the tolerant and susceptible genotypes, it was considered sufficient to analyse expression patterns in pooled cDNA, even though statistical analysis of biological replicates would not be possible. To pool cDNA from biological replicates prior to RT-qPCR would, as previously described, allow for a higher throughput analysis and was therefore used and evaluated in this study. The RT-qPCR was run on a *TaqMan 7500 Real Time PCR System* using *Applied Biosystems Power SYBR Green PCR MasterMix* and with three technical replicates per sample. The samples were run for 10 s at 95 ℃ followed by 1 min at 60 ℃, for 40 cycles. No-template controls (NTC) were included on each RT-qPCR plate to ensure that there were no contaminations and that there were no formation of primer-dimers. To ensure that only the target gene was amplified melt curves were compared between all samples and technical replicates. The genes were tested at the time point in which the RNA sequencing data previously showed them to be most differentially expressed. The generated C_t-values from the RT-qPCR runs were used to calculate the fold-change for each sample using *Pfaffl's* equation see *Figure 8*.

Sowing and sampling of test population

To determine the allelic frequency of the potential resistance genes in tolerant and susceptible lines 95 different sugar beet lines, with varying *R. solani* resistance, were sown in sterile (steamed) soil in a greenhouse at MariboHilleshög, Landskrona. A distribution of mostly susceptible and some resistant lines were originally chosen to create a testing population, but, due to unforeseen identification problems, mostly resistant lines were chosen and only very few susceptible lines. Leaf samples were taken 6 weeks after sowing with a leaf-punch. Samples were taken from 10 leaves per line and were sent to DLF's molecular lab in Stora Heddinge, Denmark for DNA extraction.

Primer design for analysis of allelic distribution

Due to time constraints, only gene 036930 was analysed for allelic variation within the testing population and was therefore the only gene to which primers were designed. Two known gene sequences (*RES*, from a resistant line, and *SUS*, from a susceptible line), both containing SNPs, each had SNP-specific primers designed for gene 036930. To distinguish between homozygous and heterozygous lines three primers were designed to amplify fragments of different length for each corresponding allele. One forward primer was designed to fit either allele, and two reverse primers were designed to fit a SNP on each of the two alleles at different lengths from the forward primer, see *Table 3*. The primers would therefore amplify products with different lengths depending on the presence of either allele in a sample. The presence of *RES* would produce a 269 base pair amplicon, and the presence of *SUS* would produce a 151 base pair amplicon. Primers were designed with the web-based SNP-specific primer designing tool *BatchPrimer3* (http://batchprimer3.bioinformatics.ucdavis.edu/cgi-bin/batchprimer3/batchprimer3.cgi; You et al. 2008), specifying *allele-specific primers* and with a modified GC interval of 40–60 % (instead of the preset GC % interval of 20–80 %). SNPs were identified by aligning both sequences with the NCBI web-based *BLAST global alignment* tool (https://blast.ncbi.nlm.nih.gov/Blast.cgi?PAGE_TYPE=BlastSearch&PROG_DEF=blastn&BLAST_PROG_DEF=blastn&BLAST_SPEC=GlobalAln&LINK_LOC=BlastHomeLink).

Table 3. Primers used to distinguish between the two alleles (from genome RES and SUS) for gene 036930.

Name	Sequence	Length	Tm	GC %	Aplicon length
F: 036930_F	CCCGACCCTGATCATAGAGA	20 bp	60 ℃	55 %	
R: 036930_R_RES	TCTCATTTGAGACCCCATAATTG	23 bp	60.2 ℃	39.1 %	269 bp
R: 036930_R_SUS	CAACCTTGCAGGAGAGGAATA	21 bp	59.3 ℃	47.6 %	151 bp

PCR and gel electrophoresis

Each DNA sample was run with all three allele-specific primers on a *GeneAmp PCR System 9700* following the *Takyon Low ROX Probe 2x MasterMix* protocol. The samples were run for 10 s at 95 ℃ followed by 1min at 60 ℃, for 40 cycles. The amplified PCR product was separated on a 1.5 % agarose gel for 1.5 h at 77 Volt using a *GeneRuler 50 bp DNA Ladder* as reference. The gel was photographed under UV-light, and the allelic frequency of *RES* and *SUS* in the testing population was determined with a *Fisher's exact test* by dividing the lines into 4 phenotypic groups depending on their tolerance score (which had been determined in previous studies), where 6 is medium tolerant, 7 is somewhat tolerant, 8 is tolerant and 9 is very tolerant.

The *Fisher's exact test* was done in the software *R*.

Results

The RT-qPCR was partly successful in replicating the results from the RNA sequencing for the genes tested. Fold-change values were irregular for some genes but expression patterns could be distinguished between tolerant and susceptible genotypes for other genes. NTC samples showed no indication of formation of primer-dimers for the gene-specific primers, but showed a slight indication of primer-dimer formation for the primers of the reference gene (*ICDH*). The C_t-values for the NTC samples with the reference gene's primers were very high (between C_t 36–40) and were therefore disregarded.

Melt curves of PCR products in technical replicates showed single peaks for every gene tested, indicating that only a single product had been amplified. Comparison of melt curves of PCR products between samples also showed single peaks for every gene tested, indicating that the same product had been amplified in samples from all genotypes. An exception to this was, however, found in the melt curve for gene 036930, as two close but distinguishable peaks could be seen between genotypes, see *Figure 9*. Within technical replicates samples showed single peaks, indicating amplification of a single product within each genotype, but between genotype 3T and 4T and genotype 1S and 2S two peaks could be distinguished. This indicates that genotype 3T and 4T amplified a fragment of slightly different length (or with different G/C content) compared to genotype 1S and 2S, when run with the same primers for gene 036930.

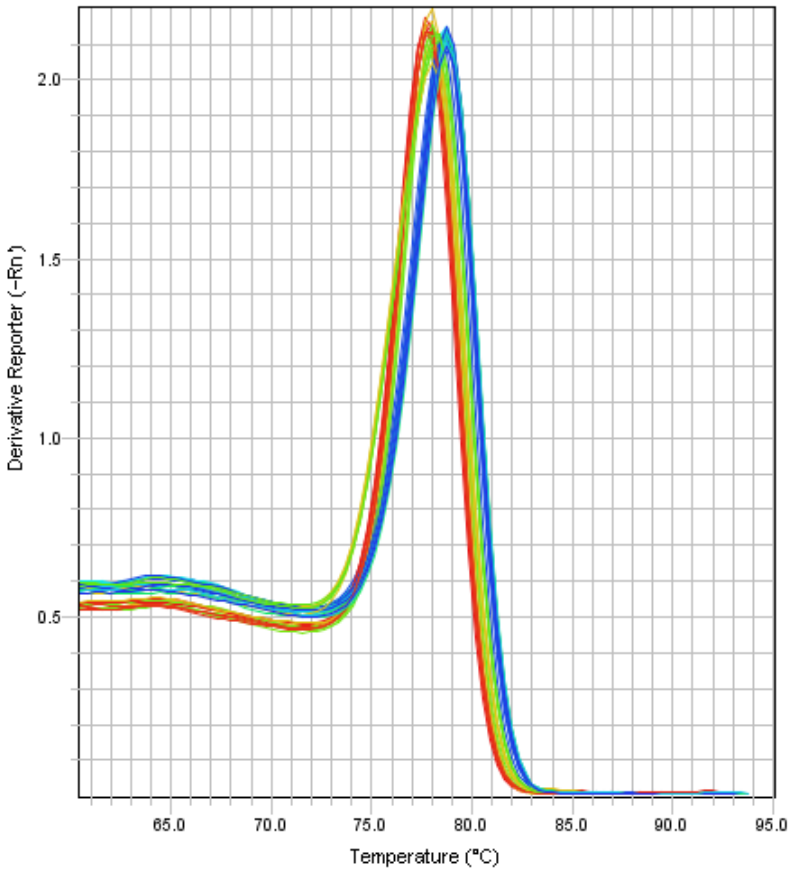

Figure 9. Melt curves for gene 036930 at DPI5 run with unpooled cDNA. The left peak (red, yellow, green) show the melt curve for samples from genotype 1S and 2S, and the right peak (cyan, light blue, blue) show the melt curve for samples from genotype 3T and 4T. Within each genotype the melt curves show a single peak, indicating that a single fragment has been amplified, but between genotypes 1S and 2S and genotypes 3T and 4T two peaks can be distinguished, indicating that the genotypes amplify fragments of slightly different lengths.

Gene-individual RT-qPCR results

The expression level of gene 18537 at DPI5 was down regulated in all genotypes, when compared to the uninoculated material (DPI0), and the expression was fairly similar between the two tolerant genotypes (3T and 4T) and the two susceptible genotypes (1S and 2S), see *Figure 10*. An indication of a pattern can be seen between tolerant and susceptible lines as there is a noticeable difference in expression between genotype 3T and 4T compared to genotype 1S and 2S. The difference between tolerant and susceptible genotypes is smaller for genotype 1S compared to genotype 2S, as genotype 2S show almost no change in expression level.

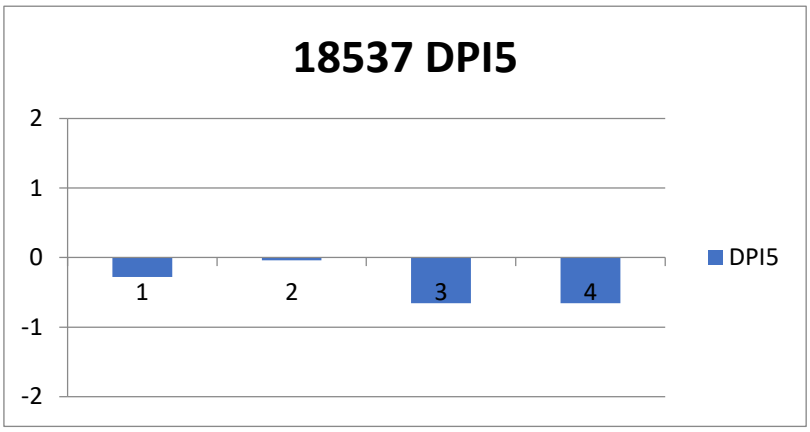

Figure 10. Fold-change values for gene 18537 at DPI5.

Gene 1973.1 at DPI5 was up regulated in all genotypes, and the expression was highest in genotype 3T and lowest in genotype 4T, see *Figure 11*. Although genotype 3T showed the highest level of expression, the expression of genotype 1S and 2S was higher than the expression of genotype 4T. No clear pattern can therefore be seen between tolerant and susceptible genotypes for gene 1973.1 at DPI5.

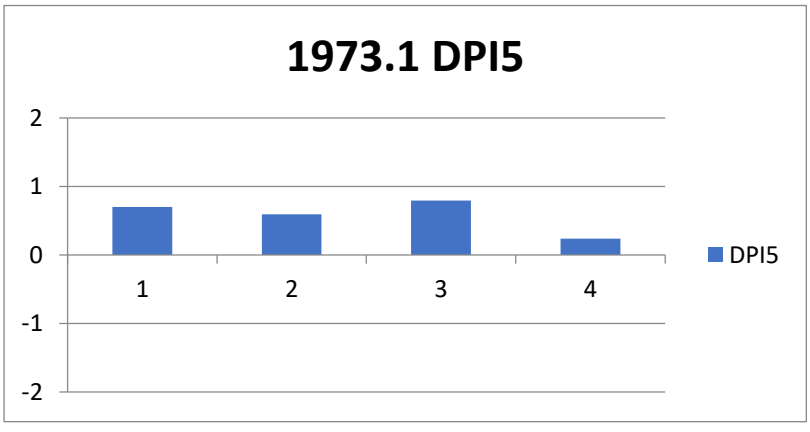

Figure 11. Fold-change values for gene 1973.1 at DPI5.

Gene 3674.1 was down regulated in genotype 3T and 4T at DPI5, see *Figure 12*. Neither genotype 1S nor genotype 2S showed any amplification in any sample or replicate. The level of expression is similar in genotype 3T and 4T, and because the gene is not expressed in genotype 1S and 2S, a pattern of expression can be seen between the tolerant and susceptible genotypes.

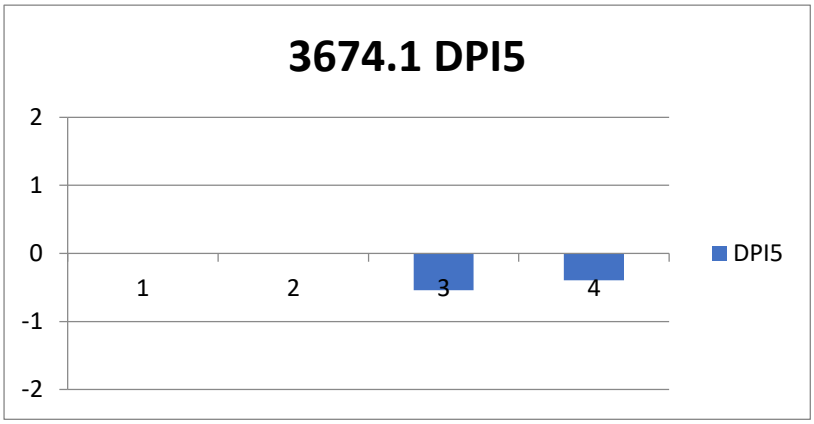

Figure 12. Fold-change values for gene 3674.1 at DPI5.

Gene 18538.1 at DPI5 was down regulated in all genotypes and showed the highest down regulation in genotype 3T and 4T and the lowest down regulation in genotype 1S and 2S. Although a pattern can be seen in the level of down regulation between tolerant and susceptible genotypes, genotype 2S and 3T showed similar levels of down regulation, making the pattern less obvious.

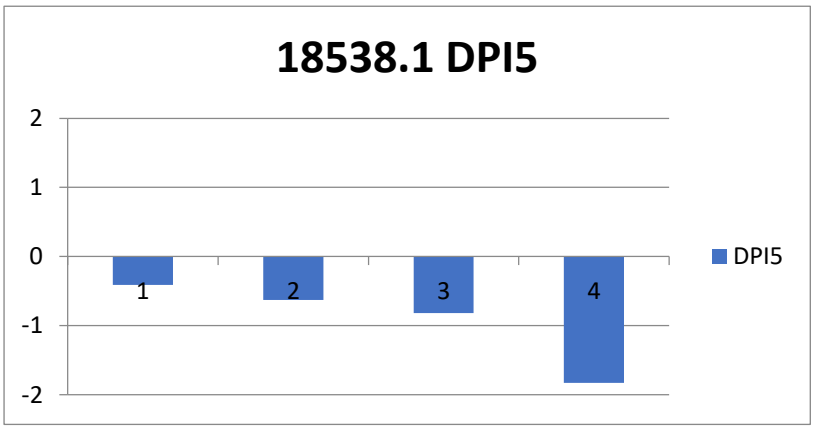

Figure 13. Fold-change values for gene 18538.1 at DPI5.

Gene 036930 was tested at DPI2 and DPI5 to determine how the expression in each genotype change over time, see *Figure 14*. The results were mostly consistent for each genotype at both DPI2 and DPI5, as genotype 1S showed down regulation at both time points, genotype 2S showed almost no change at both time points, genotype 3T showed slight up regulation at both time points, and genotype 4T showed down regulation at both time points. Genotype 1S showed approximately the same level of expression at both DPI2 and DPI5, while genotype 3T and 4T showed weaker expression at DPI5 compared to DPI2. No clear pattern can be seen between tolerant and susceptible genotypes, as both genotypes 1S and 4T showed down regulation at both DPI2 and DPI5, while genotype 3T showed up regulation at the same time points, and genotype 2S showed almost no change at either time point.

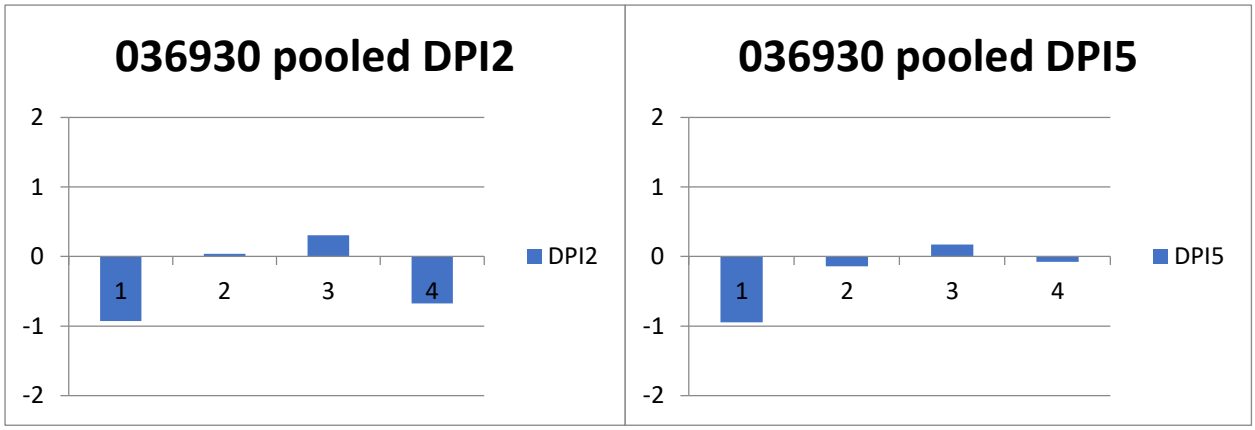

Figure 14. Fold-change values for gene 036930 at DPI2 (left) and DPI5 (right).

Since the RT-qPCR results for some genes did not align with the expected results, and to confirm that the pooling of cDNA was not the cause of the unexpected results, gene 036930 was run without pooled cDNA, as a control, to determine if the fold-change outcome would be affected by not pooling the cDNA. Not pooling the cDNA also allowed for statistical comparison of expression levels between genotypes.

Three biological replicates (A, B, and C) for each genotype at DPI5 were run in separate wells, and fold-change values are presented in *Figure 15*. The expression level between each biological replicate was very different for all genotypes, indicating high variability. Genotype 1S showed down regulation in all three replicates with a noticeable difference in down regulation between the highest (A) and the lowest (B) down regulated replicate. Genotype 2S instead showed a slight up regulation in replicate A, a slight down regulation in replicate B, and a stronger up regulation in replicate C. Genotype 3T showed up regulation in replicate A, down regulation in replicate B, and a very slight up regulation in replicate C. Genotype 4T, as with genotype 1S, showed down regulation in all three replicates but with a noticeable difference in down regulation between the highest (B) and the lowest down regulated replicate (A).

As with the results from the pooled cDNA for gene 036930, no clear pattern can be seen between tolerant and susceptible genotypes, as biological replicates are not showing consistent expression levels. To determine statistically whether there is any significant difference in expression between genotypes, the fold-change value for each replicate and genotype was used to run a *one-way ANOVA-test* (in Minitab). The *ANOVA-test* showed a p-value smaller than 0.001 indicating that there is a significant difference in the expression between the genotypes for gene 036930. Groupings between genotypes were determined with *Tukey's Pairwise Comparison* and are shown in *Table 4*. In *Table 4* we can see that there is no statistical correlation in the expression between tolerant and susceptible genotypes for gene 036930, as neither genotype 3T and 4T nor genotype 1S and 2S share the same groupings. This indicates that the expression of gene 036930 is highly variable between tolerant and susceptible genotypes and also varies between biological replicates for each genotype.

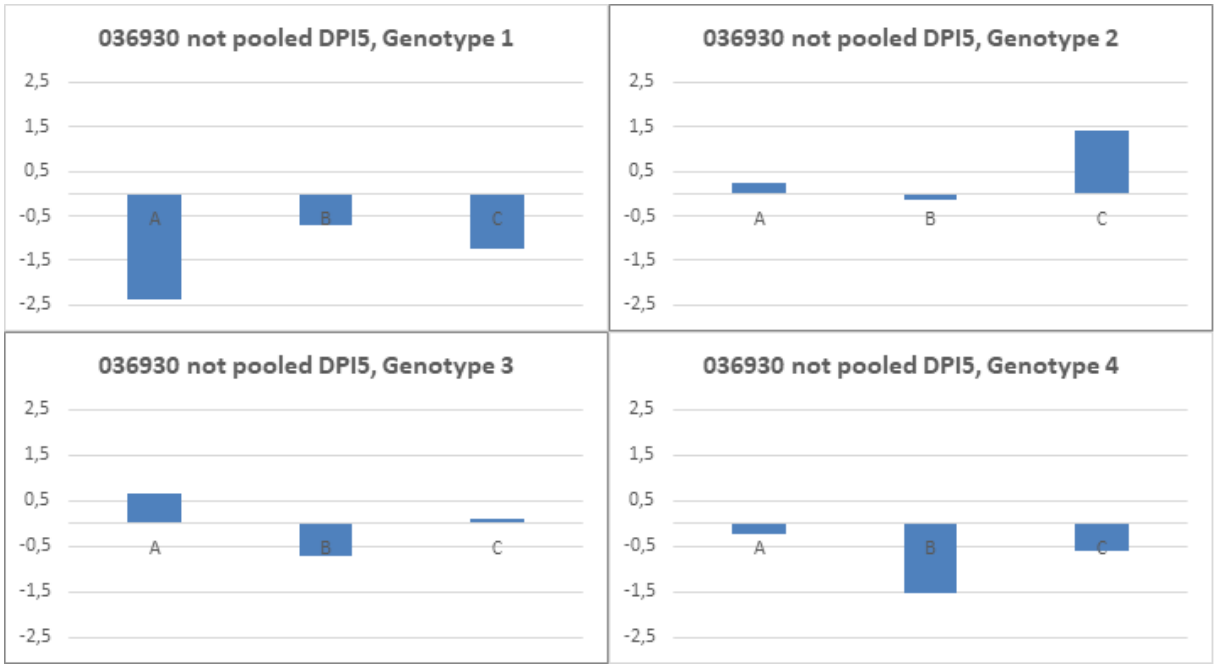

Figure 15. Fold-change values for each biological replicate for gene 036930 at DPI5.

Table 4. Groupings between genotypes for gene 036930 at DPI5, based on Tukey's Pairwise Comparison.

Genotype	N	Mean	Grouping		
2	18	1,573	A		
3	18	1,0892		B	
4	16	0,6350			C
1	18	0,4074			C

Scoring results

The scoring showed that almost all control plants in some way had been affected by *R. solani*, but also that the response between genotypes was very different. The tolerant genotypes, as expected, showed a higher overall score and had quite similar scores, as genotype 3T showed an average score of 6.6 and genotype 4T showed an average score of 8.0, see *Table 5*. The susceptible genotypes showed a lower overall score but varied more between genotype 1S and 2S, as genotype 1S showed an average score of 6.25 and genotype 2S showed an average score of 1.6, see *Table 5*. The lower score of genotype 2S was in line with the expected outcome but the fairly high score of genotype 1S was not expected. Although the average score for genotype 1S was fairly high, the lowest scored plant from genotype 1S showed a score of 3. This can be compared to the lowest scored plant from genotype 2S, 3T and 4T, which showed a score of 1, 5 and 5 respectively, see *Figure 16* and *Figure 17*. This might give a better indication of the potential degree of infection each genotype can experience, as the lowest scored plant from genotype 1S and 2S were scored lower than the lowest scored plant from genotype 3T and 4T. This also indicates that plants inoculated with barley kernels carrying *R. solani* are not uniformly infected and that variance exists between biological replicates within genotypes.

Table 5. Score for each inoculated control plant as well as the average score for each genotype.

Genotype	1S	2S	3T	4T
Score #1	7	3	9	5
Score #2	3	1	7	9
Score #3	6	1	5	9
Score #4	9	1	5	9
Score #5		2	7	
Avg. Score	**6,25**	**1,6**	**6,6**	**8,0**

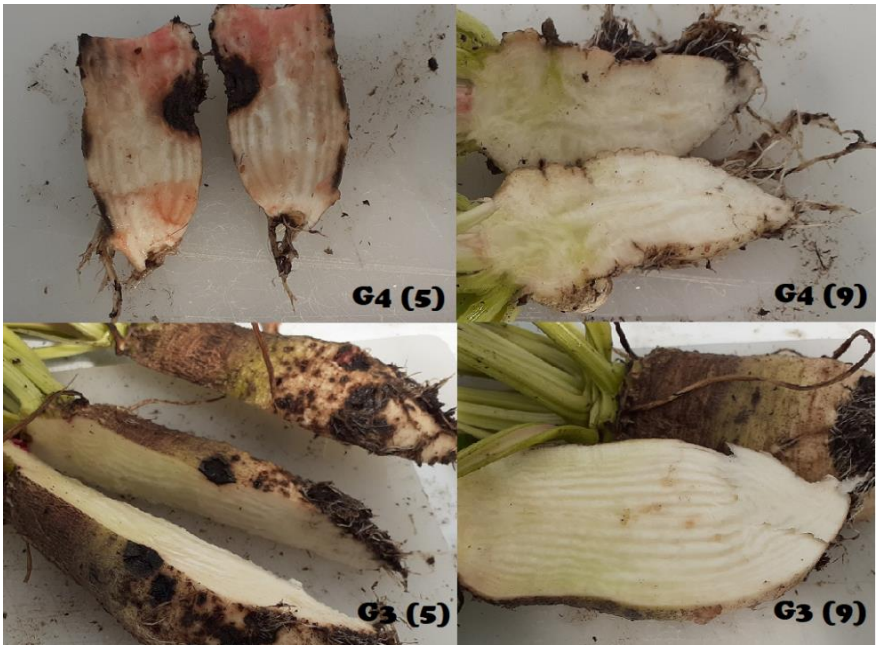

Figure 16. Highest and lowest scored plants for the tolerant genotypes, with genotype 4T above, genotype 3T below, lowest scored plants to the left, and highest scored plants to the right.

Figure 17. Highest and lowest scored plants for the susceptible genotypes, with genotype 2S above, genotype 1S below, lowest scored plants to the left, and highest scored plants to the right.

Allele frequency results

The frequency of allele *RES* and allele *SUS* was determined for gene 036930 in the test population. The electrophoresis gels showed a clear separation of two PCR products at the expected fragment lengths when photographed under UV-light, indicating that the allele-specific primers had successfully been able to distinguish between the *RES* and *SUS* alleles (*Figure 18*). The shorter *"B"* fragment (151 base pairs) is representative of the *SUS* allele and the longer *"A"* fragment (269 base pairs) is representative of the *RES* allele.

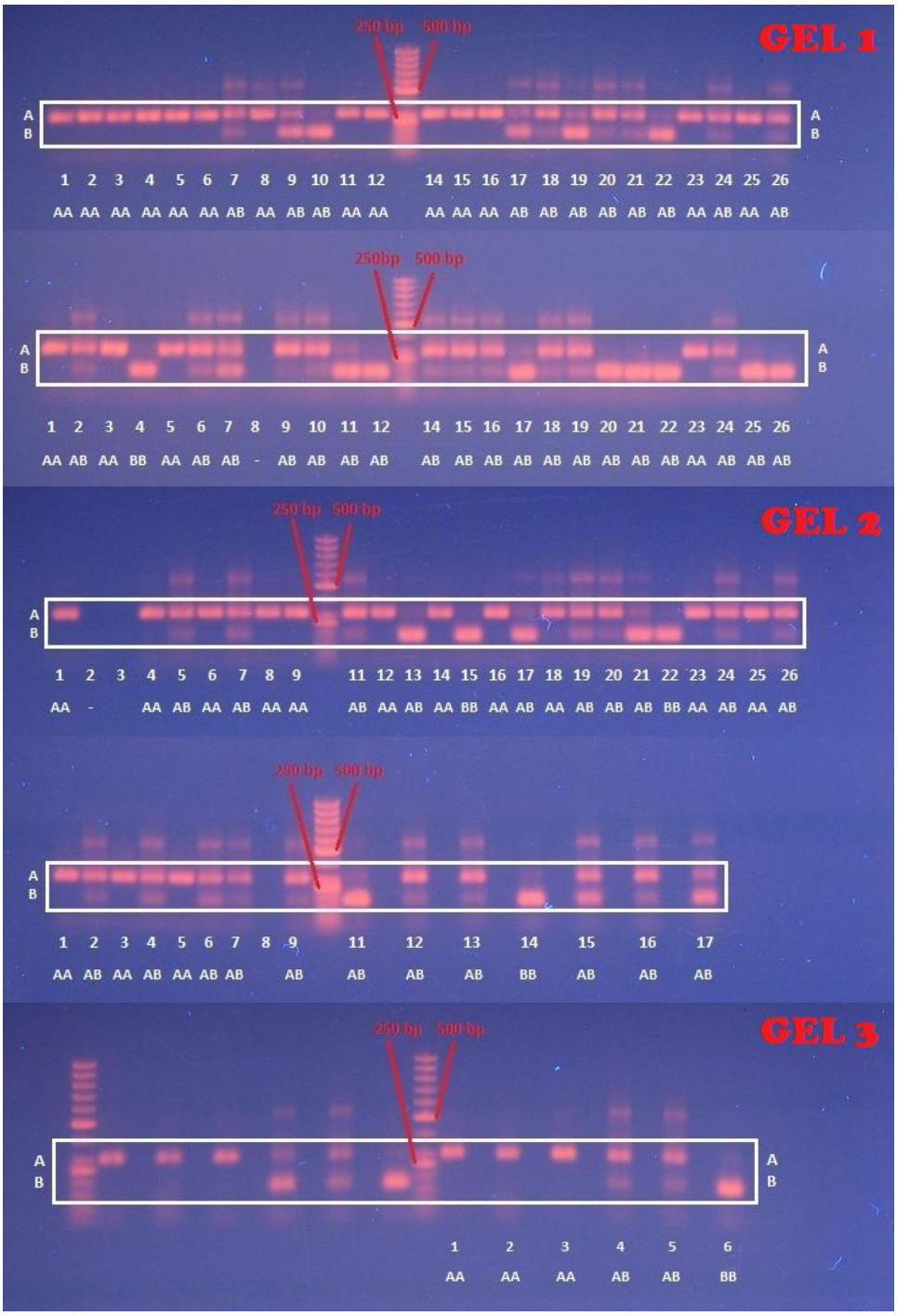

Figure 18. Electrophoresis gels showing a clear separation of two fragments of the expected sizes. Fragment A (RES) approximately 269 base pairs long and fragment B (SUS) approximately 151 base pairs long. Wells without a sample ID are empty, and wells which contained samples but didn't show any amplification are marked with a line.

We can see in the gel pictures that some individuals in the population are homozygous for allele *RES* (i.e. only contain the A fragment), some are homozygous for allele *SUS* (only contain the B fragment), and some are heterozygous (containing both alleles). The gel-data is compiled in *Table 6* and is categorized based on the phenotypic groupings of the individuals in the testing population. Although the number of individuals observed is larger in phenotype group 6 and 7, a higher frequency of heterozygosity can be seen in these phenotype groups compared to phenotype group 8 and 9.

Table 6. Number of individuals in each phenotype group containing the different allele configurations as determined from the gel electrophoresis analysis of PCR products from 95 different sugar beet lines with varying R. solani tolerance.

Phenotype Group		Homozygous for *RES*	Homozygous for *SUS*	Heterozygous
(medium tolerant)	6	5	2	15
	7	13	3	31
	8	11	2	4
(highly tolerant)	9	4	0	0

The data in *Table 6* were used to run a *Fisher's exact test*, which showed that two statistically significant groupings exist within the testing population when the individuals that are homozygous for *RES* are compared to the individuals that are heterozygous (first column in *Table 7*). Each phenotype group was compared to each other and the generated p-value for each pairing is compiled in *Table 7*. We can in *Table 7* see that when the less tolerant phenotype groups (phenotype group 6 and 7) are compared to each other (first row, first column) they generate a p-value higher than the significance level of 0.05, indicating that they are not statistically different from each other. The tolerant phenotype groups (phenotype group 8 and 9) also generate a non-significant p-value when compared to each other (last row, first column), indicating that they also are not statistically different from each other. However, when the less tolerant phenotype groups (phenotype group 6 and 7) are compared to the tolerant phenotype groups (phenotype group 8 and 9), significant p-values are generated for each pairing (row 2–5, first column), indicating that phenotype group 6 and 7 are statistically different from phenotype group 8 and 9.

We can also see that when the individuals that are homozygous for *RES* are compared to the individuals that are homozygous for *SUS* (middle column), and when the individuals that are homozygous for *SUS* are compared to the individuals that are heterozygous (last column), no statistically different p-values are generated. This indicates that what separates the four different phenotype groups from each other is the number of individuals that are homozygous for *RES* (first column *Table 6*) and the number of individuals that are heterozygous (last column *Table 6*).

Table 7. P-values generated through Fisher's exact test when comparing each phenotype group and allele pairing. Values marked in cyan are statistically significant at a 95 % confidence level.

Phenotype group pairing	Homozygous *RES* + Heterozygous	Homozygous *RES* + Homozygous *SUS*	Homozygous *SUS* + Heterozygous
6 & 7	0,773	0,6214	1
6 & 8	*0,00684*	0,5868	0,2705
6 & 9	*0,01186*	0,4909	1
7 & 8	*0,00523*	1	0,1542
7 & 9	*0,01223*	1	1
8 & 9	0,5304	1	1

Discussion

Based on the expression levels of the genes tested through RT-qPCR, the results from the RNA sequencing could not be fully replicated. The genes identified through RNA sequencing were differentially expressed in tolerant and susceptible genotypes at the time points tested and were therefore expected to show similar differences in expression level when tested through RT-qPCR. Only some of the genes tested showed an indication of an expression pattern between tolerant and susceptible genotypes, while other genes showed no consistent pattern at all. Gene 18537 (*Figure* 10) was one of the genes that showed an indication of an expression pattern between tolerant and susceptible lines, as it was more down regulated in genotype 3T and 4T compared to genotype 1S and 2S. The gene being down regulated in all genotypes and being more down regulated in the tolerant genotypes also indicates that the gene might not be involved in direct resistance against *R. solani* but might instead be involved in regulating susceptibility. This is because a resistance gene could be expected to be more up regulated in tolerant genotypes compared to susceptible genotypes, while a similar tolerance effect likely could be seen between tolerant and susceptible genotypes by down regulating susceptibility genes in tolerant genotypes. Gene 3674.1 (*Figure* 12) was another gene that showed indications of an expression pattern, based on the assumption that the gene is not expressed (or does not exist) in genotypes 1S and 2S, as no amplification was seen in any sample or replicate from the susceptible genotypes. The gene not existing does, however, seem unlikely as most genes within a population is shared between a majority of the individuals, and another explanation could therefore be that the primers accidentally were designed over a SNP, resulting in them not being able to bind to the sequence in genotypes 1S and 2S. This then instead has to be based on the assumption that two different sequences for the gene exist and that the tolerant and susceptible genotypes do not share the same sequence. This would explain why there was no amplification in any sample or replicate for either susceptible genotype, but would also make the expression pattern for the gene less clear. Another explanation could potentially be that the observed difference in expression level of the gene 3674.1 could be due to that genotype differences exist in the upstream regulatory region of this gene (i.e. in promoter regions where transcription factors bind) which can have a strong effect of the regulation of gene expression. A third gene that showed indications of an expression pattern was gene 18538.1 (*Figure 13*), which showed down regulation in all genotypes but a stronger down regulation in the tolerant genotypes compared to the susceptible genotypes. Although a large difference in expression level can be seen between tolerant and susceptible genotypes when looking at the highest and lowest down regulated genotype, the difference in expression level between the two remaining genotypes were much smaller, making the indicated expression pattern less clear. The down regulation also indicates that, as with gene 18537, gene 18538.1 might be involved in regulating susceptibility instead of direct resistance.

The other genes tested showed an irregular expression pattern, and, as was seen for gene 036930 when run with not pooled cDNA, there is a large variance in the level of gene expression between biological replicates within the same genotype. Having a larger amount of samples or repeating the experiment could possibly have reduced this variance and generated more apparent expression patterns, but the irregular expression patterns could also be a result of variance in gene expression between individuals within the same genotype (Juenger et al. 2006). The infinitesimal model, developed by Ronald Fisher in 1918, suggests that a quantitative trait is equal to the sum of the genes regulating the trait, and that each gene, as well as environmental factors, contribute an *infinitesimal* amount to the phenotype. It also states that offspring inheriting the quantitative trait will show variance independent of the

parental trait values in a large outcrossing population (Barton et al. 2016). As complex traits are regulated by multiple genes, similar phenotypes observed between individuals within the same genotype might therefore be the result of expression of different genes, each contributing a small part to the observed phenotype. Two individuals with the same genetic background might therefore show similar phenotypes but might show different gene expression profiling when examined individually. This could explain the unexpected expression patterns between genotypes for many of the genes tested and also the irregular expression patterns between the biological replicates for gene 036930. Assuming that biological variance in gene expression is the reason for the unexpected RT-qPCR results, an indication is given about the difficulty of replicating RNA sequencing data through RT-qPCR. As RT-qPCR only can examine one gene at a time it is likely that variance in expression between individuals will have a strong influence on the results.

Another possible reason for the irregular expression patterns observed between genotypes and biological replicates could be uneven infection pressure. As *R. solani* rarely produce spores, and instead is inoculated with mycelium, it is difficult to quantify the amount of inoculum used to infect plants and uniform infections therefore cannot be guaranteed. The severity of the infection caused by *R. solani* in field trials is highly affected by environmental factors such as temperature and moisture, and even if the same amount of inoculum is used each year variance can be seen in the symptoms on the plants (Scholten et al. 2001). This is also reflected in the scoring results, as we can see variance in the scores of the control plants for each genotype. Although the average scores were higher for the tolerant control plants compared to the susceptible control plants, differences could be seen between the highest and the lowest scored plant for each genotype. This was especially clear in the control plants for genotype 1S, which had a considerably higher average score compared to the control plants of genotype 2S. This could likely be because of uneven infection, and while some genotypes had fairly low variation in the scores of the control plants, uneven infection in the plants analysed through RT-qPCR could be one possible explanation for the unexpected RT-qPCR results.

Another factor that could have contributed to the irregular expression patterns is the fact that *ICDH* is not the most suitable reference gene. In most RT-qPCR runs *ICDH* showed a C_t-value between 22–25, indicating that it is not stably expressed across all biological replicates and time points. It is therefore not an optimal gene to normalize samples against and the fluctuating expression of *ICDH* is likely to have affected fold-change values. With a more precise reference gene, clearer expression patterns could possibly have been identified, as even small variance in C_t-values can have a strong impact on the fold-change. Another option would have been to use two reference genes, which would have reduced variance even further, however, due to time constraints and economic limitations only one reference gene was used.

The allele frequency experiment of the gene successfully determined the distribution of allele *RES* and *SUS* for gene 036930 within the phenotype groups of the testing population. As was seen in *Table 7*, two clear groupings emerged when individuals homozygous for *RES* were compared to individuals who were heterozygous. As no statistical groupings could be seen for any phenotype group at any other allele-configuration pairing, we can assume that what separates the less tolerant phenotype groups (phenotype group 6 and 7) from the tolerant phenotype groups (phenotype group 8 and 9) is the frequency of heterozygous individuals. This could potentially be beneficial when screening for *R. solani* resistance as markers could be developed that distinguish between homozygous and heterozygous individuals for gene 036930. If a heterozygous individual is identified, it is statistically likely (with a confidence level of 95 %) that that individual belongs to phenotype group 6 or 7, if the population only

consists of individuals from phenotype group 6, 7, 8 and 9. However, further research is needed before any stronger conclusions can be drawn, as nothing still is known about the allele frequency of the more susceptible phenotype groups (phenotype group 1–5). The results indicate that medium tolerant individuals more often are heterozygous compared to tolerant individuals, but this separation can only be used to develop markers if susceptible phenotype groups follow a similar pattern. The melt curves observed for gene 036930 during the RT-qPCR does indicate that such a pattern exists, as the two distinguishable peaks show that two fragments of slightly different size has been amplified. As both the susceptible genotypes and the tolerant genotypes show single peaks for all replicates within the genotype, we can assume that the two peaks are not a result of variation between replicates or amplification of unspecific products. As the alleles *RES* and *SUS* differ in sequence and have been shown to not be equally distributed among tolerant and less tolerant phenotype groups, the two peaks could possibly be explained by the presence of the two different alleles in the tolerant and susceptible genotypes. As both tolerant genotypes and both susceptible genotypes show single peaks together, it is not inconceivable to believe that the two tolerant genotypes are homozygous for allele *RES*, and the two susceptible genotypes are homozygous for *SUS*. This would then explain the two close but distinguishable peaks, as the fragments produced for each allele likely would not be of the exact same size.

Future perspectives

A crops yield potential is the maximum yield that can be produced without limitations to water, nutrients or sunlight and without damage caused by biotic or abiotic factors (Evans & Fischer 1999). Yield potential is a highly theoretical number, as no crop system is immune to outside factors, but under average growth conditions the yield potential of sugar beets have been estimated to be approximately 24 tonnes sugar produced per hectare (Hoffmann & Kenter 2018). Yield trials in Germany have shown a yearly increase in sugar yield of about 0.14 t/ha, approximately 0.6 %, which have sparked a debate about how much sugar yield potentially can be improved (Märländer et al. 2003). A study made by Loel et al. (2014) which compared old and new sugar beet varieties, showed that neither the number of leaves nor the number of cambial rings have increased between 1964 and 2003, indicating that yield increases are not a result of improved light absorption or storage capacity. Yield increases can instead be attributed to improved agricultural practices and breeding progress, which have increased sugar yield by more efficient use of the available resources.

Breeding has contributed to increased sugar yield in part by shifting the ratio of dry matter in the plant from non-sugar compounds, which make up the leaf and the root structures, to sugar compounds (Hoffmann & Kenter 2018). There is, however, a limit to how much non-sugar compounds that can be shifted towards sugar, as the structural integrity of the root is dependent on these compounds, and the ratio therefore cannot be improved endlessly. Future improvements in sugar yield must therefore instead come from other sources that are independent of the physical limitations of the sugar beet plant. As sugar yield is reduced by plant stress, breeding varieties better adapted to fluctuating growth conditions could improve sugar yield indirectly. Drought is a common yield reducing factor in many crop systems, and with the changing climate, precipitation is in many parts of the world expected to be reduced and temperatures are expected to increase (Jaggard et al. 2010). While higher temperatures could be beneficial for the emergence and development of sugar beet seedlings, reduced water availability could result in substantial economic losses. The changing climate might also influence the spread and damage caused by pests and pathogens. When the interactions

between species within an ecosystem are disturbed, it is difficult to predict how the surrounding environment will be affected and pests and pathogens that are common today could become less common, while pests and pathogens that today are uncommon might find new opportunities. As *R. solani* thrive under warm and wet conditions, increased temperatures might result in *R. solani* becoming a more common and more severe pathogen in sugar beet production. However, if the climate becomes drier, fungal pathogens that depend on high moisture to survive might become less severe. It is difficult to predict how future biotic and abiotic factors might affect sugar beet production, but breeding varieties better adapted for future growth conditions and better suited for the future distribution of pests and pathogens will be relevant objectives for future improvements of sugar yield. Developing more effective markers for screening *R. solani* resistance would therefore be a useful tool for future breeding of sugar beets. The work done in this thesis could possibly facilitate the development of such markers.

Conclusions

The RT-qPCR results showed that in this case it was difficult to replicate RNA sequencing data through RT-qPCR, as only one gene at a time is analysed, and large variance in expression could be seen both between genotypes but also between biological replicates. Some tested genes showed indications of the expected expression patterns, but as complex traits are regulated by multiple genes identical gene expression profiling cannot be guaranteed between genotypes of the same tolerance level. The scoring of control plants showed that another factor that could have contributed to irregular expression patterns was uneven *R. solani* infection. As inoculation is done with mycelium instead of spores, it is difficult to quantify the amount of inoculum used for each plant, and it is therefore difficult to produce uniform infection.

The allele frequency experiment showed an indication of a pattern between tolerance to *R. solani* and allele distribution for gene 036930, but further research on susceptible lines is needed before any conclusions can be drawn. If the allele distribution in susceptible lines follows the same pattern as in tolerant and medium tolerant lines, the alleles could possibly be used to develop markers for improved screening of *R. solani* resistance. Lastly, sugar yield have seen steady improvements over the years in part due to a shift in the ratio of non-sugar and sugar compounds that make up the dry matter. There is, however, a limit to how far this ratio can be shifted, and future improvements to sugar yield might therefore not come from direct increases in the sugar content of the roots but might instead come from breeding varieties better adapted to future growth conditions and more resilient to pests and pathogens.

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