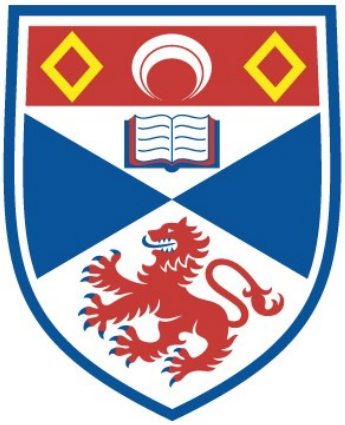
EXPLORING AND EXPLOITING THE RESISTANCE TO GLOBODERA PALLIDA IN POTATO

Ulrike Gartner

A Thesis Submitted for the Degree of PhD
at the
University of St Andrews



2023

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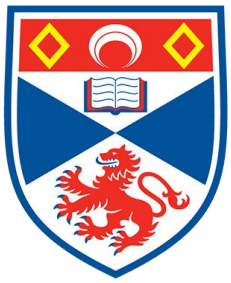

University of
St Andrews

This thesis is submitted in partial fulfilment for the degree of

Doctor of Philosophy (PhD)

at the University of St Andrews

July 2022

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I dedicate this work to Vivian Blok (1956-2022).
Her dedication and enthusiasm for her work with nematodes inspired me. Without her, none of this work would have been possible.

Acknowledgments

Many thanks to the late Vivian Blok, Glenn Bryan and John Jones, as well as Ingo Hein, for their help and support as supervisors throughout my PhD.

I would like to thank my colleagues at the James Hutton Institute and James Hutton Limited, especially the nematode lab, the potato genetics group, BIOSS, and the James Hutton Limited potato group, who taught me new methods, provided reagents and material for my research and made working at the institute enjoyable. I am also grateful for the collaboration with Benjamin Mimee and his group from Agriculture and Agri-Food Canada, St-Jean-sur-Richelieu, Canada.

I would also like to thank the glass house staff for maintaining plants; the Genome Technology Facility for the sequencing and genotyping of samples that seemed endless; the nematology group at SASA, Edinburgh for letting me use their carousel for washing out cysts from soil, especially Andrew Pitt who spent several days with me at the carousel each year.

I am very grateful to my husband Anton and my children Isabel and Tobias who always supported and encouraged me to do this PhD. A special thanks goes to my friend Emma Warbrick who listened to and discussed all my concerns and questions, and always encouraged me to proceed whenever I was doubting my work.

I also would like to thank the Globodera Alliance project (funded by USDA NIFA) for their financial support, which not only funded my work but also allowed me to go to meetings and workshops in Montreal (Canada), Ghent (Belgium), Corvallis (Oregon, USA), Antibes (France), London, Shropshire and to travel to the University of Idaho (USA).

Abstract

The potato cyst nematodes (PCN) *Globodera pallida* and *G. rostochiensis* are economically important potato pests in almost all regions where potato is grown. Studying the composition, distribution and virulence of PCN populations in fields, and finding new sources of naturally occurring resistance in wild potato species is important for the management of these pests.

In Scotland, up to three different introductions of *G. pallida*, determined by mitotyping, were found to be present in fields. To investigate whether cysts in a population show a correlation between mitotype and different virulence levels, "single cyst" lines were generated, mitotyped, and their virulence to different potato cultivars was determined. One mitotype was shown to correlate with *G. pallida* pathotype Pa3, but overall, the mitotypes are not usable as reliable virulence markers. A phylogenetic analysis was performed to determine the relationships between British *G. pallida* populations. In addition, single nucleotide polymorphisms (SNPs) in genomic DNA were identified that represent candidate virulence markers.

A screen of wild potato germplasm was undertaken to identify new resistance against *G. pallida.* The diploid species *Solanum spegazzinii* Bitter accession 7195 shows resistance to *G. pallida* pathotypes Pa1 and Pa2/3. A cross and first backcross of *S. spegazzinii* with *S. tuberosum* group Phureja cultivar Mayan Gold was performed, and the level of resistance to *G. pallida* Pa2/3 was determined in progeny. Bulked-segregant analysis using generic mapping enrichment sequencing and genotyping-by-sequencing was performed to identify SNPs that are genetically linked to the resistance, using *S. tuberosum* group Phureja clone DM1-3 516 R44 as a reference genome. These SNPs were converted into allele specific PCR assays, and the resistance was mapped using graphical genotyping. The resistance was successfully introgressed into a tetraploid potato cultivar by a tetraploid-diploid interploidy cross.

List of Abbreviations

˚C	Degree Centigrade
µl	Microlitre
µm	Micrometre
ATP	Adenosine triphosphate
Avr	Avirulence
BCA	Biological control agent
BLAST	Basic local alignment search tool
BR	Resistant bulk
BS	Susceptible bulk
CK	Cytokinins
CN	Cyst nematode
CRISPR/Cas	Clustered regularly interspaced short palindromic repeats/CRISPR-associated protein
cv.	Cultivar
CWDE	Cell wall degrading enzyme
cytb	Cytochrome b
dH_2O	Distilled water
DM	*S. tuberosum* group Phureja clone DM 1-3 516 R44
DNA	Desoxyribonucleic acid
dRenSeq	Diagnostic resistance gene enrichment sequencing
EPN	Entomopathogenic nematodes
ER	Endoplasmic reticulum
ETI	Effector triggered immunity
ETS	Effector triggered sensitivity
f1, f3	Field 1, field 3
FDR	First division restitution
g	Gram
GMO	Genetically modified organism
h	hour

HR	Hypersensitive response
IAA	Indole-3-acetic acid
ICS	Initial syncytial cell
ISR	Induced systemic resistance
J2	Second stage juvenile
KASP	Kompetitive allele specific PCR
LRR	Leucine rich repeat
m	Meter
m3	Cubic meter
min	minute
ml	Millilitre
mm	Millimetre
mM	Millimolar
mtDNA	Mitochondrial DNA
NAMP	Nematode-associated molecular pattern
NB-LRR	Nucleotide binding-leucine rich repeat
PAMP	Pathogen associated molecular pattern
PCN	Potato cyst nematode(s)
PCR	Polymerase chain reaction
PPN	Plant parasitic nematode
PR	Resistant parent
PRR	pattern recognition receptors
PS	Susceptible parent
PTI	PAMP-triggered immunity
QTL	Quantitative trait locus
R-	resistance
RFLP	Restriction fragment length polymorphism
RKN	Root-knot nematode
RLK	Receptor-like kinase
RNA	Ribonucleic acid
ROS	Reactive oxygen species

SAR	Systemic acquired resistance
scmtDNA	Small circular mitochondrial DNA
SDR	Second division restitution
sec	second
SNP	Single nucleotide polymorphism
sp.	species
SPRYSEC	Secreted Sp1a and ryanodine receptor
SSU rDNA	Small subunit ribosomal DNA
TALEN	Transcription activator-like effector nucleases
TRD	Tomato root diffusate
TRFLP	Terminal restriction fragment length polymorphism
VAP	Venom-allergen like protein
W	Watt

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1 General Introduction

1.1 Potato

Potatoes are the world's third most important staple food crop after rice and wheat (CIP, 2020), with a worldwide production of 370 million tons in 2018 (FAO, 2020). About 50% of potatoes are consumed fresh, the rest is mainly used for seed potato production, processed food, animal feed and starch production (Birch et al., 2012).With the increasing demand for food from a growing population of potentially 9.7 billion by 2050 (United Nations, 2015), pest and disease management are of increasing importance for food security. Environmentally sensitive methods of control, including natural resistance, are important for the sustainability of this crop.

The domestication process of potato started about 8,000 years ago in the South American Andes, near lake Titicaca (De Jong, 2016). Cultivated potato was introduced to Spain from South America from the second half of the 16th century and was subsequently distributed all over the world. Until the 1850s, tubers were propagated clonally mostly using inferior tubers as seed potatoes, resulting in poor crop health and low yields (Jansky and Spooner, 2018). Potato became a major staple food crop worldwide after initially not being accepted as a food crop. Most of the commercial potato varieties in Europe and North America are *S. tuberosum* group *tuberosum L*, whereas in South America a far bigger variety of different potato species are consumed. The potato cultivars used in Europe did not originally carry resistance to many pests and diseases due to a lack of genetic variation in the types originally brought to Europe. Following the potato famines in Northern Europe during the mid-1800s, caused by late blight (*Phytophthora infestans*), potato breeding developed an increasingly important role.

Potato (*Solanum tuberosum*) is a member of the *Solanaceae* family comprising more than 90 genera and 3,000-4,000 species (Machida-Hirano, 2015), including the crop species tomato (*S. lycopersicum*), peppers (*Capsicum sp*.), eggplant (*S. melongena*) and tobacco (*Nicotiana tabacum*). Wild species are mainly either diploids or allo-tetraploids or hexaploids, the commercially available potatoes are usually autotetraploid. Triploid and pentaploid species have been reported, but generally are sterile (Marks, 1966;

Graebner et al., 2019). Potatoes are mainly propagated clonally from tubers with the advantage that valuable genotypes are maintained in each generation (McKey et al., 2010). However, the use of clonal propagation alone allows the accumulation of mutations (Lian et al., 2019), pathogens and parasites over time.

1.2 Nematodes

Nematodes, are ubiquitous and diverse animals with an estimated number of species between 100,000 and 1 million (Parkinson et al., 2004), although fewer than 30,000 species have been described to date (Blaxter and Koutsovoulos, 2015; Smythe et al., 2019). Their size ranges from 0.2 mm to over 6 m (Blaxter and Koutsovoulos, 2015). Using phylogenetic analysis of the small subunit of ribosomal DNA (SSU rDNA), nematodes were separated into five clades (I to V) (Blaxter et al., 1998) and later into 12 clades (Holterman et al., 2006; van Megen et al., 2009) (Figure 1-1).

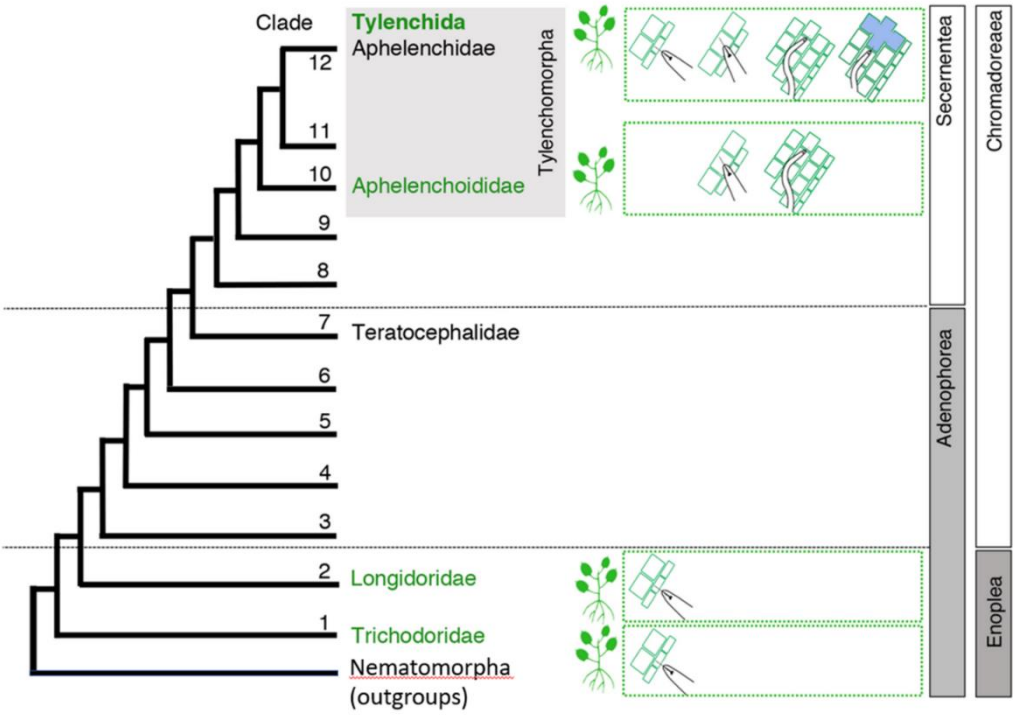

Figure 1-1 Schematic representation of relationships within the phylum Nematoda
These relationships are based on SSU rDNA (Holterman et al., 2006). In clades 1, 2, 10 and 12 plant parasites were detected (in green). The sketches on the right-hand side indicate different types of PPN: ectoparasites, semi-endoparasites, migratory endoparasites, and sedentary endoparasites. Adapted from Holterman et al. (2017). Permission to reprint/adapt by a Creative Commons Attribution International License CC BY 4.0[1].

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1.2.1 Free-living nematodes

At least 50% of all nematodes are free living species (Smythe et al., 2019). They help in maintaining ecosystems by decomposing organic material and are food for higher trophic level animals. The prevalence of the various trophic groups of free-living nematodes can be used as a bioindicator for ecosystem health (Smythe et al., 2019). The free-living nematode *Caenorhabditis elegans* serves as a model organism for studies of the basic functions and interactions of eukaryotic cells and evolution, studies of diseases, ageing, neurobiology, DNA damage repair, drug discovery, parasitism, apoptosis, genomic profile of cancer (Corsi et al., 2015). The genome sequence of *C. elegans*, published in 1998, was the first genome of any multi-cellular organism (*C. elegans* Sequencing Consortium, 1998).

1.2.2 Parasitic nematodes

Parasitism has evolved independently at least 15 times during nematode evolution in various clades (Blaxter and Koutsovoulos, 2015). Nematodes can be parasitic to animals and plants.

Animal parasitic nematodes are important pathogens of humans, livestock, domestic- and wild animals, as well as insects. An estimated 3.5 billion people are infected worldwide with about 9% of infected individuals needing medical attention and more than 125,000 people dying of nematode infections per year (Shakya and Yadav, 2020). The symptoms of the diseases caused by hookworms, Ascaris and whipworms include anaemia, diarrhoea, abdominal pain and malnutrition. Filarial nematodes, such as *Onchocerca volvulus* or *Brugia malayi*, cause African river blindness and elephantiasis, respectively (Parkinson et al., 2004; Wasmuth et al., 2008). Control of parasitic nematodes requires integrated management and relies on a small number of anthelmintic drugs. Anthelmintic resistance is widespread, particularly in parasites of sheep (Jex et al., 2019). Some animal parasitic nematodes are exploited for their beneficial impact on agriculture. Entomopathogenic nematodes (EPN) kill insects in association with insect-pathogenic bacteria (Dillman and Sternberg, 2012) and since the 1980s EPN have been used commercially as biopesticides (Koppenhofer et al., 2020).

More than 4,000 plant parasitic nematode (PPN) species have been characterised to date (Jones et al., 2013). PPN pose a serious threat to crop security; estimates of crop losses of US$ 80 billion per year are likely an underestimation (Nicol et al., 2011; Jones et al., 2013). PPN not only cause damage directly as a result of their feeding, but also because of the increased susceptibility of affected plants to other infections (*e.g.,* viral, bacterial, fungal) (Nicol et al., 2011). Some PPN are also vectors of plant viruses. PPN can feed on many plant tissues, including stem, buds and roots. The economically most important PPNs are root parasitic nematodes (Jones et al., 2013). Figure 1-1 shows a generalised overview of the 12 clades of the phylum Nematoda. Parasitism of plants has arisen independently at least four times (Baldwin et al., 2004; Smant et al., 2018) with PPN identified in clades 1, 2 10 and 12 (Holterman et al., 2017). In spite of the fact that the various clades of PPN have evolved independently, there are some shared features. For example, all PPN have a needle-like feeding structure (*e.g.,* a stylet), which is an example of convergent evolution (Smant et al., 2018), and large pharyngeal gland cells which produce proteins required for the interaction with the host plant.

PPN can be migratory or sedentary, and each of these groups includes ecto- and endoparasites. Ectoparasites live outside the plant for their entire life cycles and usually feed from outside layers of the root using their stylet. Ectoparasites are vulnerable to predators and environmental conditions, as they do not derive any protection from being enclosed by the host plant (Lambert and Bekal, 2002). Endoparasites spend at least part of their lives inside plant tissues. Being inside the plant tissue protects the nematode from environmental challenges, pathogens and predators (Lambert and Bekal, 2002); many endoparasitic nematode genomes possess fewer genes coding for components of the immune system, possibly reflecting this reduced disease pressure and the benefits of protection by the host (Kikuchi et al., 2017). Migratory endo- and ectoparasites can often feed on different plants and, where they have a broad host range, they are also able to switch between hosts (Lambert and Bekal, 2002; Moens and Perry, 2009). Migratory endoparasites migrate extensively inside host tissues, causing considerable damage to the plant. Sedentary endo- and ectoparasites form a long-term feeding structure in a single host. This feeding structure is often highly metabolically active, thus increasing their nutrient intake. However, these nematodes are vulnerable to the death of the host, or to the

programmed cell death of the feeding site as they cannot induce more than one feeding site.

The economically most important PPN are cyst nematodes (CN) (*Globodera* spp. and *Heterodera* spp.) and root-knot nematodes (RKN) (*Meloidogyne spp*.), which are both sedentary endoparasites (Lambert and Bekal, 2002; Siddique and Grundler, 2018). RKN have a broad host range, whereas most CN are highly specialised pathogens with a narrow host range (Wyss, 1997). *G. rostochiensis*, *G. pallida,* and *G. ellingtonae* are parasites of potato.

1.3 Potato cyst nematodes

Potato cyst nematodes (PCN) are highly specialised soil-borne biotrophic sedentary endoparasites of the *Solanaceae* family, and the most economically important plant-parasitic nematode of potato (Gartner et al., 2021). There are estimates that the PCN are responsible for losses amounting to about 9% of the potato crop worldwide (Turner and Subbotin, 2013). As PCN are quarantine pathogens, eradication or containment procedures need to be applied when it is detected (Pickup et al., 2018b), which imposes additional costs on growers. The two species, *G. rostochiensis* and *G. pallida*, are the predominant PCN. A third species, *G. ellingtonae* (Handoo et al., 2012), has been detected in the USA in Oregon and Idaho (Skantar et al., 2011), and in the Andean region of South America (Lax et al., 2014). However, it is not consistently pathogenic to potato plants (Zasada et al., 2019) and has not been identified elsewhere to date. In the early 20th century, it was suggested that cyst nematodes seen on potato plants were the beet cyst nematode *Heterodera schachtii* (Jones, 1970). Later, in 1923, Wollenweber distinguished PCN from *H. schachtii,* and from 1972, *G. pallida* and *G. rostochiensis* were separated into two different species (Stone, 1972). More recently, Thevenoux et al. (2020) identified two potentially new crypto species of *G. pallida*, "*pallida* Chilean type" and group 4, when investigating the genetic diversity within *G. pallida* in the Andean region of South America.

1.3.1 Origin and introduction to Europe

PCNs are probably native to Andean Cordillera in South America (Canto Saenz and Mayer de Scurrah, 1977; Stone, 1985; Grenier et al., 2010). Analysis of the

mitochondrial cytochrome b (*cytb*) gene and microsatellites suggest that *G. pallida* originates from the region around Lake Titicaca in the south of Peru from where their range has expanded northwards. During this time, *G. pallida* co-evolved with their solanaceous hosts, a process that is estimated to have taken place over 20 million years (Picard et al., 2004; Picard et al., 2007).

In the search for blight resistant potatoes following the Irish potato famine , both PCN species *G. rostochiensis* and *G. pallida* were probably introduced to Europe in the 1850s when blight resistant tubers with contaminated soil attached were imported (Evans et al., 1975). As there have been a very restricted number of introductions of PCN into Europe, only a small proportion of the total genetic diversity of these species is represented outside South America (Plantard et al., 2008; Grenier et al., 2010). It is this restricted genetic variation that allows a potato breeding approach using natural host resistance for their control (Gartner et al., 2021).

About three decades after the introduction of PCN to Europe, from the 1880s, PCN symptoms were seen in potato fields in Germany, with similar reports about 20 years later in the UK (Evans et al., 1975). PCN have spread to almost all potato growing countries with reports from 126 countries to date (CABI, 2020a; 2020b) where potato is grown. The continued spread of PCN is reflected by the recent discoveries of these nematodes in Kenya (Mwangi et al., 2015) and China (Jiang et al., 2022).

1.3.2 Life cycle

Cysts from PCN, that are located in the soil, are formed from the dead body wall of a female PCN and contain several hundred eggs, each of which contains an embryo that develops into the first stage juvenile and moults to a second stage (J2) nematode inside the egg. The cyst encloses the eggs in the soil and the J2 are protected by the eggs. Unhatched J2 can survive for years in the eggs, depending on the environmental conditions. The J2 nematodes in the eggs are dehydrated and immersed in a perivitelline fluid containing a high concentration of trehalose, which protects the nematodes against low temperatures (*e.g.,* reviewed by Perry (2002)). *G. pallida* and *G. rostochiensis* usually have an obligatory diapause in the first months after their development (Moens et al., 2018). Hatching of J2 from the eggs is dependent on environmental factors, such as temperature, moisture and the presence of root

exudates of host plants. This dependence on the presence of root exudates allows PCN to coordinate the life cycle with the presence of a host. Before hatching, the metabolic activity of the dehydrated J2 animals is reactivated due to the change in the eggshell permeability induced by the hatching factors, allowing water to enter the egg (Clarke and Perry, 1985). The J2 subsequently cut the eggshell with the stylet to allow eclosion (Doncaster and Seymour, 1973).

Once hatched, the nematodes locate the roots by following chemical gradients of compounds exuded by physiologically active roots. It is thought that different compounds serve as long-distance, short-distance and local attractants. During this time, the animals do not feed (Perry and Curtis, 2013). When the J2 nematode reaches a host root, it penetrates it and migrates through root cells to the inner cortical cells. This process is facilitated by the production of a cocktail of plant cell wall degrading enzymes (CWDE). The genes encoding these endogenous nematode enzymes are thought to have been acquired by horizontal gene transfer from bacteria (reviewed in Kikuchi *et al.,* 2017). Once the J2 nematode reaches the inner cortical layer of the root, it selects a suitable cell (initial syncytial cell (ICS)), from which to form a syncytium as a feeding site. The syncytium is formed as a result of the fusion of the protoplasts of many plant root cells. The nematode carefully inserts its stylet into a ICS candidate and waits for a cell response: an adverse response, such as collapse of the protoplast or deposit of callose on the stylet, leads to the retraction of the stylet and probing of another cell until a suitable ICS is found (Sobczak and Golinowski, 2011). Virulence factors (Section 1.4.4) are injected into the ICS, causing massive changes in the cell (Sobczak and Golinowski, 2011). Morphological changes occur, such as protoplast-fusion between merging cells at the cell wall openings leading to a syncytium of up to several hundred cells, or the disappearing of the central vacuole and enrichment of rough endoplasmic reticulum (ER) and other organelles in the cytoplasm. These changes derive from changes in the expression patterns of thousands of plant genes (Sobczak and Golinowski, 2011; Moens et al., 2018; Kooliyottil et al., 2019; Price et al., 2021). It is vital for the nematode to keep this site alive for the duration of the nematode's life cycle as the nematode can only generate one syncytium and it relies on this syncytium as its only food source. Feeding from the syncytium occurs with a

feeding tube, produced during each feeding cycle, probably acting as a molecular filter to prevent damage to the syncytium while feeding (Eves-van den Akker et al., 2015a).
Sex determination of the nematode occurs at the end of the J2 stage and is dependent on environmental conditions. Nematodes that produce a small syncytium due to the resistance response of the plant or competition for feeding sites due to overcrowding will develop primarily as males (Trudgill, 1967; Moens et al., 2018). By contrast, those that induce a large active feeding structure develop into females. The nematodes moult through J3, J4 and adult stages while feeding. The male nematodes revert to the wormlike shape, detach from the feeding site, leave the root and search for females guided by pheromones. The female nematodes stay at the feeding site and grow until their bodies emerge from the root tissues. Females are fertilized, sometimes by multiple males. The female nematode produces eggs, and its body swells as it turns into a cyst that eventually detaches from the root (Turner and Subbotin, 2013).

1.3.3 Virulence and pathotypes

The capability of nematodes to reproduce on a host plant that contains specific genes otherwise conferring resistance to these nematodes is called virulence. Nematodes that cannot reproduce on a host plant carrying a specific resistance source are termed avirulent (Vanderplank, 1978; Turner et al., 1983; Blok et al., 2018). Virulence is promoted by excretory/secretory products (Bobardt et al., 2020), called virulence factors or effectors (Siddique and Grundler, 2018; Spallek et al., 2018; Bobardt et al., 2020) (Section 1.4.4). Some effectors were discovered as triggering the HR response in resistant plants and were called *avirulence* proteins. Later, it was discovered that the avirulence response is induced by a matching R-protein, that is activated by the recognition of the avirulence protein directly or indirectly (guard hypothesis). If no effective R-protein is present (in susceptible plants), these proteins contribute to virulence. (Hogenhout et al., 2009). Virulence is a characteristic of individual nematodes, therefore a nematode population can and will contain individuals that are virulent and avirulent; the proportion of virulent/avirulent individuals is dynamic. For example, the use of the same *G. pallida* resistant potato genotypes over several generations can lead to an increasing proportion of virulent to avirulent nematodes (Whitehead, 1991; Hockland et al., 2012; Beniers et al., 2019; Varypatakis et al., 2019).

This increase in virulence can lead to resistance breaking, which has been reported for a initially highly resistant potato cultivar, Innovator, in Germany (Niere et al., 2014).

In an attempt to classify different levels of virulence by growing them on various potato genotypes with different sources and levels of resistance, a pathotype scheme for *G. rostochiensis* and *G. pallida* was developed (Kort et al., 1977). For *G. rostochiensis*, five pathotypes Ro1 to Ro5, were defined by their reproduction rate on different potato genotypes (Kort et al., 1977; Gartner et al., 2021). In the UK so far only one pathotype, Ro1, has been described and is possibly the result of a single introduction (Evans et al., 1975; Bendezu et al., 1998). Ro1 is the dominant pathotype outside South America. The European *G. pallida* populations were categorised into the pathotypes Pa1, Pa2 and Pa3 by Kort et al. (1977), whereas South American PCN populations were grouped into the groups R_1A, R_1B, R_2A and R_3A for *G. rostochiensis*, which corresponds to Ro1, Ro4, Ro2 and Ro3 in the European scheme respectively and P_1A (Pa1), P_1B, P_2A, P_3A, P_4A (Pa2) and P_5A (Pa3) for *G. pallida* populations with the corresponding European pathotype in brackets (Canto Saenz and Mayer de Scurrah, 1977). Pa1 reproduces very poorly on *S. multidissectum* hybrid P55/7 whereas Pa2 and Pa3 can reproduce, slightly less than on potato genotypes without any resistance gene, and growth on *S. vernei* (VTn)2 62.33.3 allows differentiation between Pa2 (poor reproduction) and Pa3 (only slight decrease in reproduction compared to growth on susceptible potato genotype). Trudgill (1985) showed, that Pa2 and Pa3 were not reliably distinguishable and suggested that these two pathotypes were rather a continuum which was termed Pa2/3. The disadvantages of both the Kort and the modified pathotype schemes are that it is based on specific potato genotypes, which might not be relevant or available for the determination of the virulence. EPPO (2006) developed a method to categorise potato genotypes/cultivars into different levels of resistance based on the multiplication of the cysts relative to a susceptible cultivar (Desirée). This method will be used in this study to show differences in the virulence of *G. pallida* populations and is described in more detail in Chapter 2.

1.3.4 Resistance and tolerance to PCN

Two different strategies, resistance and tolerance, are used by plants and animals to cope with pests and pathogens. These two traits are independent from each other

(Evans and Haydock, 1990; Trudgill, 1991). For PCN, resistance to a pathogen is defined as a plant's ability to inhibit or limit reproduction relative to a susceptible plant (Blok et al., 2018; Pagán and García-Arenal, 2020), whereas tolerance of PCN refers to the ability of the plant to tolerate infection by nematodes with no or only a minor reduction in yield (Evans and Haydock, 1990; Blok et al., 2018). The use of tolerant potato cultivars without resistance in the presence of PCN allows for a high multiplication rate and consequently the build-up of very high levels of cysts in the field. Ideally, resistance and tolerance should be combined to get potato cultivars which provide acceptable yields in areas with high PCN population densities, while reducing PCN population levels (Trudgill, 1991). Tolerance is a complex trait, depending on factors such as root responses and root growth characteristics, plant stress responses and initial nematode population density (Blok et al., 2018) as well as environmental conditions. Therefore, it is very difficult to get reliable assessments of this trait. The existing nematode population density determines the extent of damage (Evans and Haydock, 1990). A way to assess tolerance is to grow potatoes in a field that is uniformly infested with PCN and one that is not infested and compare the tuber yield and the plant- and root growth. Pot bioassays for tolerance were developed for single plants in one pot with many replicates, which showed a good correlation with field tests (*e.g.,* Trudgill and Cotes (1983); Arntzen and Wouters (1994)) that allowed for earlier assessment of tolerance in the breeding process. The genetic basis of tolerance has not been elucidated, it is a quantitative trait and factors such as enhanced root growth after pathogen attack, or more efficient ways of using water are thought to contribute to tolerance (Evans and Franco, 1979). Pagán and García-Arenal (2020) conclude that "tolerance to pathogens is a plant defence as effective and widespread as resistance and may play an important role in plant disease control, the dynamics of pathogen infection, and plant–pathogen coevolution".

1.3.5 Strategies to manage and control PCN levels

Potatoes are an important staple food for an increasing world population; therefore, it is important to control pests to ensure food security. PCN are very important pathogens of potato and cause significant economic losses each year. PCN are quarantine organisms and therefore phytosanitary regulations are applied for the

prevention of the introduction or spread of the pathogen. Once PCN are detected, eradication or containment procedures ideally need to be applied (Pickup et al., 2018b), which cause additional costs. Eradication programs can be useful when the infestation of PCN is very localised. For example, *G. rostochiensis* was detected in an area of 35ha in Western Australia in the 1980s; after an eradication program this area was declared PCN free in 2010 (reviewed in Gartner et al. (2021)). In the USA, a containment and eradication program[2] is ongoing, after the detection in 2006 of two potato fields infested with *G. pallida* in Idaho (Hafez et al., 2007; USDA, 2007; Dandurand et al., 2017). The situation in the UK is different, as PCN is widespread and established. In this case, the eradication of PCN is impossible because cysts are viable for many years in the soil, even in absence of host plants (Turner, 1996) and moreover, the detection of new PCN infestations may take many years, during which cysts can spread and infest new land. Therefore, the focus of strategies for the control of PCN in the UK is to prevent the spread into new land and to keep population densities within acceptable levels to minimize the damage that they cause. Models that allow the prediction of expected potato yields and the PCN population development when using different control measures or potato cultivars were developed and are available as online tools, such as NemaDecide[3] (Been et al., 2007) and PCN Calculator[4] (Trudgill et al., 2014).

Each method for controlling PCN levels has its limits and applied on their own will not provide sustainable and effective control. An effective integrated pest management (IPM) strategy must combine different methods that can act in a synergistic way. (Back et al., 2018).

The chemicals used to control PCN can be categorised into two groups, fumigant and non-fumigant nematicides. A third group, seed treatment, is only used for the soybean cyst nematode *Heterodera glycines* control (reviewed in Back et al. (2018)). A successful application of nematicides depends on using the correct concentration at the right time and often the right depth in the soil. Due to the potential harm of synthetically produced nematicides to the environment, their use is strictly regulated,

[2]https://www.aphis.usda.gov/aphis/ourfocus/planthealth/plant-pest-and-disease-programs/pests-and-diseases/nematode/pcn/pcn-home
[3]http://www.nemadecide.com
[4]https://pcncalculator.ahdb.org.uk/

and many products have recently been banned in many countries (Back et al., 2018). This reduction in available chemicals for PCN control has resulted in the development of alternatives, such as bio-fumigation, which refers to the application of naturally occurring biocidal compounds from brassica green manure to the soilborne pests and pathogens (Lord et al., 2011; Back et al., 2018). Furthermore, other plant products, such as oils, extracts and biomass, from *e.g.,* acacia, eucalyptus, garlic, tobacco and the neem tree, have been investigated for their potential suppressive effects on cyst nematode populations. Mostly, these studies have only been performed in *in vitro* or in controlled environment experiments and not in field conditions (Back et al., 2018). In the UK, one garlic extract-based product to control PCN and other PPN, Nemguard DE, is registered (Gillbard, 2021). Very recently, Ochola et al. (2022) developed a renewable eco-friendly "wrap-and-plant" technology which uses banana-paper for wrapping seed potatoes before planting as protection from *G. rostochiensis*. The wrap reduces the communication between nematode and plant by adsorbing PCN hatching factors. Furthermore, the banana-paper can be coated with very low doses of nematicide which will be slowly released directly into the region surrounding the developing potato root system.

Biological control of PPNs uses living organisms (biological control agents - BCAs) to reduce the nematode population below an economic damage threshold. Different fungi and bacteria, such as endophytic or filamentous fungi, rhizobacteria or root endophytic bacteria have been used as BCAs. They can work by attacking the PPN directly, for example by parasitising or paralyzing the nematodes, they can be predators, or they can be antagonists and compete for example for colonization or infection sites. An indirect way to provide some control of PPN can be provided by the induction of plant defences, such as the activation of systemic acquired resistance (SAR) or induced systemic resistance (ISR). Such priming agents include *Trichoderma* or Chitosan, a naturally occurring polymeric deacetylated derivative of chitin (Aranega-Bou et al., 2014). Arbuscular mycorrhizal fungi may also be used to enhance tolerance of plants to PPN damage (reviewed in Poveda et al. (2020)). When using biological control, the PPN are not considered in isolation, but part of an ecosystem (Back et al., 2018).

A third approach for the control of PCN are the so-called cultural control methods, in which no chemicals or biological agents are used to manage nematode numbers. An example of this approach is crop rotation. This is an old and efficient method of managing PCN, as long as the rotation cycle is long enough. As PCN have a narrow host range, this method of control is well suited. A rotation of 6 to 8 years of potato crops by the Incas is thought to have been used to limit potato yield losses (Picard et al., 2007). In absence of a host, up to a third of the nematodes in a cyst will die or hatch spontaneously each year, depending on soil type and environmental conditions (Trudgill et al., 2014). The required length of rotation to reduce PCN levels in the soil below the damage threshold may be too long to be used by many potato growers. Using trap crops in the rotation increases the reduction of PCN levels per year significantly (Evans, 1993). Non-host trap crops stimulate hatching of the J2 nematodes, but they do not allow the nematodes to reproduce (Dandurand and Knudsen, 2016). *S. sisymbriifolium* (also known as sticky nightshade or litchi tomato) is an example of a non-host trap crop for PCN which was shown induce the hatching of the PCN J2 nematodes nearly at the level of potato and does not allow further development of *G. pallida* and *G. rostochiensis*, resulting in the death of the J2 nematodes (Scholte, 2000; Dandurand and Knudsen, 2016). Resistant potato genotypes can also be used as trap crops (Viaenne et al., 2013).

1.4 Interactions of PPN with their hosts

PPN and their hosts maintain a complex long-term relationship in which the pathogen tries to establish in or on the host to complete their life cycle whereas the host plant constantly seeks to combat the pathogen attack.

1.4.1 Plant responses to pathogen attacks

Plants are constantly under threat from a range of pathogens, including viruses, bacteria, nematodes, oomycetes and insects. In contrast to mammals, plants do not have mobile defender cells; they rely on the innate immune present in each plant cell and systemic signals of the wounded or infected cells (Jones and Dangl, 2006).

1.4.1.1 Innate immune system

The innate immune system is activated upon detection of potential pathogens or other cell damage. Jones and Dangl (2006) proposed the "Zig-Zag" model which illustrates the interactions of pathogens and plants and consists of two levels of inducible defences that have been established due to plant/pathogen co-evolution (Figure 1-2).

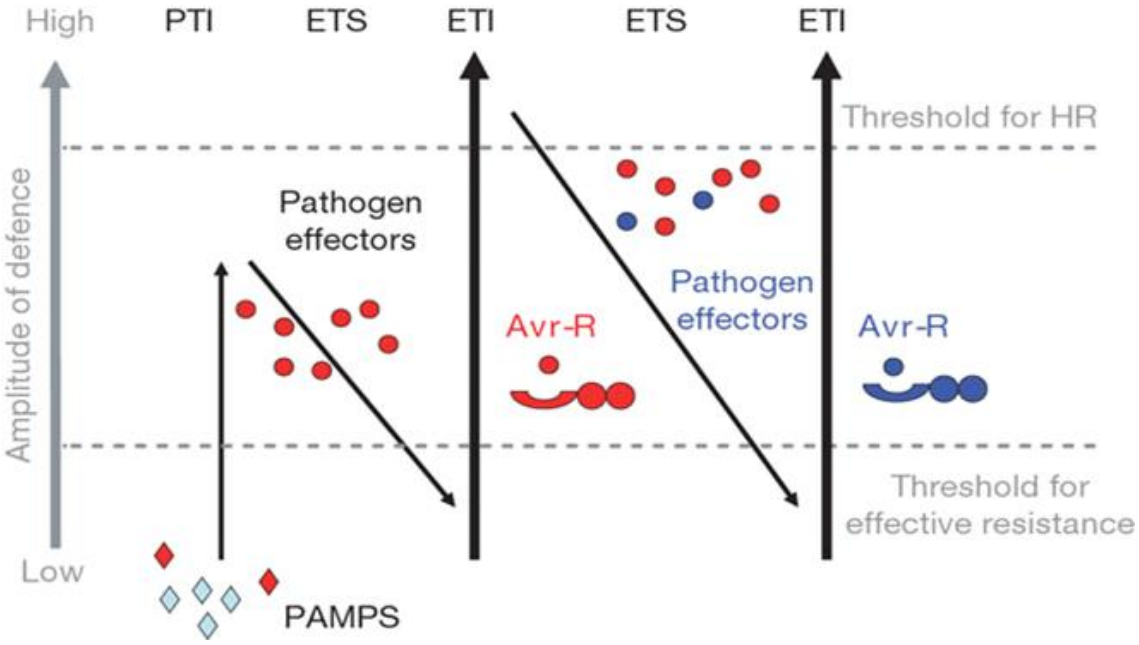


Figure 1-2 "Zig-Zag" model of the plant immune system
Reprinted from Jones and Dangl, 2006. Permission to reprint/adapt by RightsLink®[5], licence no. 5415790618413; see main text for explanation.

The first layer is activated by the recognition of a broad spectrum of pathogens. Pathogen associated molecular patterns (PAMPs) are highly conserved molecules derived from pathogens (Ingle et al., 2006) that are recognised by pattern recognition receptors (PRRs) that have an extracellular leucine rich repeat (LRR) and a transmembrane domain, which anchors them to the cell surface (Zipfel, 2008). Activation of PRRs results in PAMP-triggered immunity (PTI) or basal resistance. Examples of PAMPS include bacterial flagellin or fungal chitin. Within minutes of the recognition of PAMPs, the plants activate different pathways with the aim to prevent the growth of the pathogen by activating apoplastic defences to inhibit microbial enzymes, strengthen the cell wall (*e.g.,* depositing of callose or lignin) or to poison the pathogen (production of reactive oxygen species (ROS)) (Huckelhoven, 2007; Couto and Zipfel, 2016). PTI provides protection against non-adapted pathogens. However, many pathogens have adapted to PTI and secrete proteins, known as effector proteins,

[5] https://www.copyright.com/solutions-rightslink-permissions/

that promote susceptibility of the plants to the pathogens by the suppression of PTI (Jones and Dangl, 2006; Spoel and Dong, 2012). This is called effector-triggered susceptibility (ETS) (Jones and Dangl, 2006).

The second layer of plant defences is called effector-triggered immunity (ETI); a pathogen effector protein (translated from an avirulence (*avr*) gene) is recognised by a corresponding (or "matching") resistance (R) protein (translated from an *R*-gene) in the plant, inducing a hypersensitive response (HR) in the infected cell. The HR is characterised by rapid and localised programmed cell-death, which differs from developmental programmed cell-death, and a subsequent cell-death lesion (Heath, 2000). Pathogens are under high selection pressure to evade recognition by the plant immune system; therefore, new or modified effector proteins that are not recognised by R-proteins of the host plants need to evolve or be gained. So plants, in turn, have to adapt to the new situation and are under high selection pressure as well (Dangl and McDowell, 2006). This co-evolution of plants and pathogens is described in a mathematical model referred to as "arms race" (Bergelson et al., 2001).

Different concepts of *R*-gene-mediated resistance have been suggested over the years. A direct mechanism, in which the R-protein physically binds to the recognised effector protein, was proposed as the "gene-for-gene" concept (Flor, 1971; Van der Biezen and Jones, 1998; Dodds and Rathjen, 2010); this hypothesis has been supported by the characterisation of *R-Avr* gene pairs, an example is the flax rust fungus, where different alleles of the *AvrL567 effectors* are directly recognized by the L5, L6, and L7 R-proteins of flax (Dodds et al., 2006; Caplan et al., 2008). More often, however, an indirect mechanism occurs to activate R-proteins to deliver resistance to the pathogen, in which no physical interaction between the effector and R-proteins is observed. In the "guard" strategy, R-proteins monitor the status of other proteins (guardees). Guardees are frequently involved in signalling for defence and therefore are often targets of diverse pathogen effectors. Plants may also deploy decoys, which are similar to targeted proteins but do not play a role in defence reactions but are linked to a resistant response (Dangl and McDowell, 2006; van der Hoorn and Kamoun, 2008; Jones et al., 2016; Deng et al., 2020). When an effector changes the structure of the guardee/decoy, the corresponding R-protein is activated. With this strategy a small number of R-proteins can protect against diverse pathogen effectors that target the

same host proteins. Cf-2 is an example for an RLP immune receptor from tomato, conferring resistance to both *G. rostochiensis* and the leaf mold fungus *C. fulvum* (Lozano-Torres et al., 2012).

In the "integrated decoy" strategy (Cesari et al., 2014) an R-protein includes an additional sequence, the "decoy" that is targeted by a pathogen effector protein, this complex then activates a signalling cascade and leads to resistance (Figure 1-3).

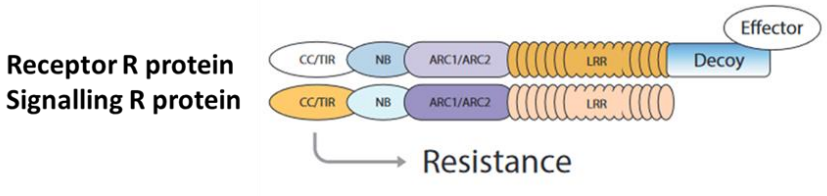


Figure 1-3 "Integrated decoy" model to obtain resistance to pathogens
The decoy for the pathogen effector is integrated into the structure of the receptor component of an R-protein pair, allowing the effector recognition by direct binding. Adapted from Cesari et al. (2014). Permission to reprint/adapt by a Creative Commons Attribution International License CC BY 4.0.

1.4.1.2 R-proteins

Pattern recognition proteins (PRRs) are cell surface receptors that recognise PAMPs. They include receptor kinases (RKs) and receptor-like proteins (RLPs) (Boutrot and Zipfel, 2017). The majority of the intracellular R-proteins from plants involved in ETI are the highly diverse and rapidly evolving nucleotide binding leucine rich repeat (NB-LRR) proteins (Spoel and Dong, 2012). This protein family belongs to the nucleotide binding domain leucine rich repeat containing (NLR) proteins, that are important for the detection of pathogens and the activation of the innate immune system. This leads to programmed cell death, in both plant- and animal cells, where they are called NOD-like receptors (Eitas and Dangl, 2010; Maruta et al., 2022). Despite their similarity, it is thought that plant and animal NLRs evolved independently (Urbach and Ausubel, 2017). Plant NLRs have been found to be more diverse than animal NLRs (Maruta et al., 2022). Their common basic structure consists of three domains, firstly, the N-terminal domain, which consists in plants either of the Toll/Interleukin-1 receptor (TIR) domain, a coiled-coil (CC) domain, or a resistance to powdery mildew 8-like (RPW8 or CC-R) coiled-coil domain (Maruta et al., 2022) and is important for signal transduction (Bentham et al., 2017). Secondly, the central domain is a conserved ATPase domain from the superfamily signal transduction ATPases with numerous domains (STAND),

which possibly evolved from a bacterial ancestor of plants and animals. The third domain, the C-terminal LRR domain, is responsible for pathogen recognition (Jones et al., 2016; Bentham et al., 2017); many plant NLRs have additional domains that can be important for the effector recognition and the regulation of NLRs. For *R*-genes and PCN resistant potato genotypes see Sections 4.1 and 5.1, respectively.

1.4.1.3 Role of small RNA molecules in plant pathogen defence

Another route of plant defence is host induced gene silencing (HIGS), in which small RNA molecules produced in the plant silence post-transcriptionally the expression of targeted pathogen RNA in the cytoplasm (Weiberg and Jin, 2015; Siddique and Grundler, 2018; El-Sappah et al., 2021). Although there is currently no information on the role of HIGS in plant-nematode interactions, Siddique and Grundler (2018) suggest that the exchange of RNA molecules between host plants and pathogens could be common in plant-pathogen interactions. A role for small RNAs in the control of gene expression in cyst nematode feeding structures has been identified (Hewezi et al., 2008).

1.4.1.4 Induction of PAMP-triggered immunity upon PPN infection

PTI can be induced during nematode infection either as a result of detection of nematode PAMPs or through detection of damage- associated molecular patterns (DAMPs) released as cyst nematodes damage the roots when moving intracellularly. Both types of molecule can be detected by PRRs leading to the induction of PTI (1.4.2). The *Arabidopsis* leucine-rich repeat (LRR) receptor-like kinase (RLK) NILR1 is the only cyst nematode PRR so far reported (Mendy et al., 2017), although the identity of the molecule that this PRR recognises remains unknown. Recently, a compound termed Asc#18 from the conserved family of the ascaroside plant pheromones was identified as a NAMP which induces PTI in plants (Manosalva et al., 2015). Moreover, PTI induced by Asc#18 increases the resistance of viral, bacterial, oomycete, fungal and nematode infections in both monocot and dicot plants (Manosalva et al., 2015).

1.4.1.5 Induction of ETI by PPN

Effector recognition by R-proteins can occur extra- and intracellularly. Extracellular R-proteins have a similar structure to the PRRs (Boutrot and Zipfel, 2017). However, most

effectors are recognised intracellularly by NB-LRRs thereby activating ETI. The interaction of GpRB1-Gpa2 is one example of a well-studied effector—R-protein pair. Virulent and avirulent variants of the SPRYSEC effector protein Gp-RBP1 have been described that differ only in a single amino acid exchange in the SPRY domain (Sacco et al., 2009). The avirulent variant GpRB1 that is recognized by Gpa2 has a proline at position 187, and recognition of this protein induces ETI. Virulent forms of GpRB1 have a mutation in this position and are not recognized by Gpa2, therefore they avoid the immune response of the plant (Sacco et al., 2009). While RBP1 conforms to the model for recognition and evasion common to other plant pathogens, the Cg-1/Mi-1 pair clearly operates differently. Tomatoes carrying the gene *Mi-1* confer resistance to some species of RKN, such as *Meloidogyne javanica*, that have the *Cg-1* gene. RKN in which *Cg-1* has been deleted become virulent on tomatoes with the gene *Mi-1* (Gleason et al., 2008; Gross and Williamson, 2011; Sato et al., 2019) strongly suggesting a role in recognition. However, the *Cg-1* locus encodes only a very short potential open reading frame, and the function of this transcript remains uncertain.

1.4.1.6 R-protein independent mechanisms for resistance to H. glycines in soybean

The mechanisms that lead to resistance involving R-proteins as described above have been intensely studied. In soybean, two loci *rhg1* and *rhg4*, conferring resistance to *H. glycines* with a different mechanism were described. Protein products from the allele *rhg1b,* from soybean accession PI 88788 are cytotoxic and are accumulated in the nematode feeding site thereby conferring full resistance to *H. glycines*, whereby the level of resistance is dependent on the copy number of the genes on locus *rhg1* (Cook et al., 2012). This resistance is broadly used in the USA. Another resistance, from a Peking accession, requires low copy numbers of the allele *rhg1a* and the resistance allele of the dominant quantitative trait locus (QTL) *Rhg4* (Shaibu et al., 2020). A serine hydroxy methyl transferase has been identified as responsible for the resistance to *H. glycines* (reviewed in Siddique and Jones (2021)). For resistance to PCN no similar mechanism is known, however Butler et al. (2019) showed that potatoes transformed with the soybean *Rhg1* genes become resistant to PCN.

1.4.2 PPN control host plant hormone pathways

Phytohormones are important factors in plant development and stress responses. By manipulating these pathways, pathogens can suppress defence responses, or they can take control of plant development pathways (Ma and Ma, 2016; Gheysen and Mitchum, 2019).

Auxins are a class of plant hormones, with indole-3-acetic acid (IAA) being the major auxin, that are crucial in many growth and developmental processes such as cell division, root development and organogenesis (Gheysen and Mitchum, 2019; Oosterbeek et al., 2021). IAA, and possibly other auxins as well, are accumulated at the nematode infection side at initiation of feeding sites in both RKN and CN, this higher level of auxin leads to secondary root formation (*de novo* organogenesis) (Olmo et al., 2017; Oosterbeek et al., 2021) and is provoked by the nematode. Plants with an auxin mutation are less susceptible to PPN infection (Oosterbeek et al., 2021). Auxin biosynthesis genes are upregulated by 75% upon RKN infection of *A. thaliana* (Oosterbeek et al., 2021) whereas auxin repressor genes are turned off (Gheysen and Mitchum, 2019). Auxin transport plays an important role in the initiation of the feeding site. PIN1, an auxin transporter, is down regulated in cells infected with CN. This leads to a local accumulation of auxin by preventing efflux of auxin out of the cell. Inoculation of *H. schachtii* in *pin1* mutant plants resulted in 40% fewer cysts (Grunewald et al., 2009). After the initiation, the auxin is active mainly in the periphery of the feeding site, probably to facilitate the radial expansion of the syncytium. In order to achieve this the auxin transporters PIN3 and PIN4 are relocalised to the lateral cell membranes which promotes lateral auxin flow. Consistent with this, when *H. schachtii* is inoculated in *pin3* or *pin4* mutant plants, the cysts are smaller (Grunewald et al., 2009). *A. thaliana* with mutations in the influx proteins AUX1 and LAX3 show smaller galls when infected with RKN, (Kyndt et al., 2016). Two effector proteins have been identified in *H. schachtii* that are involved in the manipulation of the auxin transport, 19C07, which targets LAX3 and 10.A.07 which interacts with auxin regulator protein IAA16 (Gheysen and Mitchum, 2019). Furthermore, auxin that is produced and secreted by the nematode has been detected in RKN and CN (De Meutter et al., 2005),

the role of this nematode produced hormone in forming of a new feeding site is not clear yet (Gheysen and Mitchum, 2019).

Cytokinins (CK) are another class of phytohormones that control cell division and differentiation in plants, together with auxins; they are also involved in nutrient mobilisation. Chemically, they are N6-substituted adenine derivatives (Spallek et al., 2018; Gheysen and Mitchum, 2019). CK were detected in the secretion of *H. schachtii* and *M. incognita* (De Meutter et al., 2003). Siddique et al. (2015) identified a gene for CK synthesis in *H. schachtii* that is expressed in early infection stages. Plants with mutations that have lower endogenous CK levels or high levels of cytokininoxidase show smaller syncytia than wildtype which results in less susceptible plants (Dowd et al., 2017; Spallek et al., 2018; Gheysen and Mitchum, 2019). Therefore, both plant and nematode CK are important for the formation of syncytia.

1.4.3 Suppression and evasion of the plant immune response

Many pathogens have evolved strategies to suppress or evade the plant immune system, in order to fulfil their life cycle. Several examples of proteins secreted by PCN that enable suppression of the immune system are now known.

Venom allergen-like proteins (VAPs) are a large family of effector proteins that are present in both animal parasitic nematodes and PPN; they are secreted abundantly, and they are necessary for the start of parasitism. One example is *Gr*VAP1 from *G. rostochiensis*, which is secreted by infectious juvenile nematodes. VAP1 suppresses plant immune responses by targeting the extracellular protease RCR3. This protease is a target for many other plant pathogens including *P. infestans* and *Cladosporium fulvum*. RCR3 is by the R-protein Cf2 (Lozano-Torres et al., 2014).

Another important family of effectors consists of a **SP**1a domain and three mammalian Ca^{2+} release channel **ry**anodine receptors (first identified in *Dictyostelium discoideum*) that are secreted. Therefore, this effectors family is called SPRYSEC. The SPRYSEC effector GpSPRY-414-2 from dorsal gland cell of J2 *G. pallida* nematodes can suppress PTI by suppressing the ROS production that is induced by flg22, the mechanism involves the manipulation of the microtubule bundles by direct interaction with StCLASP a CLIP associated protein (CLASP) from potato, that is necessary for the dynamics of the microtubule network (Galjart, 2005; Lawrence et al., 2020). This

effector suppresses ETI that is activated by Gpa2 and its matching effector RBP1 (Mei et al., 2018).

1.4.4 Virulence factors

Plant pathogens excrete/secrete molecules, so-called virulence factors that enable them to infect and colonise host tissues (Spallek et al., 2018). The vast majority of virulence factors are secreted proteins and peptides, also known as effectors. However, plant hormone analogues, produced by the pathogen have also been reported in PPN. Many animal parasitic nematodes transfer molecules such as small RNAs, lipids or toxins to the host to facilitate parasitism via extracellular vehicles (*e.g.*, Bobardt et al. (2020); Vanhamme et al. (2020)). These molecules manipulate host plant cells to the benefit of the nematode, such as the initiation and maintenance of the feeding site or changes in the plant defence response (Quentin et al., 2013). Virulence factors can be divided in two main groups, firstly factors that are involved in establishing and maintaining of the feeding site and secondly factor that enable the evasion/suppression of the plant immune response.

1.4.4.1 Effectors

There are many different definitions of effectors in the literature. Some consider effectors solely as molecules that suppress plant defence responses. However, for plant-nematode interactions, other functions such as feeding site induction are included (Diaz-Granados et al., 2016) Effector proteins of PCN are mainly produced from three oesophageal gland cells (one dorsal, two subventral) and are secreted into the host via the stylet.

The selection pressure on effector molecules has led to large effector gene families that have evolved by duplication and diversification of genes (Lilley et al., 2018; Vieira and Gleason, 2019). Before the wide availability of high throughput sequencing, effector candidates were identified by looking for genes that were highly expressed in J2s and less in other stages, that were expressed in the oesophageal glands and had a signal peptide for secretion. The availability and analysis of the *G. pallida* genome has allowed much larger scale identification of effectors. Many effectors are encoded by large gene families (Cotton et al., 2014; Thorpe et al., 2014; Eves-van den Akker et al., 2016).

With the sequencing of the genomes of both potato cyst nematodes, motifs in the promoter region of effector genes were discovered which can be used to find other potential effector genes that are associated with the promoter. For example, a promoter region found in most of the *G. rostochiensis* effector families secreted from the **do**rsal **g**land cell contain a conserved promoter region called the DOG box (Eves-van den Akker and Birch, 2016; Eves-van den Akker et al., 2016) . A similar element was found in the soybean CN *H. glycines*, which was used for the prediction of new effector genes (Masonbrink et al., 2019). A similar strategy led to the identification of the STATAWAARS box associated with effector genes of the pinewood nematode *Bursaphelenchus xylophilus* (Espada et al., 2018) and another promoter motif in the root lesion nematode *Pratylenchus penetrans* associated with effector genes was discovered (Vieira et al., 2018) using this approach.

The large and diverse family of SPRYSEC effectors are present in CN but not in RKN (Vieira and Gleason, 2019). The first SPRYSEC effectors were cloned by Qin et al. (2000) (*G. rostochiensis*) and Grenier et al. (2002) (*G. pallida*), followed by the detection of another 35 SPRYSEC effector candidates by analysis of expressed sequence tags of transcripts (Diaz-Granados et al., 2016). SPRY domain proteins can be found in animals, plants and fungi and this domain is thought to be involved in protein-protein interactions (Perfetto et al., 2013). Whereas most nematode species encode up to 25 SPRY domain proteins, *G. pallida* has over 300 with a subset of about 30 having a N-terminal secretion peptide that are thought to be effector proteins (Mei et al., 2015; Diaz-Granados et al., 2016). SPRYSEC effector proteins are known to activate ETI as well as to suppress the plant immune response.

CLAVATA-like (or CLE peptides) are another well studied effector protein family in CN. These effectors are mimics of plant CLE peptides and manipulate plant developmental signalling pathways to establish a feeding site. Plant CLE peptides are important in multiple signalling pathways, such as determination of the cell fate, proliferation and differentiation, and meristem development. CLE peptides have only been described in nematodes and plants (Guo et al., 2017).

Lilley et al. (2018) present a theory of “effector gene birth”. Most plants and animals have a housekeeping gene called glutathione synthetase (GS) gene and the corresponding protein protects cells from redox stress. Some PPN show a huge

increase in number of GS-like genes which are expressed in the oesophageal glands during the parasitic life stage, and by acquiring a secretion signal peptide are secreted into the host plant during the infection period, becoming “GS-like effectors”.

CWDE and cell wall modifying proteins are effectors that play an important role in the early stages of nematode infection and in migration within the roots (Haegeman et al., 2012). Many are secreted into the apoplast but also cell cytoplasm and facilitate infection and migration (Vieira et al., 2011). β-1,4-endoglucanase, a cellulase, was the first effector identified from PPN (Smant et al., 1998). Cellulases from this family, the glycosyl hydrolase family 5 (GHF5), have been identified in different nematode genera including, but not limited to *Meloidogyne, Globodera, Heterodera, Pratylenchus, Radopholus, Ditylenchus, Rotylenchulus and Aphelenchus* (Haegeman et al., 2012). Other plant CWDE have been identified in PPN, such as pectate lyases which cleaves the internal alpha-1,4-linkages of pectate and more (reviewed in Haegeman et al. (2012)). Most of the CWDE genes are expressed are expressed during the migratory life stages in the subventral gland cells of the nematode (Haegeman et al., 2012). The similarity of CWDE effectors of PPN with bacterial genes led to the hypothesis that PPN have acquired these genes by multiple horizontal gene transfer from bacteria (Haegeman et al., 2011; Vieira and Gleason, 2019).

1.4.4.2 Phytohormone analogues produced by parasitic nematodes

Various plant pathogens, including sedentary PPN, produce and release plant hormone analogues of *e.g.*, auxin, gibberellic acid (GA), ethylene, abscisic acid, or CK into the plant cells. These may take over hormone signalling pathways of the host to increase susceptibility by reducing the plant defences to the host plant and to activate the cell cycle in and around the syncytium (Siddique and Grundler, 2018; Spallek et al., 2018). However, this is not the only mechanism for controlling plant hormone pathways. In many cases, the nematodes secrete effectors, which lead to the accumulation of a hormone by the host plant, and both plant and nematode produced hormones seem to be necessary for the formation of syncytia (Gheysen and Mitchum, 2019).

1.5 Scope of the thesis

The project focuses on resistance of potato cultivars to *G. pallida* populations and on work to identify molecular markers that can distinguish the level of virulence to given potato cultivars. In the first part of this project *G. pallida* populations are investigated (Chapters 2 and 3). In Chapter 2, selected British *G. pallida* populations are characterised for their ability to grow on different potato genotypes and their composition regarding the introduction from South America through their mitotypes is examined. No functional association between mitotype and virulence is expected. However, in European there is a very small number of introductions. Each introduction (assuming it is composed of a relatively small number of individuals) will have a defining mitotype and will have a certain virulence status. In this work it will be determined whether there is a link between mitotype and virulence profile that has the potential to be used as pathotype markers for British *G. pallida* populations. Over time, if populations are mixing, this link is expected to break down. Chapter 3 also focuses on *G. pallida*, with the aim to find potential molecular pathotype markers suitable for high throughput screening and the genetic relationship of different European *G. pallida* populations. In Chapters 4 and 5 the focus shifts to the potato plants, with the aim to map a potentially new type of resistance in the wild potato species *S. spegazzinii* (Chapter 4) with the attempt to introgress this resistance to commercially important potato cultivars (Chapter 5).

2 Characterisation of historical and current British *G. pallida* populations

2.1 Introduction

Knowing the composition and virulence status (pathotypes) of *G. pallida* populations in fields is one aspect of targeted management strategies to control this pathogen. For example, knowledge of the virulence status of *G. pallida* in a field could impact the choice of potato cultivar being grown in the field. Furthermore, knowing how the composition changes over time, and the possible generation of new hybrid populations with different genotypes and virulence against resistance sources is invaluable information for the generation of potato cultivars containing the resistance needed for current and evolving *G. pallida* populations.

2.1.1 Distribution of PCN in the UK

In the UK, PCN symptoms were first reported 1904 by a farmer in Yorkshire, probably about 30 to 50 years after PCN was introduced into Europe/UK and become established there. In the 1910s, occurrences were reported in Scotland and East England. *G. rostochiensis* was the dominant species early on, but after the deployment of the *H1* resistance gene in cultivars such as Maris Piper, *G. pallida* has become prevalent in the UK. Minnis et al. (2002) showed in a survey in England and Wales that almost 2/3 of the tested land were infested with PCN, with 67% having *G. pallida*, 8% *G. rostochiensis* and 25% both species. In a more recent survey from England, Wales and Scotland, Dybal (2018) confirmed the continuous shift towards *G. pallida* with almost 89% of PCN infested land having only *G. pallida,* 5% only *G. rostochiensis* and 6% containing both species. Figure 2-1 shows a J2 nematode, female nematodes and mature cysts of *G.pallida*.

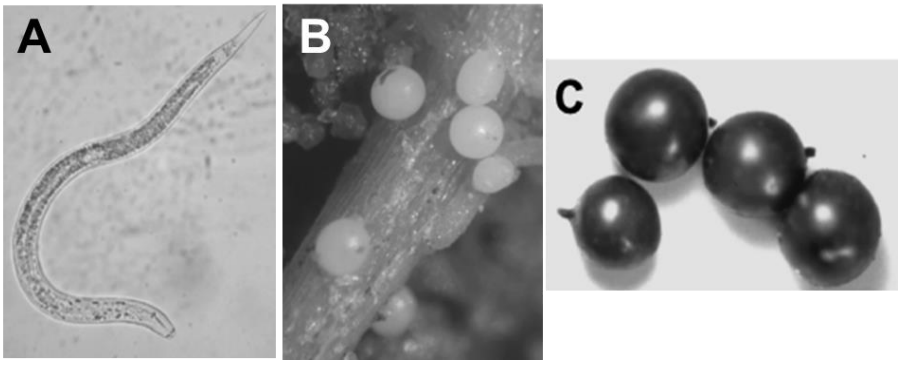


Figure 2-1 Different life stages of *G. pallida*
Picture (A) shows one hatched J2 nematode. (B) shows adult female nematodes parasitizing a potato root and (C) shows spherical *G. pallida* cysts. This figure is adapted from Lilley et al. (2005). Permission to reprint/adapt by RightsLink®, licence no. 5418000086301.

In Scotland, the incidence of *G. pallida* is doubling every six to seven years, whereas the spread of *G. rostochiensis* is increasing at slightly less than 1% per year (Pickup et al., 2018a). In 2010, about 13,500 Ha were found to have *G. rostochiensis*, in 2017, 14,200 Ha were found to be contaminated. By contrast, the infestation for *G. pallida* was 2,400 Ha in 2010 and 5,200 Ha in 2017 (Pickup et al., 2018a). As seed potato production is very important for Scotland and land used for seed production needs to be free of PCN, this development is of concern for the protection of the land. Testing of all seed land prior to planting and performing a survey of 0.5% of ware potato land per year is a requirement of the EU Council Directive 2007/33/EC (Pickup et al., 2012).

2.1.2 Mitotyping as tool to detect genetically distinct populations of a species

Mitochondria are cytoplasmic organelles that carry some genetic information on their own DNA. They are thought to have evolved from bacterial endosymbionts and they are present in almost all eukaryotes (Gray, 2012; Ladoukakis and Zouros, 2017). Generally, mitochondrial DNA (mtDNA) is inherited through the maternal germline and is therefore non-Mendelian (van den Ameele et al., 2020). However, in cucumber, for example, mitochondria are inherited paternally (Havey et al., 2004). Transmission of mitochondria from both parents in animals by doubly uniparental inheritance is found in several species of molluscan bivalves (Zouros, 2013). In addition, in some organisms which were considered as having strictly maternal inheritance, paternal leakage has been reported at a low frequency, *e.g.,* Wang et al. (2021) (fish), Polovina et al. (2020) (*Drosophila*) or Hoolahan et al. (2011) (*G. pallida*). Paternal leakage of mtDNA in

humans is controversial (Luo et al., 2018; Lutz-Bonengel and Parson, 2019). Uniparental inheritance facilitates the tracing of lineages. Recombination, though at a low rate, has been reported in different organisms, including *G. pallida* (Armstrong et al., 2007; Gibson et al., 2007b; Hoolahan et al., 2012b; Kern et al., 2020; Schwartz, 2021). Paternal leakage, recombination and mutagenesis, leads to heteroplasmy; that is the occurrence of different mtDNA circles within a cell or an organism. Despite this, using mtDNA to investigate relationships within and between species and to distinguish introductions of non-indigenous species has become an established approach. However, exceptions to the paradigm of maternal inheritance and recombination need to be borne in mind.

2.1.2.1 mtDNA of PCN

In most metazoans, mtDNA consists of a single circular DNA molecule, which is present in multiple copies per mitochondrion and encodes 12 or 13 proteins involved in electron transport and oxidative phosphorylation as well as ribosomal RNAs (rRNAs) and transfer RNAs (tRNAs). However, PCN contain more than one circle of mtDNA in a multipartite conglomeration. *G. pallida* was shown to have at least six small circular mtDNA molecules (scmtDNA) with a size ranging from ~6 to 9 kb (Armstrong et al., 2000). Similarly, Gibson et al. (2007a) describe six circles of mtDNA in *G. rostochiensis*. More recently, Phillips et al. (2016) reported that the mitochondrial genome of *G. ellingtonae* consists of two circles of mtDNA. Only four animal phyla (nematodes, dicyema, arthropods and rotifers) are known to have such multiple mitochondrial chromosomes (Kern et al., 2020).

The multipartite structure, gene organization and mosaic structure of mtDNA in *G. pallida* were investigated in more detail. Gibson et al. (2007b) and Armstrong et al. (2000) completely sequenced and mapped coding regions onto scmtDNAs I-V. There is a high content of duplicated genes, sometimes with one functional copy and multiple pseudogenes with a different levels of divergence; scmtDNA I and IV contain either functional genes or highly degenerate pseudogenes, whereas scmtDNA II, III and V contain pseudogenes, except *cytb* on scmtDNA III (Gibson et al., 2007b). Some of the scmtDNA molecules have been shown to be mosaics, *i.e.,* they comprise a combination of different scmtDNA molecules. For example, scmtDNA II has one section derived

from scmtDNA I and another from scmtDNA IV. Such mosaic patterning is unusual for animal mtDNA but common in plant mtDNA (Gibson et al., 2007b). It is thought that this organization of the scmtDNA is due to inter-mitochondrial recombination.

The scmtDNA molecules present in *G. pallida* show differences in frequency in different populations. For example, the Luffness population which scores as pathotype 3 in the Kort-scheme and which is virulent against both *H3* and *GpaV* resistance sources, was found to have predominantly scmtDNA I and shows no detectable scmtDNA II and III with Southern blotting, whereas population Gourdie (Pa2/3) shows a predominance of scmtDNA II and III and an absence of scmtDNA I on Southern blots, although scmtDNA I can be detected with PCR (Armstrong et al., 2007). The Peruvian *G. pallida* population P4A shows scmtDNA I and III as found in populations Gourdie and Luffness, as well as different variants of scmtDNA IV (Armstrong et al., 2007). This could indicate that *G. pallida* lineages can segregate and retain their mitochondrial structure.

G. pallida mtDNA also has large non-coding regions, which is unusual for animal mtDNA. These regions share high similarity between the different scmtDNA molecules (Gibson et al., 2007b). Despite the above-described differences to mtDNA from the majority of metazoan, scmtDNA sequences from *G. pallida* have proven to be useful in phylogenetic studies.

2.1.2.2 Mitochondrial cytb marker

Armstrong et al. (2000) reported two copies of *cytb* on scmtDNA I and III, that are highly similar. When Picard et al. (2007) used a subsequence of 872 bp of the *cytb* gene for their phylo-geographic studies of *G. pallida* in the Andes, performed on individual cysts, they saw a distinct pattern of the *cytb* mitotypes that were geographically structured. This and subsequent studies allowed the *G. pallida* populations in Peru, from which the *G. pallida* introduced to Europe is derived, to be identified.

Eves-van den Akker et al. (2015b) used *cytb* for their studies on the composition of *G. pallida* populations in Scottish fields. For this work they identified a set of five SNPs within the *cytb* sequence that can distinguish between *G. pallida* introductions in the region of 281-590 bp downstream from the start codon (Table 2-1).

Table 2-1 *cytb* allele polymorphisms in *G. pallida* "single cyst" lines
In this table the five allele polymorphisms for *cytb* to distinguish different *G. pallida* introductions to Britain are shown. Adapted from Eves-van den Akker et al. (2015b). Permission to reprint/adapt by a Creative Commons Attribution International License CC BY 4.0. Note that in the paper the positions are mislabelled, the corrected position are in brackets. The different mitotypes are associated with different *G. pallida* populations in the collection.

	Relative SNP position					Associated with population
GenBank	**61**	**118 (117)**	**137 (135)**	**158 (157)**	**228 (225)**	
DQ631812.1	**G**	**A**	**A**	**A**	**T**	
Mitotype 1	G	A	A	A	T	Luffness JHI f1
Mitotype 2	G	G	A	A	C	Pa1 JHI
Mitotype 3	A	G	G	C	T	Lindley JHI
Sequence no. 7	G	G	G	C	T	--

2.1.2.3 Mitochondrial marker s222 from a non-coding region on scmtDNA II and IV

Hoolahan et al. (2012a) described a different mitochondrial marker from a non-coding region of scmtDNA IV, called x222 (Armstrong et al., 2000), concluding that the sequence diversity within this region could be used as a phylogenetic marker to distinguish different introductions of *G. pallida* into Britain (and Europe). The sequence diversity in this non-coding region of scmtDNA is higher than in *cytb* (Armstrong et al., 2007). This is almost certainly due to the fact that *cytb* encodes an essential protein in the mitochondrial electron transport chain, so only limited genetic mutations are tolerated.

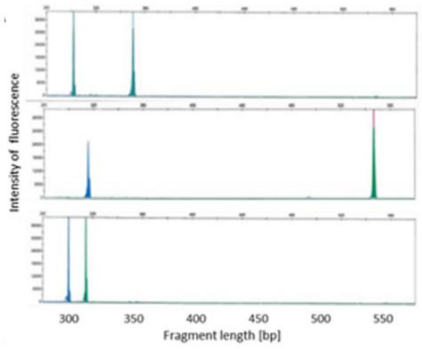
Based on these findings, Grujic (2010) developed a terminal restriction fragment length polymorphism (TRFLP) assay, with the aim of providing a marker to distinguish groups of different *G. pallida* populations and to detect possible new introductions or hybridisations of *G. pallida* in Europe with a sensitive and (semi) high-throughput method. Part of the s222 non-coding region from scmtDNA II and IV were amplified and three groups each of *G. pallida* populations representing scmtDNA II and IV respectively were detectable (Grujic, 2010). Table 2-2 shows the terminal fragments of different *G. pallida* population types in an *in-silico* digest of s222 with *Taq*1.

Table 2-2 TRFLP obtained from different *G. pallida* populations
MtDNA fragments s222 were digested with the restriction enzyme *Taq*1 and showed differences in the DNA fragment size for different *G. pallida* populations.

	Mitotype	**Fragment length of s222 digested with *Taq1* [bp]**	
		5'	**3'**
scmtDNA II	1 (two subgroups)	297	637
		297	320
	2	304	318
	3	308	318
scmtDNA IV	1 (two subgroups)	167	625
		171	630
	2	298	355
	3	311	555

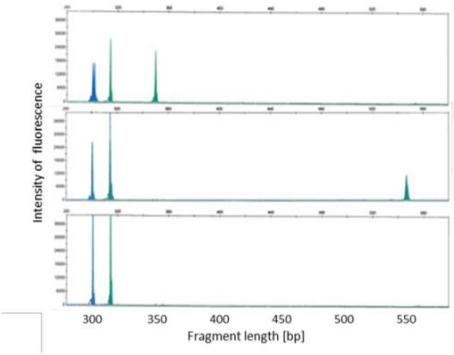
Mróz and Blok (personal communication) then investigated the s222 TRFLP pattern (*Taq*1 digest) of three British populations, Lindley, Luffness and Pa1, which match the three different Kort scheme pathotypes Pa2, Pa3 and Pa1 respectively. They cloned the s222 fragments of these populations and performed a TRFLP assay with the *Taq*1 digested fragments. Figure 2-2 panel A shows the three different TRFLP patterns obtained. When total DNA from the same populations was analysed with this assay, population Lindley JHI showed a mixture of patterns 1 and 3 called mitotype A, population Luffness JHI field1 (f1) showed a mixture of patterns 2 and 3 called mitotype B, and Pa1 JHI was only pattern 3 called mitotype C (Figure 2-2 panel B). So far, cysts from all but one population showed one of the three pattern combinations (1&3, 2&3, 3 only); the new TRFLP pattern observed in one cyst looked like 1&2 (Gartner and Blok unpublished). This observation could be an artefact, however, Eves-van den Akker et al. (2015b) also reported one *G. pallida* population with a different *cytb* mitotype, named sequence no. 7, from the same region in Scotland.

A TRFLP patterns of cloned fragments



Pattern
1
2
3

B TRFLP patterns of *G. pallida* populations



Pattern combination	s222 mitotype	Associated with *G. pallida* population
1&3	A	Lindley JHI
2&3	B	Luffness JHI f1
3 only	C	Pa1 JHI

Figure 2-2 s222 mitotype patterns in three British *G. pallida* populations
Panel A shows the different TRFLP patterns obtained when the s222 PCR amplification was cloned from three British *G. pallida* populations. In panel B the TRFLP patterns from the same *G. pallida* populations are shown, without cloning first. Panel B shows the chromatograms of the s222 fragment lengths when digested with the restriction enzyme *Taq*1 in these three populations. Fragments in blue are from the 5' terminus, and fragments in green are from the 3' terminus.

2.1.3 Composition of British *G. pallida* populations:

Some 50 years ago, cysts from PCN populations all over the UK were collected and have been maintained at the JHI since this time. Three genetically distinct *G. pallida* populations, which correspond to the three detected introductions to the UK were described and are represented by the populations called Lindley JHI, Luffness JHI f1 and Pa1 JHI. These three distinct populations also differ in their ability to multiply on different potato genotypes with different levels of resistance.

In order to determine the composition of *G. pallida* infested fields in respect to their mitotype, Eves-van den Akker et al. (2015b) analysed *G. pallida* DNA obtained from cysts collected from Scottish potato growing land during annual surveys of ware land. Using next generation sequencing, they determined a set of five SNPs, that allowed discrimination of genetically distinct populations (Table 2-7). Three mitotypes, 1, 2 and 3, were found all over Scotland; in one single field, another mitotype, called sequence 7 was detected, at 8% frequency in a mixture with mitotype 2. Sequence 7 resembles mitotype 3, but at position 61 there is a different SNP, usually found in mitotype 1 and 2 (Table 2-1). About 80% of the tested fields contained only one mitotype, 18% contained a mixture of two mitotypes (1 & 2, 2 & 3, and 1 & 3) and 2.3% a mix of all three mitotypes 1, 2, and 3. Figure 2-3 shows the distribution of the different mitotypes in Scottish fields. Type 3 is most widespread, followed by type 1.

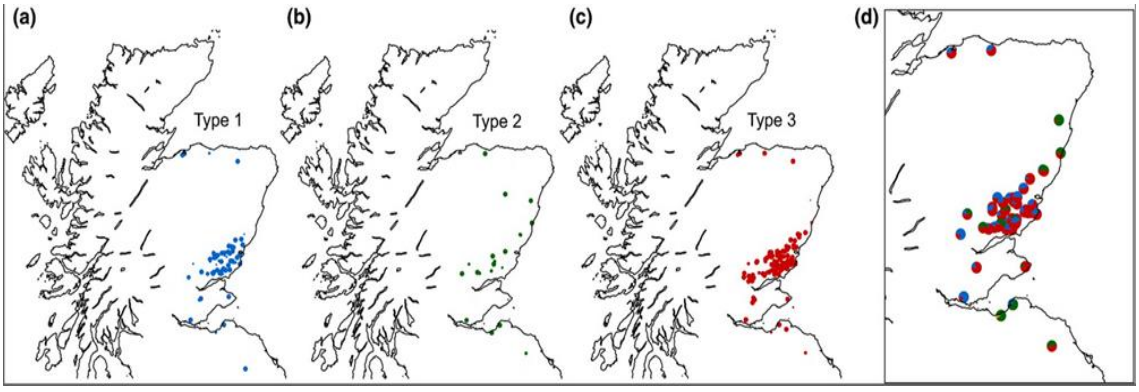


Figure 2-3 Distribution of distinct *G. pallida* populations in Scotland
Panels (a) to (c) show the incidence of the different *G. pallida* mitotypes. Type 1 (blue) is associated with population Luffness, type 2 (green) with population Pa1 and Type 3 (red) with population Lindley. Panel (d) shows the super-imposed pictures of all three mitotypes. In East Lothian, a fourth mitotype, called sequence 7 was detected in a field together with mitotype 2. Adapted from Eves-van den Akker et al. (2015b). Permission to reprint/adapt by a Creative Commons Attribution International License CC BY 4.0.

In a different approach, Grujic's TRFLP assay based on the noncoding s222 mtDNA region was used to compare individual cysts from different historical *G. pallida* populations with recently collected field cysts from UK field (Table 2-3) (Gartner, Mróz and Blok, personal communication). All cysts of the historical populations except one (Newton) show only one s222 mitotype with at least 80% frequency, whereas the results for the current *G. pallida* field populations indicate that they are typically composed of more than one introduction from South America. Historically, the

composition of *G. pallida* populations seems to be less complex than in some more recently sampled *G. pallida* field populations, especially from near Harper Adams University in Hertfordshire (HA) and Luffness in East Lothian, which show cysts of each of the three mitotypes. One population, East Lothian 3, showed a new mitotype, which looks like a combination of mitotypes A, B and C. This is in accordance with Eves-van den Akker et al. (2015b), who observed a new mitotype of *cytb* in one sample (sequence 7) which was from the same region as the one in this study. For the *G. pallida* population Luffness f1 cysts from the JHI collection and recently collected field cysts were available for this current analysis. These observations indicate that UK fields contain multiple introductions of *G. pallida,* so they might also be mixtures with different levels of virulence.

Table 2-3 s222 mitotype on selected historical and recent field UK *G. pallida* populations
Single cysts from field and collection populations were analysed with the s222 TRFLP assay. Empty cells represent 0% frequency (Gartner, Mróz and Blok, personal communication)

			s222 mitotype				
	Population	**Origin**	**A**	**B**	**C**	**New type**	**No. of cysts**
Historical JHI collection	Lindley	England	96%	4%			26
	Luffness f1	Scotland	6%	94%			35
	Pa1	Scotland	10%	5%	85%		20
	Bedale	England	86%	14%			7
	Farcet	England	91%	9%			11
	Halton	England	100%	0%			9
	Newton	England	50%	50%			10
Recently collected field samples	Luffness f1	Scotland	20%	70%	10%		20
	Luffness (other fields)	Scotland	60%		40%		5
	HA	England	43%	48%	9%		23
	East Lothian 1	Scotland		100%			9
	East Lothian 2	Scotland	50%	50%			6
	East Lothian 3	Scotland	38%	50%		13%	8
	East Anglia	England	100%				6
	Crow	England	33%		67%		3
	Bourne	England	92%	8%			13
	Legge	England	67%	33%			6
	Chinn	England	80%	20%			5
	Spalding	England	70%	30%			10
	Ash	England	50%	50%			2

2.2 Scope of this chapter

In this chapter it will be investigated whether individual cysts in a population show different levels of virulence. Firstly, in order to determine the composition of British *G. pallida* populations, "single cyst" lines, which are sub-populations derived from a single cyst of *G. pallida* populations generated from the historical JHI PCN collection and from field populations collected in the UK between 2010 and 2014 were generated. Then, wo different mitotype markers were applied and the virulence on six different potato genotypes was determined with the aim of investigating whether there is a relationship between the virulence and mitotype and if so, to determine the validity of using mitotypes as a proxy for virulence (pathotypes). This would allow the potential for development of mitochondrial pathotype markers. Furthermore, cyst diameters from cysts growing on different potato genotypes were analysed to determine whether there was a correlation between cyst size and virulence on a particular potato genotype.

Although no functional association between mitotype and virulence of a *G. pallida* population is expected, the different introductions to Europe (i.e., mitotypes) show to have a link between mitotype and virulence profile on population level. This could be explained by an introduction of a small number (3 are described so far) of only a few individual cysts. When these introductions are hybridising, this link is expected to disappear over time.

To investigate whether cysts from different introductions in a field also show different virulence to potato genotypes, populations developed from single cysts from selected *G. pallida* collection and field populations were generated ("single cyst" lines) by multiplying one cyst of a population on the susceptible potato genotype Desirée. The following years, 10 cysts from the previous multiplication were used to further multiply cysts, until at least 500 cysts were available. These "single cyst" lines were then assessed for their virulence phenotype against six potato genotypes with different sources of resistance (Table 2-5) using pot bio-assays (Section 2.3.5). Two mitotyping methods, one based on the s222 non-coding region and the other on the *cytb* gene, were used with these subpopulations to determine if the mitotype can be used as a consistent marker for the virulence of a population.

2.3 Materials and Methods:

2.3.1 *G. pallida* populations:

Table 2-4 lists the *G. pallida* populations investigated in this chapter. They are a combination of cysts recently collected from fields in East Lothian (Luffness), East Anglia (AB) and near Harper Adams University (HA) in Shropshire (Kaczmarek et al., 2019) as well as some from the JHI PCN collection which have been maintained for ~50 years.

Table 2-4 British *G. pallida* populations used in this chapter

Name	Year sampled	Geographical origin	Notes
Lindley JHI	In the 1970s	West Yorkshire, England	
Luffness JHI f1		East Lothian, Scotland	
Pa1 JHI		Duddingston, Scotland	
Luffness f1	2011 & 2014	East Lothian, Scotland	Kaczmarek et al. (2019) & this project
Luffness field 3 (f3)			
HA	2011 & 2012	Shropshire, England	Kaczmarek et al. (2019)
AB field A	2013	East Anglia, England	Blok, unpublished
AB field PB			

2.3.2 Potato genotypes

Six potato genotypes with different sources of resistance to *G. pallida* were used to assess the level of virulence (phenotype) of the *G. pallida* populations. The tubers were stored at RT two weeks before planting them to let them sprout. Table 2-5 shows the potato genotypes with their sources of resistance.

Table 2-5 Potato genotypes used and their source of resistance to *G. pallida*

Potato genotype	Source of *G. pallida* resistance
S. tuberosum cv. Desirée	None
S. tuberosum cv. Maris Piper	None, presence of *H1* resistance to *G. rostochiensis*
S. tuberosum differential clone P55/7	*H2, S. multidissectum*
S. tuberosum cv. Vales Everest	*H3, S. tuberosum* group *andigena* CPC2802
S. tuberosum differential clone 62.33.3	*GpaV, S. vernei* V24/20
S. tuberosum cv. Innovator	*GpaV, S. vernei* LGU8

2.3.3 Multiplication of PCN cysts

To obtain sufficient numbers of cysts from the "single cyst" lines to perform all the required experiments and also to maintain the lines, 10 cysts from each single cyst line were multiplied in 400 g 1:1 sterilized sand/loam mixture on the susceptible potato *S. tuberosum* cv. Desirée in a heated, but not air-conditioned greenhouse, with a daylength of 16 h, shading applied at a light intensity over 450 W/m^2 and with a temperature of 20˚C during the day and 15˚C during the night. The pots were irrigated from above, liquid feed was applied every 2 weeks and biological pest (insect) control was applied if required. After 12 weeks watering was stopped, and the soil was allowed to dry out. The cysts were then washed out from the dry sand/loam mixture with water using 700 and 250 µm sieves, and dried and stored at 4˚C for at least 3 months before they were counted. Their viability was then assessed using a hatching test.

2.3.4 Hatching tests

Three replicates of five cysts of each population were placed into wells of a 24-well petri-dish containing 2 ml tomato root diffusate (TRD: made by soaking roots from 2 tomato plants (cv. Moneymaker) in dH_2O for 2 h at RT and filtering through a paper filter, (Palomares-Rius et al., 2016)). The plate was incubated at 20˚C in the dark for 28 days in total; after 1 week, the juveniles with TRD were transferred to a petri dish with gridlines and counted with a low-power microscope; fresh TRD was added to the cysts, and they were further incubated. The counting and top up with TRD was repeated once to twice a week.

2.3.5 Determination of PCN virulence:

For the determination of the PCN virulence, a pot bio-assay (2.3.5.1) was performed in which the multiplication rate of a PCN population was determined for 6 potato genotypes with different (or no) sources of resistance to PCN (Table 2-5). These different multiplication rates were then assessed for their virulence and pathotype (2.3.5.2).

2.3.5.1 pot bio-assay

Clay pots were filled with 400 g 1:1 sterilized sand/loam mixture and sunk into a sand

bed in an unheated greenhouse. Bags with 15 cysts (made by sealing them in nylon mesh (125 µm) obtained from Normesh Limited) to ensure that only newly formed cysts were counted, were added to the pots. The historical populations Lindley JHI, Luffness JHI f1 and Pa1 JHI were used as controls for the determination of the virulence and pathotypes, as they correspond to Pa2, Pa3 and Pa1, respectively. Pots were laid out according to a randomized block design, using 24 bags per cyst line, as each sample was tested in 4 replicates. One potato piece containing one eye, cut out with a melon scoop, was put into each pot. After 12 weeks, the plants were no longer watered and, when the soil was dry, the cyst bag and plant material were removed. The cysts were collected by the floatation method using a MEKU nematode carousel (MEKU Pollähne) on 24 cm round filter papers (Macherey-Nagel MN 751) at SASA, Edinburgh (Reid et al., 2015). The cyst number for each sample was counted under a low-power microscope.

2.3.5.2 Evaluation of the virulence/pathotype

The evaluation of the phenotype assessment to determine the virulence was performed using the relative resistance score of potato genotypes to *G. pallida* populations, which was introduced by EPPO (2006). This scheme is now used to assess potato varieties for resistance in a standardized way in the EU and UK. Here it was used to assess the virulence of *G. pallida* populations, without classifying them into rigid pathotypes. The cyst counts of each *G. pallida* population tested were normalized to the cyst count on the potato cultivar Desirée in the same experiment, to get a susceptibility value relative to this susceptible cultivar. This relative susceptibility was then categorized into 9 groups, called the resistance score. Table 2-6 shows the relative susceptibility to Desirée in % and the corresponding resistance score. Score 9 is the highest level of resistance, a score of 1, 2 or 3 is considered susceptible. Pathotypes were assigned by determining the multiplication rates of the potato genotypes Desirée, P55/7 and 62.33.3 (Kort et al., 1977).

Table 2-6 Resistance score for the assessment of the resistance level of potatoes to *G. pallida* Reprinted from EPPO (2006). Permission to reprint/adapt by RightsLink®, licence no. 5418020289726. Resistance scores of 1 to 3 are considered as susceptible to *G. pallida*, the level of resistance increases the higher the resistance score with level 9 being the highest level.

Relative susceptibility (%)	Resistance score
<1	9
1.1-3	8
3.1-5	7
5.1-10	6
10.1-15	5
15.1-25	4
25.1-50	3
50.1-100	2
>100	1

2.3.6 Cyst measurements

When only few cysts are growing on potato genotypes (*i.e.,* resistant potatoes), it is important to take the egg numbers into account for the determination of the resistance score. In this study, the cyst volume, which was estimated by measuring and averaging the cyst diameter of up to 30 cysts per sample, was used as a proxy for the egg number (Stelter and Gaur, 1969). Pictures of cysts (up to 35 cysts per picture) with a scale bar were taken with a camera attached to a low-power microscope (Olympus). The diameter of the cysts was then measured using ImageJ software and statistically analysed with the software Genstat 20th edition.

2.3.7 TRFLP-assay for mitotype marker s222

This assay consists of the extraction of total DNA of individual cysts or pools of cysts, the PCR amplification of DNA of the mitochondrial s222 with primers that are labelled with two different fluorophores The individual steps of the method are described below.

2.3.7.1 DNA extractions from cysts

Individual cysts were selected under a low-power microscope with tweezers and placed into a 1.5 ml Eppendorf tube. Single cysts were crushed with a plastic pestle in 30 µl of 1x PCR buffer (Promega) supplemented with 1.5 mM $MgSO_4$ (if not already in PCR buffer), centrifuged at 13,000 rpm for 30 sec and the supernatant was transferred

to a 0.2 ml PCR tube and heated to 95˚C for 15 min. It was then cooled on ice and 3 µl of 10 mg/ml Proteinase K (Roche, P6556) was added and incubated at 65˚C for 2 h, followed by 15 min at 95˚C, then stored at -20˚C, unless used for PCR immediately.

2.3.7.2 PCR amplification of the DNA s222

PCR-reactions containing 1.5 µl 10x HF buffer (Invitrogen), 0.6 µl primers F3-mtDNA-222(FAM) (5'-ATTAGACCGATAAGTTTACACCTTG-3') and SCMT4-8(HEX) (5'-GACTAGGTCCATCAATCTGAACC-3') (10 µM), respectively, 0.6 µl $MgSO_4$ (50 mM), 1.0 µl dNTPs (2 mM), 0.6 µl BSA (10 mg/ml), 0.2 µl Platinum *Taq* Polymerase (Invitrogen), 8.9 µl H_2O and 1.0 µl DNA were heated to 94°C for 2 min, then for 40 cycles at 94°C for 30 sec, 55°C for 30 sec, 68°C for 60 sec, and finally 68°C for 10 min.

2.3.7.3 Digestion with restriction enzyme

A 1 µl aliquot of master mix comprised of 0.1 µl MULTI-CORE® buffer, 0.8 µl H_2O, 0.1 µl restriction enzyme *Taq*1 (Promega Cat No. R615A, 10 units/µl) per reaction was transferred to each well of a 96 well PCR plate, then 5 µl fluorescent PCR product, which was kept in the dark, was added to each well, mixed, and briefly centrifuged. Reactions were digested for 4 h at 65°C in the dark, then frozen at -20°C or processed to the next step.

2.3.7.4 TRFLP electrophoresis and evaluation

A 9 µl aliquot of master mix (895 µl formamide (Sigma) and 5 µl ROX1000 marker (GeneScan™ 401098) was added to each well of a 96 well plate (AB600); then 1 µl of the TRFLP digestion product was added to each well. The plate was transferred to the JHI Genome Technology Facility and run on an ABI micro-capillary gel sequencer (Applied Biosystems) with laser detection. The fluorescent reads were analysed using Peak Scanner 2 software from Applied Biosystems.

2.3.8 *cytb* mitotyping

Eves-van den Akker et al. (2015b) describe a diagnostic region of *cytb* which can serve to identify the three known introductions from South America to the UK. The SNP patterns are summarized in Table 2-1. DNA which was used for s222 mitotyping was PCR amplified with the primers described in Eves-van den Akker et al. (2015b) to amplify the region 281-590 GenBank DQ631912.1 (*cytb* gene). PCR reactions

containing 4 µl 5x Phusion HF buffer (NEB), 0.6 µl primers F1 (5'-CTTGAAGACCTTCTGTAAAAATG-3') and R1 (5'-CGAGCTACCGTCTTAAGAG-3') (10 µM), respectively, 1.2 µl $MgSO_4$ (25 mM), 1.25 µl dNTPs (2 mM), 0.2 µl Phusion DNA Polymerase (NEB M0530S), 11.15 µl H_2O and 1.0 µl DNA were heated to 98°C for 30 sec, then 35 cycles with 98°C for 30 sec, 57°C for 30 sec and 72°C for 60 sec and a final extension at 72°C for 5 min was performed. All samples were checked by electrophoresis (2% agarose gel), then the PCR amplifications were cleaned by adding 2 µl of ExoSAP-IT (Thermo Fisher 78200), which removes excess primers and nucleotides, incubating for 15 min at 37°C and then inactivating the enzyme for 15 min at 80°C. The samples were then sent to the JHI Genome Technology Facility and sequenced in both directions with the F1 and R1 primers shown above. They were analysed with Sequencher 5.4.6 software from GeneCodes.

2.4 Results

2.4.1 Generation of "single cyst" lines

Table 2-7 lists the PCN "single cyst" lines that were used in at least one of the following experiments: genotyping (Chapter 3), mitotyping or resistance phenotyping in a pot bio-assay (Chapter 2), as well as the PCN population they are derived from and shows the cyst numbers, obtained in two to four multiplication rounds. Single cysts from population Luffness JHI f1 did not produce enough cysts after four years to perform phenotyping experiments. In contrast, field populations Luffness and HA produced up to 80 and 120 cysts, respectively, in the first year from individual cysts.

Table 2-7 "Single cyst" lines and their source PCN populations used in this project
Genotyping was used for phylogenetic analysis (Chapter 3), mitotyping and phenotyping for resistance is described in this chapter. The asterisks indicate in which assays the populations were used.

G. pallida population	"Single cyst" line	Cyst no.	Genotyping	Mitotyping	Phenotyping
Lindley JHI		In stock		*	*
	Lindley JHI 3	2110	*	*	*
	Lindley JHI 11	4060	*	*	*
	Lindley JHI 12	780	*		
Luffness JHI f1		In stock	*	*	*
	Luffness JHI 4	44	*		
	Luffness JHI 13	55	*		
	Luffness JHI 15	64	*		
Pa1 JHI		In stock	*	*	*
	Pa1 JHI 3	1550		*	*
	Pa1 JHI 12	1510	*		*
AB		~100	*		
	AB A8-2	1970			*
	AB PB3	1480			*
	AB PB12	1000			*
HA		~100	*		
	HA 9	2560	*	*	*
	HA 10	274	*		
	HA 12	1890	*	*	*
	HA 27	1210	*	*	*
	HA 32	840	*	*	*
	HA 34	1860	*	*	*
	HA 39	1859	*	*	*
	HA 41	435	*		
	HA 43	145	*		
	HA 47	320	*		
	HA 49	1000	*	*	*
	HA 50	189	*		
	HA 51	129	*		
	HA 52	143	*		
Luffness f1		~100	*		
	Luffness 1-4	1040	*	*	*
	Luffness 1-12	3560	*	*	*
	Luffness 1-14	40	*		
	Luffness 1-19	1200	*	*	*
	Luffness 1-26	155	*		
	Luffness 1-30	1500	*	*	*
Luffness f3		~100	*		
	Luffness 3-8	1300	*	*	*
	Luffness 3-17	1530	*	*	*
	Luffness 3-18	1680	*	*	*

2.4.2 Mitotyping of the “single cyst” lines

2.4.2.1 Mitochondrial marker cytb

The SNP patterns at five loci that determine the mitotype (Table 2-1) were identified in *G. pallida* populations from the JHI collection. Population Lindley JHI is associated with the SNP pattern AGGCT, Luffness JHI f1 with GAAAT and Pa1 JHI with GGAAC. Sequence no. 7 was reported by Eves-van den Akker et al. (2015b) as well as Gartner, Mróz and Blok (personal communication) but was not observed in this work. 218 individual cysts of 21 “single cyst“ lines were analysed for the mitotype defining SNP pattern; 104 of these were mitotype 1 (48%), 3 (1%) had mitotype 2 and 111 (51%) were mitotype 3. 15 of the 21 lines showed a “pure” mitotype, that was defined by having >80% one mitotype at the cyst level, otherwise they were defined s being of “mixed” mitotype. The “single cyst” lines with “mixed” mitotype 1&3 were Lindley 3 JHI, HA 34, HA 39 and HA 49, mitotype 2&, 3 Pa1 3 JHI and a mixture of all 3 mitotypes was Luffness 1-4. In order to further investigate this result, more cysts per line would need to be analysed. The three cysts with mitotype 2 were detected in line Pa1 3 JHI (2 cysts), which is expected, and Luffness 1-4 (1 cyst) (Table 2-8).

Table 2-8 *cytb* allele polymorphisms in *G. pallida* "single cyst" lines

This table shows the number of cysts with the different mitotypes in the "single cyst" lines. * The numbers in bold indicate the dominant mitotype, the numbers in brackets indicate that the dominant mitotype has an occurrence of >80%. # with this low cysts number analysed the result is not robust to determine the mitotype(s) composition of the population.

	No. of cysts with mitotype					
"Single cyst" line	**1 [GAAAT]**	**2 [GGAAC]**	**3 [AGGCT]**	**No. 7 [GGGCT]**	**No. of cysts analysed**	**Mitotype(s)***
Lindley JHI 3	1	0	3	0	4	1 & **3**
Lindley JHI 11	0	0	10	0	10	**3**
Pa1 JHI 3	0	2	4	0	6	2 & **3**
Pa1 JHI 12	0	0	11	0	11	**3**
AB A8-2	0	0	8	0	8	**3**
AB PB3	1	0	13	0	14	**3 (&1)**
AB PB12	0	0	3	0	3	**3**
HA 9	6	0	0	0	6	**1**
HA 12	23	0	0	0	23	**1**
HA 27	5	0	0	0	5	**1**
HA 32	1	0	5	0	6	**3**
HA 34	1	0	1	0	2	#
HA 39	2	0	5	0	7	1 & **3**
HA 49	4	0	2	0	6	**1** & 3
Luffness 1-4	9	1	2	0	12	**1** & 2 & 3
Luffness 1-12	23	0	2	0	25	**1** (& 3)
Luffness 1-19	5	0	0	0	5	**1**
Luffness 1-30	18	0	0	0	18	**1**
Luffness 3-8	0	0	18	0	18	**3**
Luffness 3-17	0	0	13	0	13	**3**
Luffness 3-18	2	0	11	0	13	**3** (& 1)

2.4.2.2 Mitochondrial TRFLP marker s222

All “single cyst” lines were also tested with the TRFLP marker s222. Figure 2-2 panel B shows a summary of the different TRFLP patterns in different British *G. pallida* populations. Table 2-9 shows the mitotypes found in individual cysts of the same 21 “single cyst” lines used in 2.3.1 and additionally, of the field populations HA and Luffness f1 and the collection populations Lindley JHI, Luffness JHI f1 and Pa1 JHI. In addition to the “single cyst” lines, 390 individual cysts of 21 “single cyst“ lines and 5 pooled populations were mitotyped for s222 with TRFLP; 212 of these were mitotype A (54%), 157 (40%) had mitotype B and 21 (5%) were mitotype C. 16 of the 21 “single cyst” lines showed a “pure” mitotype, the two field populations showed a mixture of all three mitotypes and the JHI populations were “pure” mitotypes with Lindley associated to mitotype A, Luffness to B and Pa1 to C. All “single cyst” lines with “mixed” mitotype were from the HA group, with HA 9, HA 27 and HA 34 having A and B, HA 49 B and C, and HA 39 having all three mitotypes. The cysts with mitotype C were detected in lines HA 12 (1 cyst), HA 39 (2 cysts) HA 49 (3 cysts), and the populations HA (2 cysts), Luffness f1 (2 cysts) and Pa1 JHI (10 cysts).

Table 2-9 s222 mitotype for selected *G. pallida* "single cyst" lines and populations
This figure shows the number of individual cysts detected with the three mitotypes and the consensus mitotype. The letters in bold indicate the dominant mitotypes. * The numbers in bold indicate the dominant mitotype, the numbers in brackets indicate that the dominant mitotype has an occurrence of >80%

"Single cyst" line/population	No. of cysts with mitotype A	B	C	No. of cysts analysed	Mitotype(s)*
Lindley JHI 3	12	1	0	13	**A** (& B)
Lindley JHI 11	10	0	0	10	**A**
Pa1 JHI 3	10	0	0	10	**A**
Pa1 JHI 12	11	0	0	11	**A**
AB A8-2	10	0	0	10	**A**
AB PB3	10	0	0	10	**A**
AB PB12	2	0	0	2	**A**
HA 9	2	4	0	6	A & **B**
HA 12	0	11	1	12	**B** (& C)
HA 27	11	10	0	21	A & B
HA 32	10	2	0	32	**A** (& B)
HA 34	5	4	0	9	A & B
HA 39	5	6	2	13	A & B & C
HA 49	0	6	3	9	**B** & C
Luffness 1-4	2	20	0	22	**B** (& A)
Luffness 1-12	1	12	0	13	**B** (& A)
Luffness 1-19	0	10	0	10	**B**
Luffness 1-30	0	9	0	9	**B**
Luffness 3-8	27	3	0	30	**A** (& B)
Luffness 3-17	24	2	0	26	**A** (& B)
Luffness 3-18	13	1	0	14	**A** (& B)
HA	10	10	2	22	A & B & C
Luffness f1	4	14	2	20	A & **B** & C
Lindley JHI	27	0	1	28	**A** (& C)
Luffness JHI f1	4	32	0	36	**B** (& C)
Pa1 JHI	2	0	10	12	**C** (& A)

2.4.2.3 Comparison of the mitotypes s222 and cytb

The data generated from the s222 and *cytb* analysis were compared to firstly determine if the two mitotypes provide the same results regarding the composition of the populations, as both s222 and *cytb* markers were used to distinguish different introductions of *G. pallida* in Britain (see Table 2-1 and Figure 2-2). Secondly, by analysing the mitotypes from cysts which have been tested for both markers I analysed whether the data for individual cysts were correlated.

In Table 2-10 the mitotyping results for both markers on the 21 "single cyst" lines are listed together for comparison. In the "single cyst" lines with "pure" mitotypes s222

mitotype A correlates a 100% to *cytb* mitotype 3 and s222 B to *cytb* 1. In the 6 lines with "mixed" *cytb* mitotype, s222 shows 3 "pure" mitotypes (Lindley JHI 3, Pa1 JHI 3 and Luffness 1-4), with the dominant mitotype correlating to A-3 and B-1 as seen above. HA 34 shows *cytb* mitotypes 1 and 3, which is correlating to s222 mitotypes A and B, HA 39 as well show *cytb* mitotypes 1 and 3, the s222 mitotypes however, show A and B and C. HA 49 shows *cytb* mitotypes 1 and 3, s222 B and C, however, in both the dominant mitotypes are 1 and B respectively. There are also lines which show a "pure" *cytb* mitotype with a "mixed" s222 mitotype; HA 9 and HA 27 show *cytb* mitotype 1 and s222 mitotypes A and B. The two mitotyping assays correlate in general, mitotype A (s222) is linked to 3 (*cytb*), B is linked to 1. There are not enough samples with mitotype C and 2 link these mitotypes in a statistically significant way.

Table 2-10 Comparison of the markers s222 and *cytb* on "single cyst" lines
Individual cysts of the lines were analysed with both markers and compared to see if they provide the same introduction pattern on a single cyst level (see also Tables 2-8 and 2-9). Mitotypes in bold indicate the dominant mitotypes.

"Single cyst" line	Consensus mitotype(s)	
	s222	***cytb***
Lindley JHI 3	**A**	1 & **3**
Lindley JHI 11	**A**	**3**
Pa1 JHI 3	**A**	2 & **3**
Pa1 JHI 12	**A**	**3**
AB A8-2	**A**	**3**
AB PB3	**A**	**3**
AB PB12	**A**	**3**
HA 9	A+**B**	**1**
HA 12	**B**	**1**
HA 27	A+B	**1**
HA 32	**A**	**3**
HA 34	A+B	1 & 3
HA 39	A+B+C	1 & **3**
HA 49	**B**+C	**1** & 3
Luffness 1-4	**B**	**1** & 2 & 3
Luffness 1-12	**B**	**1**
Luffness 1-19	**B**	**1**
Luffness 1-30	**B**	**1**
Luffness 3-8	**A**	**3**
Luffness 3-17	**A**	**3**
Luffness 3-18	**A**	**3**

Next, cysts with both mitotypes *cytb* SNP pattern and s222 TRFLP, when available, were compared to each other and are shown in Table 2-11. Panel A shows the mitotypes obtained per “single cyst” line and panel B shows the number of cysts obtained for each mitotype combination. Both mitotypes were available from 146 cysts in 21 “single cyst” lines. 88 showed mitotype A for s222, 87 (99%) of which showed *cytb* mitotype 3, one had *cytb* mitotype 2 (Pa1 JHI 3). 56 cysts showed mitotype B, 55 (98%) of which had also *cytb* mitotype 1, 1 showed mitotype 3 (Luffness 3-8). One of the two cysts showing s222 TRFLP mitotype C shows *cytb* mitotype 1 (HA49) the other mitotype 2 (Pa1 JHI 3). This means that a correlation between s222 mitotype A and *cytb* mitotype 3 and s222 mitotype B and *cytb* mitotype 1 could be seen, although this was not 100%. s222 mitotype C was present in too few cysts to draw a conclusion.

Table 2-11 Comparison of *cytb* and s222 mitotypes from the same cysts

DNA from individual cysts were analysed for both s222 and *cytB* mitotypes, and the number of cysts detected with the s222/*cytb* combinations are listed in panel A. Panel B shows total number of mitotype combinations detected in the 21 "single cyst" lines.

A

"Single cyst" line	No. of cysts	Mitotype s222	Mitotype cytb	No. of cysts analysed with both markers
Lindley JHI 3	4	A	3	4
Lindley JHI 11	9	A	3	9
Pa1 JHI 3	1	A	2	2
	1	A	3	
Pa1 JHI 12	8	A	3	8
AB A8-2	5	A	3	5
AB Pb-3	12	A	3	13
	1	B	1	
AB Pb-12	2	A	3	2
HA 9	4	B	1	4
HA 12	4	B	1	4
HA 27	4	B	1	4
HA 32	4	A	3	5
	1	B	1	
HA 34	1	A	3	2
	1	B	1	
HA 39	4	A	3	6
	2	B	1	
HA 49	2	B	1	3
	1	C	1	
Luffness 1-4	2	A	3	12
	9	B	1	
	1	C	2	
Luffness 1-12	2	A	3	12
	10	B	1	
Luffness 1-19	4	B	1	4
Luffness 1-30	12	B	1	12
Luffness 3-8	11	A	3	12
	1	B	3	
Luffness 3-17	12	A	3	12
Luffness 3-18	10	A	3	11
	1	B	1	

B

Mitotype	s222 A	s222 B	s222 C
1	0	55	1
2	1	0	1
3	87	1	0

2.4.3 Phenotyping for virulence on potato genotypes with different levels of resistance

The “single cyst” lines were assessed for virulence using a pot test. The historical collection populations, Lindley JHI, Luffness JHI f1 and Pa1 JHI, which show different virulence phenotypes (pathotypes) on a panel of potato genotypes were included as controls. Prior to the pot test hatching assays were performed to ensure that the cysts were viable and that living nematodes were hatching (2.3.4). The experiment was performed four times between 2016 and 2019. In 2018 very few cysts were produced even on the susceptible cultivars Desirée and Maris Piper, so phenotyping was not possible. This may be the result of exceptionally hot and dry conditions during the hatching phase. In total, two sets of data for each population are available.

Figure 2-4 shows the resistance scores for the tested *G. pallida* lines. In panel A, the average cyst diameter is included in the analysis, panel B only considers the cyst number in the analysis. The susceptibility scores differ slightly in A and B, with the susceptibility score usually, but not always scoring higher (better resistance of the potato) when the cyst size is included; however, the overall picture stays the same. An exception is HA 12 on Innovator, where the susceptibility score changes from 7 to 9 when including the cyst diameter, indicating smaller cysts compared to cysts grown on Desirée.

Innovator shows a high level of resistance with all lines tested. Vales Everest shows lower but still good resistance but was more susceptible to the Lindley “single cyst” lines and the two HA lines HA 12 and HA27. P55/7. Potato line 62.33.3 showed a higher variability of scores, this being consistent with partial resistance. Both Maris Piper and Desirée were susceptible to all *G. pallida* lines tested. The biggest difference in the phenotypes of “single cyst” lines between cysts of the same source population occurred in the HA lines. The Lindley “single cyst” lines had a similar phenotype that was more virulent than Lindley JHI, the population of origin, much more like the collection population Luffness JHI.

A

Name	Desirée	Maris Piper	P55/7	62.33.3	Vales Everest	Innovator	Kort pathotype
Source of Resistance#	none	*H1*	*H2*	H3	*GpaV*	*GpaV*	
Lindley 3	2	1	2	5	4	9	Pa3
Lindley 11	2	2	2	5	6	9	Pa3
Pa1 3	2	2	8	8	8	9	Pa1
Pa1 12	2	3	7	7	9	9	Pa1
AB 8-2	2	2	3	7	7	9	Pa2
AB PB3	1	2	4	7	8	8	Pa2
AB PB12	2	1	2	9	8	8	Pa2
HA 9	1	1	2	6	8	9	Pa3
HA 12	1	2	4	5	6	9	Pa3
HA 27	2	2	4	6	6	9	Pa3
HA 32	2	2	5	6	7	9	Pa3
HA 34	2	2	4	7	8	8	Pa2
HA 39	2	2	2	8	8	8	Pa2
HA 49	1	2	4	6	7	9	Pa3
Luffness 1-4	2	1	4	6	7	8	Pa3
Luffness 1-12	2	2	6	5	8	9	Pa3
Luffness 1-19	2	2	4	6	7	8	Pa3
Luffness 1-30	2	3	3	4	7	8	Pa3
Luffness 3-8	2	2	6	9	9	9	Pa2
Luffness 3-17	2	2	3	7	7	9	Pa2
Luffness 3-18	1	3	5	9	9	9	Pa2
Lindley JHI	2	2	3	7	8	9	Pa2
Pa1 JHI	2	1	7	7	7	9	Pa1
Luffness JHI	2	3	4	5	8	9	Pa3

B

Name	Desirée	Maris Piper	P55/7	62.33.3	Vales Everest	Innovator	Kort pathotype
Source of Resistance#	none	*H1*	*H2*	H3	*GpaV*	*GpaV*	
Lindley 3	2	1	2	5	5	9	Pa3
Lindley 11	2	2	3	4	5	9	Pa3
Pa1 3	2	2	8	8	7	9	Pa1
Pa1 12	2	3	7	7	9	9	Pa1
AB 8-2	2	2	4	7	7	8	Pa2
AB PB3	1	2	4	6	7	8	Pa2
AB PB12	2	2	2	8	8	8	Pa2
HA 9	1	2	2	6	7	9	Pa3
HA 12	1	2	3	5	5	7	Pa3
HA 27	1	2	4	5	5	9	Pa3
HA 32	2	1	5	5	7	9	Pa3
HA 34	2	2	4	7	8	8	Pa2
HA 39	2	2	2	7	8	8	Pa2
HA 49	1	2	4	6	7	9	Pa3
Luffness 1-4	2	1	4	6	7	8	Pa3
Luffness 1-12	2	2	6	5	8	8	Pa3
Luffness 1-19	2	2	4	6	7	8	Pa3
Luffness 1-30	2	3	3	4	7	9	Pa3
Luffness 3-8	2	2	5	9	9	9	Pa2
Luffness 3-17	2	2	3	7	7	9	Pa2
Luffness 3-18	1	2	5	9	9	8	Pa2
Lindley JHI	2	2	3	7	8	9	Pa2
Pa1 JHI	1	2	7	7	8	9	Pa1
Luffness JHI	2	3	5	5	6	9	Pa3

Figure 2-4 Resistance scores of British *G. pallida* populations
Six different potato genotypes were inoculated with various *G. pallida* lines and the level of resistance was evaluated. The resistance scoring was performed according to EPPO (2006). The colour scheme allows for a quick visual assessment of the resistance levels. The "greener" the potato genotype the more resistant, and the "redder" the more susceptible. # More info about the potato resistance can be found in Section 2.3.2. Panel A shows the resistance scores, including the average cyst diameter on each potato genotype in the analysis as a proxy of egg numbers. Panel B only considers the cyst number in the analysis.

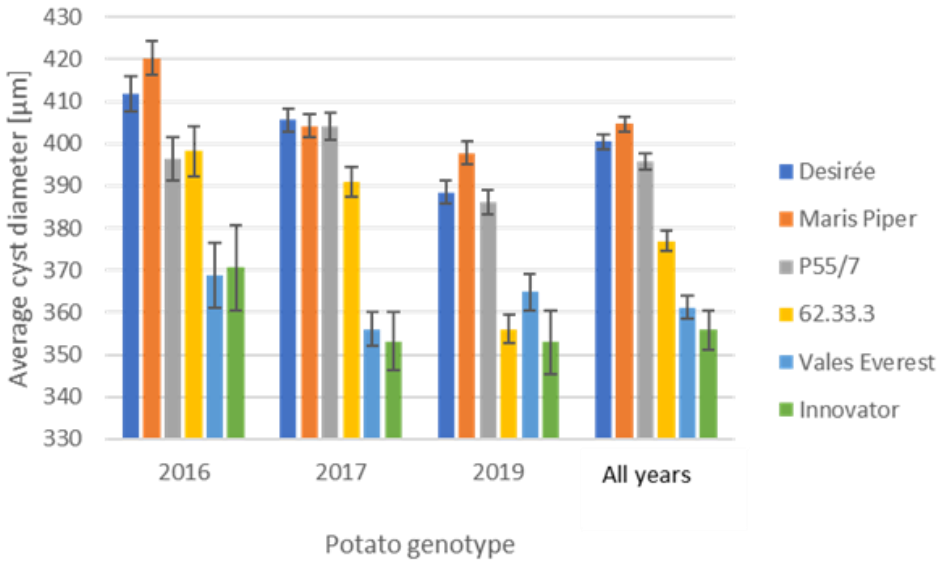
2.4.3.1 Cyst measurements

When measuring cyst diameters for the phenotyping experiment, it was noticed that cyst diameters showed considerable variation. To determine if potato genotypes have an influence on cyst sizes, cyst diameters were statistically analysed with an unbalanced ANOVA test. Figure 2-5 panel A shows the average cyst diameter in µm on the different potato genotypes for all *G. pallida* populations combined, for each year separately and all years combined. The absolute average cyst diameters vary from year to year. However, the trend in the potato genotypes is the same independent of the year. Panel B shows the relative diameter compared to potato genotype Desirée in percent. The diameter is about 95% in 62.33.3 and 90% in both Vales Everest and Innovator compared to the diameter in Desirée, with a higher variation in size, but this

could be due to lower cyst numbers. Cysts on Maris Piper were larger in 2016 and 2019, those on P55/7 were smaller in 2016.

The cyst diameters from Desirée and Maris Piper are not significantly different from each other, P55/7 shows a p value of <0.05 in respect to Maris Piper but not to Desirée, 62.33.3, Vales Everest and Innovator produced significantly (p <0.01) smaller cysts, with cysts from 62.33.3 being significantly larger than the other two potato genotypes.

A



B

	Relative cyst diameter compared to Desirée			
Potato genotype	2016	2017	2019	All years
Desirée	100%	100%	100%	100%
Maris Piper	102%	100%	102%	101%
P55/7	96%	100%	99%	99%
62.33.3	97%	96%	92%	94%
Vales Everest	90%	88%	94%	90%
Innovator	90%	87%	91%	89%

Figure 2-5 Cyst diameters of *G. pallida* populations grown on different potato genotypes
Panel A shows the average diameter of cysts grown on different potato genotypes per year. Panel B shows the relative cyst diameter in relation to potato cv. Desirée.

When looking at average cyst diameters on different potato genotypes at the population level, Pa1 shows the biggest reduction in size on P55/7 of all *G. pallida* populations, AB produced big cysts on Desirée, Maris Piper and P55/7. However, the sizes on the other potato genotypes was greatly reduced. HA only showed a reduced

size with Vales Everest and Innovator, not with 62.33.3. Luffness and HA show the least reduction on 62.33.3. Figure 2-6 shows the different diameters in cysts for different *G. pallida* populations.

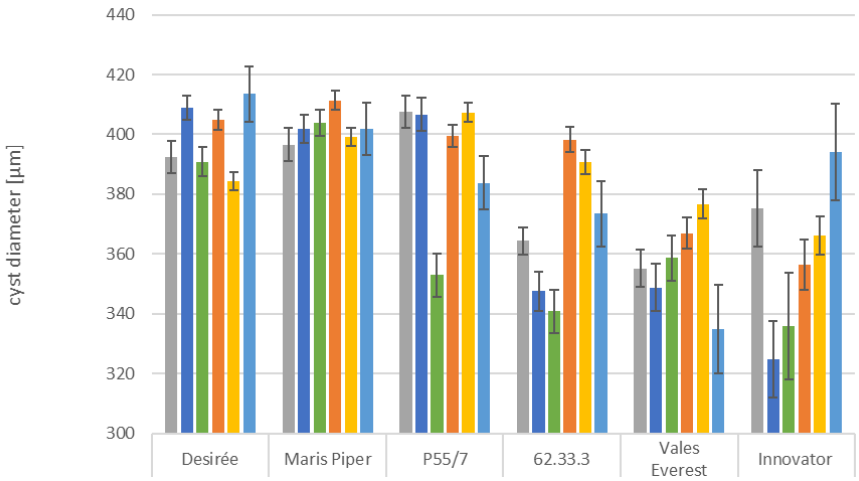


Figure 2-6 Cyst diameters of different potato genotypes from cysts grouped by populations
Grey stands for population Lindley JHI, dark blue for AB green for Pa1 JHI, orange for HA, yellow for Luffness f1 and light blue for Luffness f3.

Next, the cyst diameters were analysed as a function of the susceptibility score. The results are shown in Figure 2-7. There is a linear relationship between the resistance score and the average cyst size, independent of the potato genotype the cysts are grown on. Linear regression analysis was performed. The linear regression factor R^2 is 0.93. An initial one-way ANOVA analysis showed that there are highly significant ($p=1.2*10^{-19}$) differences between the susceptibility groups (degree of freedom between groups =1, within groups =16). Then, a Fisher's least significance difference test was performed to pairwise compare the size differences between susceptibility scores and showed that the size differences between scores 1 and 2, 4 and 5, 5 and 6, 7 and 8, 7 and 9, as well as 8 and 9 are not significant ($p>0.05$). All other cyst size differences between two scores are either significantly different ($p<0.05$) (2 and 4, 3 and 6) or very highly significant ($p<0.001$).

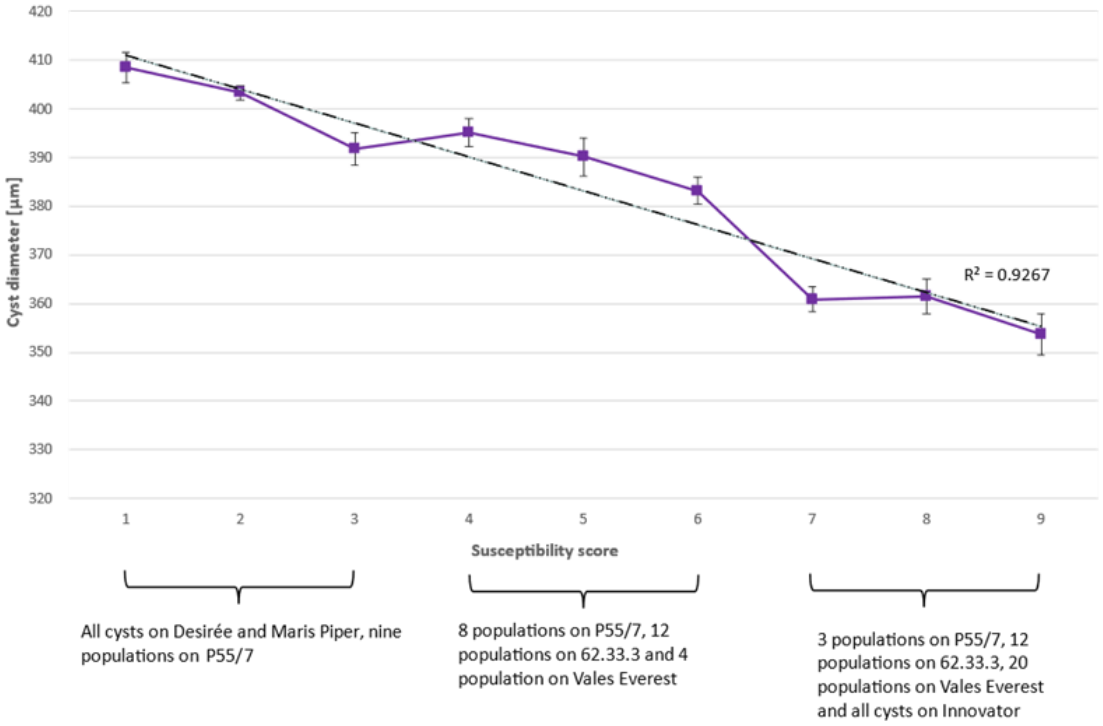


Figure 2-7 Cyst size as a function of the susceptibility score
The susceptibility score and the cyst diameter are in a linear correlation.

2.4.4 Is there a correlation between mitotype and virulence to potato genotypes with different sources of resistance in British *G. pallida* populations?

The TRFLP mitotypes and phenotypes of the "single cyst" lines on potatoes with different sources of *G. pallida* resistance were analysed together to determine whether the TRFLP assay could potentially be used as virulence (pathotype) marker (Figure 2-8).

Name	P55/7	62.33.3	Vales Everest	Innovator	s222 Mitotype
Lindley 3 JHI	2	5	4	9	A
Lindley 11 JHI	2	5	6	9	A
Pa1 3 JHI	8	8	8	9	A
Pa1 12 JHI	7	7	9	9	A
AB 8-2	3	7	7	9	A
AB PB3	4	7	8	8	A
AB PB12	2	9	8	8	A
HA 9	2	6	8	9	A+B
HA 12	4	5	6	9	B
HA 27	4	6	6	9	A+B
HA 32	5	6	7	9	A
HA 34	4	7	8	8	A+B
HA 39	2	8	8	8	A+B+C
HA 49	4	6	7	9	B+C
Luffness 1-4	4	6	7	8	B
Luffness 1-12	6	5	8	9	B
Luffness 1-19	4	6	7	8	B
Luffness 1-30	3	4	7	8	B
Luffness 3-8	6	9	9	9	A
Luffness 3-17	3	7	7	9	A
Luffness 3-18	5	9	9	9	A
Lindley JHI	3	7	8	9	A
Pa1 JHI	7	7	7	9	C
Luffness JHI	4	5	8	9	B

Figure 2-8 Combination of mitotypes and level of resistance to different potato genotypes
This figure combines the results from Figures 2-4 **A** and Table 2-9

If mitochondrial markers can be taken as indicative of phenotype then, according to the Kort-scheme (Kort et al., 1977), mitotype A would correspond to pathotype Pa2, mitotype B would correspond to Pa3, and mitotype C would correspond to Pa1.

The cyst lines used here with pathotype Pa1 were Pa1 JHI (the control population with s222 mitotype C) and the two "single cyst" lines were Pa1 3 JHI and Pa1 12 JHI. Comparison of the s222 mitotype between "single cyst" lines of Pa1 JHI and the source showed that Pa1 JHI has about 83% mitotype 2 and 17% mitotype 3, while the two "single cyst" lines Pa1 3 JHI and 12 JHI both showed mitotype A.

The control population Lindley JHI shows pathotype Pa2 with mitotype A; however, the derived lines Lindley 3 and 11 both show pathotype Pa3 and have mitotype A.

All "single cyst" lines derived from AB and Luffness field 3 have mitotype A and similar phenotypes to the control population Lindley JHI. All "single cyst" lines with mitotype B show the same pathotype as the control population Luffness JHI f1. Mitotype A occurs

in “single cyst” lines with both pathotypes Pa2 and Pa3, mitotype B is linked to pathotype Pa3 and mitotype C was only detected in a combination with mitotype A or B, but not on its own in a “single cyst” line. This means that the mitotype cannot be used as proxy for the virulence phenotype.

2.5 Discussion

Eves-van den Akker et al. (2015b) used polymorphism differences in the mitochondrial *cytb* to determine the *G. pallida* composition of Scottish fields. In this study, a TRFLP based assay using a non-coding region of mtDNA was used as an alternative, with the aim of generating a semi-high throughput marker for determining the virulence state of *G. pallida* on potato genotypes. In this study, amplicons from individual cysts were Sanger sequenced, whereas in Eves-van den Akker et al. (2015b), the samples underwent deep sequencing with MiSeq next generation sequencing and the DNA from was from pools of cysts. Both sequencing techniques led to the same SNP pattern for *cytb*.

The two mitochondrial markers are clearly linked to each other, as 87 out of 88 cysts tested showed the marker pair A (*cytb*) 3 (TRFLP), 54 of 55 cysts showed B (*cytb*) 1 (TRFLP), one cyst showed each A (*cytb*) 2 (TRFLP) and B (*cytb*) 3 (TRFLP). There are too few cysts with mitotype C or 2 to make a statistically significant statement about the third mitotype and any possible correlation. However, individual cysts of population Pa1 JHI show mainly the mitotypes C (TRFLP) and 2 (*cytb*), which suggests a correlation, even if both mitotypes were not determined in the same cysts.

The comparison between cysts collected from the same field, but some 50 years apart, Luffness JHI f1 and Luffness f1 indicate that the composition of historical *G. pallida* populations was likely less complex than the composition of current field populations, which contain nematodes representative of the three introductions of *G. pallida* into the UK that have taken place to date. This increasing complexity in field populations of *G. pallida* raises the possibility of hybridisation between different genotypes of *G. pallida* that coexist in the same field. Such hybridisation events allow the generation of novel genotypes with new combinations of *virulence* and *avirulence* genes that may lead to virulence phenotypes that need new sources of resistance genes or stacking of different known resistances to *G. pallida* into one potato genotype.

Interestingly, the “single cyst” lines of population HA consist of mixed consensus mitotypes, despite only one cyst being the origin of these sub-populations. MtDNA is usually inherited by the maternal germline; therefore, a pure mitotype would be expected in a line that is derived from a single cyst, in which all nematodes share the same maternal parent, but not necessarily the same paternal parent. The occurrence of a mixed mitotype in a “single cyst” line suggests that the different *G. pallida* introductions hybridize, and the occurrence of paternal leakage (Hoolahan et al., 2011). Other possibilities explaining a change of mitotype are mutation in the relevant scmtDNA molecule or recombination (Armstrong et al., 2007), which is also a rare event. It was instead presumed that different “single cyst” lines from one population would have different mitotypes, which was not the case except in the HA line.

The resistance scores with and without considering the cyst diameter as proxy for the number of eggs does not change the overall picture for the resistance performance although scores in resistant potato genotypes can change slightly (Figure 2-4). The virulence of “single cyst” lines varies the most in HA derived lines. When comparing “single cyst” lines with the corresponding source populations, they do not always behave in the same way. However, most “single cyst” lines derived from population HA behave like Luffness f1 in terms of virulence (pathotype). It would be interesting to test on more potato genotypes with a broader range of sources of resistance, e.g., as described in (Canto Saenz and Mayer de Scurrah, 1977) to determine whether an already described South American pathotype can be detected. This can be seen in population Lindley JHI and its “single cyst” lines Lindley 3 JHI and Lindley 11 JHI. These lines behave more like population Luffness from the JHI collection. This fact supports the reclassification of the pathotypes Pa2 and Pa3 to Pa2/3 (Trudgill, 1985), as no clear and reliable boundaries between Pa2 and 3 could be established. This is consistent with the PCN populations having a certain proportion of virulent and avirulent individuals for the distinction between Pa2 and Pa3, which can change depending upon selection pressure. Although there is a clearly a difference in virulence between the *G. pallida* populations Lindley and Luffness, there is no reliable method to determine it reliably with phenotypic assays. Typically, in these bio-assays, the results are quite variable with high standard errors.

In this work a resistance-level scheme instead of the rigid pathotype scheme from Kort et al. (1977) was used to determine the virulence of *G. pallida* populations/"single cyst" lines, as it is a more flexible approach that provides results which are independent of testing with specific potato genotypes, which may currently not be used and which may be difficult to obtain.

Canto Saenz and Mayer de Scurrah (1977) investigated races of PCN in the Andean region and proposed a new classification system. It would be very interesting to see if especially population HA could be characterised as a "South American" pathotype.

This work shows that mitotyping mtDNA with TRFLP of s222 is partly successful in predicting the phenotype of *G. pallida* populations from the UK. If a population has mitotype B, it is very likely to be highly virulent to the potato genotype 62.33.2, which differentiates between Pa2 and Pa3 in the Kort scheme, as was observed in population Luffness. However, the presence of mitotype A is not linked to a specific virulence phenotype of the *G. pallida* population. There were not enough cysts with mitotype C to draw statistically significant conclusions.

When the cyst sizes for the assessment of virulence (phenotypes) of the different single cyst lines as a proxy for egg mass were measured, it was noticed that cysts produced on potato cultivars with high resistance levels were generally smaller as well as having fewer cysts, with exceptions only where, for example, one very large cyst was produced. An explanation could be that syncytia are restricted in potato genotypes with high resistance levels, leading to the formation of smaller cysts as less food is available. The more virulent the *G. pallida* population on a potato genotype, the bigger the cyst size, *i.e.,* the more eggs per cyst, in addition to more cysts. The cysts analysed here were produced under controlled greenhouse conditions and cysts were also multiplied in a greenhouse; an interesting next step would be to determine if this correlation is robust in field cysts, where other environmental factors can influence the cyst size. Mwangi et al. (2019) have described a new *G. pallida* population from Germany. They reported an increase of more than 25% in diameter of cysts in virulent *G. pallida* populations compared with avirulent *G. pallida* populations. This result fits well into the findings of this work, in that the average size of the *G. pallida* cysts are dependent on the resistance level of the potato they are grown on. If the relative sizes of cysts grown on the different potato genotypes are taken into account, a trend for

smaller cysts on potatoes with resistance to the *G. pallida* population can be seen, for example *G. pallida* population Pa1 grown on P55/7 containing the *H2* resistance shows a 10% reduced cyst diameter compared to Pa1 cysts grown on Desirée, which corresponds to 25% fewer eggs. In this study, the size differences for *G. pallida* populations according to their origin is about 12%; however, Luffness f1 shows a difference of 30% in the cyst sizes between Desirée and Vales Everest, the cysts on Innovator are only 10 % smaller than on Desirée. The results for individual "single cyst" lines are not presented here, as the low number of cysts for the potato genotypes with high resistance level do not allow for statistically significant conclusions. In short, the cyst size depends on the potato cultivar they are growing on. On resistant cultivars, not only the number of cyst is reduced, but also the average cyst size. This was shown on different cultivars with different sources of resistance.

3 Genetic composition of British *G. pallida* populations

3.1 Introduction

In the previous chapter, selected British *G. pallida* populations and "single cyst" lines were characterised. I found that when the mitochondrial markers s222 and *cytb* show mitotype B and type 1 respectively, the PCN population is virulent to potato genotypes containing the resistances *H2* from *S. multidissectum, H3* from *S. tuberosum* group *andigena* CPC2802 or *GpaV* from *S. vernei* V24/20, whereas *G. pallida* populations with mitotype A for marker s222 (3 for *cytb*) is not indicative for any level of virulence. Therefore, these markers have only limited value as pathotype markers for PCN populations. In addition, diagnostic molecular markers for the quick and reliable discrimination between different levels of virulence (pathotypes), and which are easy to run in a high-throughput format are not available yet. Such markers would help to identify more routes of introduction and facilitate the determination of the relationships between different populations. Obtaining data about virulence is still a very elaborate and time-consuming process with bio-assays on defined potato genotypes in a greenhouse (2.3.5); moreover, due to *G. pallida* being a quarantine organism, safety measures and licences are needed.

To find such diagnostic molecular markers, the genetic variation between *G. pallida* populations with different pathotypes has been intensely studied. Before next generation sequencing, this was performed using various molecular approaches including restriction fragment length polymorphism (RFLP) (Phillips et al., 1992), random amplified polymorphic DNA (RAPD) (*e.g.,* Folkertsma et al. (1994); Blok et al. (1997)) and analysis of the internal transcribed spacer (ITS) region of the rDNA (*e.g.,* Blok et al. (1998); Subbotin et al. (2000); Pylypenko et al. (2005)). Grenier et al. (2001) used microsatellites (also known as simple sequence repeats or SSRs) and the mitochondrial *cytb* gene (*e.g.,* Picard et al. (2004)) to describe genetic variation. However, using these different methods, no linkage between any differences detected and different pathotypes were found that allowed them to be used as reliable pathotype markers.

The genotypes of 27 Peruvian and 15 European *G. pallida* field populations were analysed using 7 genomic microsatellite markers (Picard et al., 2004) and the mitochondrial *cytb* marker (Picard et al., 2007) to determine routes of introduction of *G. pallida* from South America to Europe (Plantard et al., 2008). Microsatellite markers were used to analyse populations of *G. rostochiensis* from all over the world into genetic clusters (Boucher et al., 2013). Eves-van den Akker et al. (2015b) used NGS data on mitochondrial *cytb* to determine the distribution and composition of *G. pallida* populations in Scotland. More recently, Thevenoux et al. (2020) studied the phylogeographic pattern of 119 PCN populations in the region of South America from where European populations were derived in more detail by using the intron length polymorphism of the cathepsin L gene as a marker. The cathepsin L gene consists of 12 introns and the three species *G. pallida*, *G. rostochiensis* and *G. ellingtonae* can be distinguished by their PCR amplification length polymorphisms in introns 4 and 5. Some of the populations tested showed different length polymorphisms and it was suggested that two cryptic species of *G. pallida* may exist.

SNPs are distributed throughout the genome, occur in high density and have been shown to be useful for genetic differentiation of nematodes. Mimee et al. (2015) identified numerous SNPs in *G. rostochiensis* in the search for molecular diagnostic pathotype markers using the Pool-Seq method, which is a modified GBS method, comprising the use of pooled samples that can be, according to Futschik and Schlotterer (2010), more effective in discovery of SNPs and provide better estimates for allele frequencies than analysis of many individual samples while reducing the costs and workload (Schloetterer et al., 2014). However, there are also some limitations which come with pooling sequences. The information about haplotypes and heterozygosity of individuals from a population are lost, sometimes rare alleles, which can be important, might be ignored as result of corrections for sequencing errors, and, especially when the read depth is <10, differences in the molar amount of the individual DNAs can give errors in SNP frequency determination (Cutler and Jensen, 2010; Rellstab et al., 2013). However, Pool-Seq has been used successfully to address different biological questions. Turner et al. (2010) investigated if *Arabidopsis lyrata* locally adapts to serpentine soils with two pools each of 25 plants of serpentine and granite soil. About 8.4 million SNPs were found with 96 having an allele frequency

difference of >80% between soil types in the exons, introns or within 1 kb of the start or stop codons of 81 genes. Ren et al. (2021) investigated feral and domestic geese of different colours with Pool-Seq and identified 26 genes potentially involved in the regulation of feather colour. Benevenuto et al. (2019) identified significant SNPs that could be involved in the resistance to the synthetic auxinic herbicide 2,4-dichlorophenoxyacetic acid in red clover by Pool-Seq. This is only a very small fraction of successful applications of Pool-Seq analyses but provides an example of the range of biological processes that have been analysed using this approach.

Recently, Varypatakis et al. (2020) investigated *G. pallida* populations which were selected for several generations on potato cultivars carrying the resistances *GpaV* (from *S. vernei*) or *H3* from *S. tuberosum* ssp. *andigena* CPC 2802 (Section 4.1.2). This selection led to increased virulence in the nematode lineages specific to the genetic background. When combining two next generation sequencing approaches, pathogen enrichment sequencing (Pen-Seq) (Thilliez et al., 2019) and scanning of the whole-genome (re-sequencing, ReSeq) of these host adapted *G. pallida* populations, 11 putative avirulence (*Avr)* gene candidates were identified.

3.2 Scope of the chapter

In this chapter, population genetics from *G. pallida* is studied. The data were obtained by PoolSeq and genome wide frequency analysis[6] The aim was to investigate and understand the genetic diversity within and interrelationships between the 36 British *G. pallida* populations and "single cyst" lines derived from these populations that were characterised in Chapter 2 for their virulence on six potato genotypes with different sources of resistance, as well as 2 populations from USA (Idaho). Allele frequencies from 1505 loci throughout the genome were obtained and the relationship between the *G. pallida* populations are illustrated in a phylogenetic tree. It was not possible yet, possibly because of the small number of markers involved, to link genomic sequences with complex phenotypes such as pathotypes. SNPs are abundant and widespread markers that raise the hope to develop diagnostic virulence markers in *G. pallida*.

[6] Pierre-Yves Vérroneau from Benjamin Mimee's lab at Agriculture and Agri-Food Canada, St-Jean-sur-Richelieu, Canada extracted DNA from all *G. pallida* populations and performed the PoolSeq analysis with the initial SNP calling as part of a collaboration (Sections 3.3.2 and 3.4.1).

Therefore, the allele frequencies from the 1505 loci were screened for SNP candidates which might be useful as molecular markers to distinguish between phylogenetic groups and/or virulence levels (pathotypes).

3.3 Materials and methods

3.3.1 *G. pallida* populations

Table 3-1 shows the *G. pallida* populations and "single cyst" lines used in this analysis. Some populations were taken from the James Hutton PCN Collection where the cysts were collected from fields about 50 years ago and multiplied in pots in a greenhouse, whereas the other populations were collected more recently. From both sources populations and "single cyst" lines derived from one cyst of a population, designated as p and s respectively, were used. The "single cyst" lines were generated as described in 2.3.1 and are listed in Table 2-7. The lines Idaho 1 and 2 were provided by Louise-Marie Dandurand, with Idaho 1 being used as outgroup.

Table 3-1 List of *G. pallida* populations used in this chapter

Source indicates if the PCN are recent field samples (f) or from the historical JHI collection (c). Population type distinguishes between population (p) and "single cyst" line (s).

Population/ "single cyst" line	Code in tree	Source	Population type	Reference
Pa1 JHI	Pa1_JHI	c	p	Chapter 2, Phillips and Trudgill (1998)
Pa1 12	Pa1_12 JHI	c	s	Chapter 2
Pa1 Ireland 1	Pa1_Ire1 JHI	f	p	
Pa1 Ireland 2	Pa1_Ire2 JHI	f	p	
Pa1 Ireland 3	Pa1_Ire3 JHI	f	p	C. Fleming, pers. comm.
Pa1 Ireland 4	Pa1_Ire4 JHI	f	p	
Luffness JHI f1	Luff_JHI_f1	c	p	Chapter 2, Phillips and Trudgill (1998)
Luffness 4	Luff_4 JHI	c	s	Table 2-7
Luffness 13	Luff_13 JHI	c	s	Table 2-7
Luffness 15	Luff_15 JHI	c	s	Table 2-7
Luffness 1-4	Luff_1-4	f	s	Chapter 2
Luffness 1-12	Luff_1-12	f	s	Chapter 2
Luffness 1-14	Luff_1-14	f	s	Table 2-7
Luffness 1-19	Luff_1-19	f	s	Chapter 2
Luffness 1-26	Luff_1-26	f	s	Table 2-7
Luffness 1-30	Luff_1-30	f	s	Chapter 2
Luffness field 3	Luff_f3	f	p	Kaczmarek (2014)
Luffness 3-8	Luff_3-8	f	s	Chapter 2
Luffness 3-17	Luff_3-17	f	s	Chapter 2
Luffness 3-18	Luff_3-18	f	s	Chapter 2
Bedale JHI	Bedale_JHI	c	p	Phillips and Trudgill (1998)
Farcet JHI	Farcet_JHI	c	p	Phillips and Trudgill (1998)
Halton JHI	Halton_JHI	c	p	Phillips and Trudgill (1998)
HA	HA	f	p	Kaczmarek (2014)
HA 9	HA_9	f	s	Chapter 2
HA 10	HA_10	f	s	Table 2-7
HA 12	HA_12	f	s	Chapter 2
HA 27	HA_27	f	s	Chapter 2
HA 34	HA_34	f	s	Chapter 2
HA 41	HA_41	f	s	Table 2-7
HA 43	HA_43	f	s	Table 2-7
HA 47	HA_47	f	s	Table 2-7
HA 49	HA_49	f	s	Chapter 2
HA 50	HA_50	f	s	Table 2-7
HA 51	HA_51	f	s	Table 2-7
HA 52	HA_52	f	s	Table 2-7
Idaho 1	Idaho_1	f	p	P. Véronneau, personal
Idaho 2	Idaho_2	?	p	communication

3.3.2 Pool-Seq and initial SNP calling

50 cysts of each *G. pallida* population and "single cyst" lines shown in Section 3.3.1 were sent to B. Mimee's lab, where 30 cysts were used to extract DNA with subsequent Pool-Seq analysis and a *de novo* SNP-calling pipeline UNEAK (Universal Network Enabled Analysis Kit) developed by Lu et al. (2013). This method was chosen, as no well assembled *G. pallida* genome is available, although a newly assembled genome should be available shortly (Thorpe et al., 2021). DNA was extracted, digested with the restriction enzymes *Pst*I/*Msp*I and barcoded (Elshire et al., 2011), and sequencing was performed on an Ion Proton™ platform which provided 100 bp single end reads. For the analysis of the Pool-Seq sequences, all reads were trimmed to 64 bp. Pairwise alignment of the sequences provides sequence groups with only one bp mismatch, this corresponds to one putative SNP. The SNPs were filtered using the criteria: no missing data, minimum minor allele frequency (MAF) threshold 0.01, and a minimum number of 5 reads at each locus (see Mimee et al. (2015)).

3.3.3 Phylogenetic tree

In a phylogenetic tree evolutionary relationships of different species, organisms, genes etc. from a common ancestor are illustrated in a tree diagram (Baum, 2008). A rectangular phylogenetic tree is one layout to visualize the relationships. In this work, branch lengths have a meaning, with longer branches indicating more genetic change has occurred. The allele frequency data of the 1505 loci for the *G. pallida* populations were used to generate phylogenetic trees using the software package Phylip-3.695[7]. Firstly, Seqboot, which is bootstrapping software that allows the generation of multiple data sets by resampling the input data set, was applied. The settings were default except: D (type of data): gene frequencies, R (number of repeats): 1000, 0 (termination type): none. The input file contained the list of the 1505 gene frequencies for the *G. pallida* populations. Then, the program Contml was used on the output file from Seqboot. Contml is a program that estimates phylogenies by the restricted maximum likelihood method based on Brownian motion using an expectation-maximisation algorithm (Felsenstein, 1981). The conditions that differ from the default were M (replicates): 1000, T (termination type): none. Next, consensus tree of the

[7] http://evolution.genetics.washington.edu/phylip/

1000 trees obtained by Contml was calculated with the program Consense. Lastly, the output tree file from Consense was run with the software FigTree v1.4.3[8] to visualize the consensus tree.

3.3.4 BLAST search

BLAST (Basic local alignment search tool) searches of the 64 bp sequences containing the potentially informative SNPs, were performed on the platform WormBase ParaSite[9] (Howe et al., 2017) using the following conditions: DNA sequences searched against the DNA databases of *Globodera pallida* (PRJEB123 - Lindley) and *Globodera rostochiensis* (PRJEB13504 - Ro1) (Cotton et al., 2014) with the search tool BLASTN and search sensitivity optimised for short sequences. All other conditions were default.

3.3.5 DNA extraction for Sanger sequencing

30 cysts were crushed after selecting them under a low power microscope, and DNA was extracted as described in 2.3.7.1 but with increased volumes of reagents: 900 µl 1x PCR buffer (Promega) with 1.5 mM MgSO4, 100 µl 10mg/ml Proteinase K.

3.3.6 Sanger Sequencing

The primers for sequencing were designed using the retrieved sequence from the BLAST search with the online primer design program Primer3web[10] (Koressaar and Remm, 2007; Untergasser et al., 2012; Kõressaar et al., 2018)). All samples were checked by electrophoresis (2% agarose gel), then the PCR amplifications were cleaned by adding 2 µl of ExoSAP-IT (Thermo Fisher 78200), which removes excess primers and nucleotides, by incubating for 15 min at 37°C and then inactivating the enzyme for 15 min at 80°C. The samples were then sent to the JHI sequencing facility and sequenced in both directions with the respective forward and reverse primers shown in Table 3-5. They were analysed with Sequencher 5.4.6 software from GeneCodes.

3.4. Results

3.4.1 Determination of allele frequencies in *G. pallida* populations

The determination of allele frequencies of 38 *G. pallida* populations and the derived "single cyst" lines (Table 3-1) and their analysis was performed as described in Mimee

[8] http://tree.bio.ed.ac.uk/software/figtree/
[9] https://parasite.wormbase.org/Multi/Tools/Blast?db=core
[10] http://primer3.ut.ee/

et al. (2015) by P. Véronneau using the UNEAK pipeline (Lu et al., 2013). 436,008,390 reads were analysed resulted in the calling of 14,957 SNPs. After applying the filtering criteria, 1505 SNP candidates remained. An excel spreadsheet, which contains the candidate SNPs with read counts for each allele by population and allele frequency, respectively was provided by P. Véronneau. This allele frequency table was then used in for the generation of a phylogenetic tree and determination of potential diagnostic pathotype marker candidate SNPs.

3.4.2 Phylogenetic tree of British populations and derived "single cyst" lines

The allele frequencies of the *G. pallida* populations/"single cyst" lines were used to generate a phylogenetic tree (Figure 3-1A and 3-2) to visualize their relationships based on the allele frequencies. Figure 3-1A shows a rectangular phylogenetic tree, where population Idaho 1 was used as outgroup. The 2 Idaho populations separate from the rest of the populations. The British populations were divided into 3 groups numbered I, II and III. Group I was subdivided into Ia, containing field population HA and all derived "single cyst" lines, and Ib, consisting of the historical populations Bedale JHI, Halton JHI and Farcet JHI from the JHI collection and population Luffness field 3 and its 3 derived "single cyst" lines Luffness 3-8, 3-17 and 3-18. Group II contains the five populations Pa1 JHI, Pa1 Ire1, 2, 3 and 4 as well as the "single cyst" line Pa1 JHI 12. Group III is comprised of the historical population Luffness JHI field 1, the "single cyst" lines Luffness 4 JHI, Luffness 13 JHI and Luffness 15 JHI derived thereof and the "single cyst" lines from the field population Luffness field 1, Luffness 1-4, 1-12, 1-14, 1-26 and 1-30. The *G. pallida* populations are labelled in blue. The "single cyst" lines derived from a population are closely related to the source population. Figure 3-1B shows the pathotypes/virulence levels of the *G. pallida* populations and "single cyst lines" on different potato genotypes, where known. The resistance scoring was performed in this project and is described in Chapter 2. The Kort- and the modified Kort pathotype scheme are described in Kort et al. (1977) and Phillips and Trudgill (1998). All populations from group II show pathotype Pa1, with the populations coming from different places in Northern Ireland and Scotland. No other populations/"single cyst" lines in other groups have pathotype Pa1. As expected, the clustering of these Pa1 populations corresponds with avirulence against the *H2* resistance source. The

“single cyst” lines of Ia are generally more virulent on potato genotype 62.3.33 than the ones from Ib, which is the defining distinction between Pa2 and 3 in the Kort scheme. They are also slightly more virulent on Vales Everest than Ib lines. Group III lines are all from one place, Luffness field 1, and can consistently multiply on all potato genotypes tested except Innovator. Figure 3-2 shows a simplified tree with no “single cyst” lines, so that each population is represented only once. If this phylogenetic tree is robust, similar results as in the tree shown in Figure 3-1A is expected, as is the case for all populations.

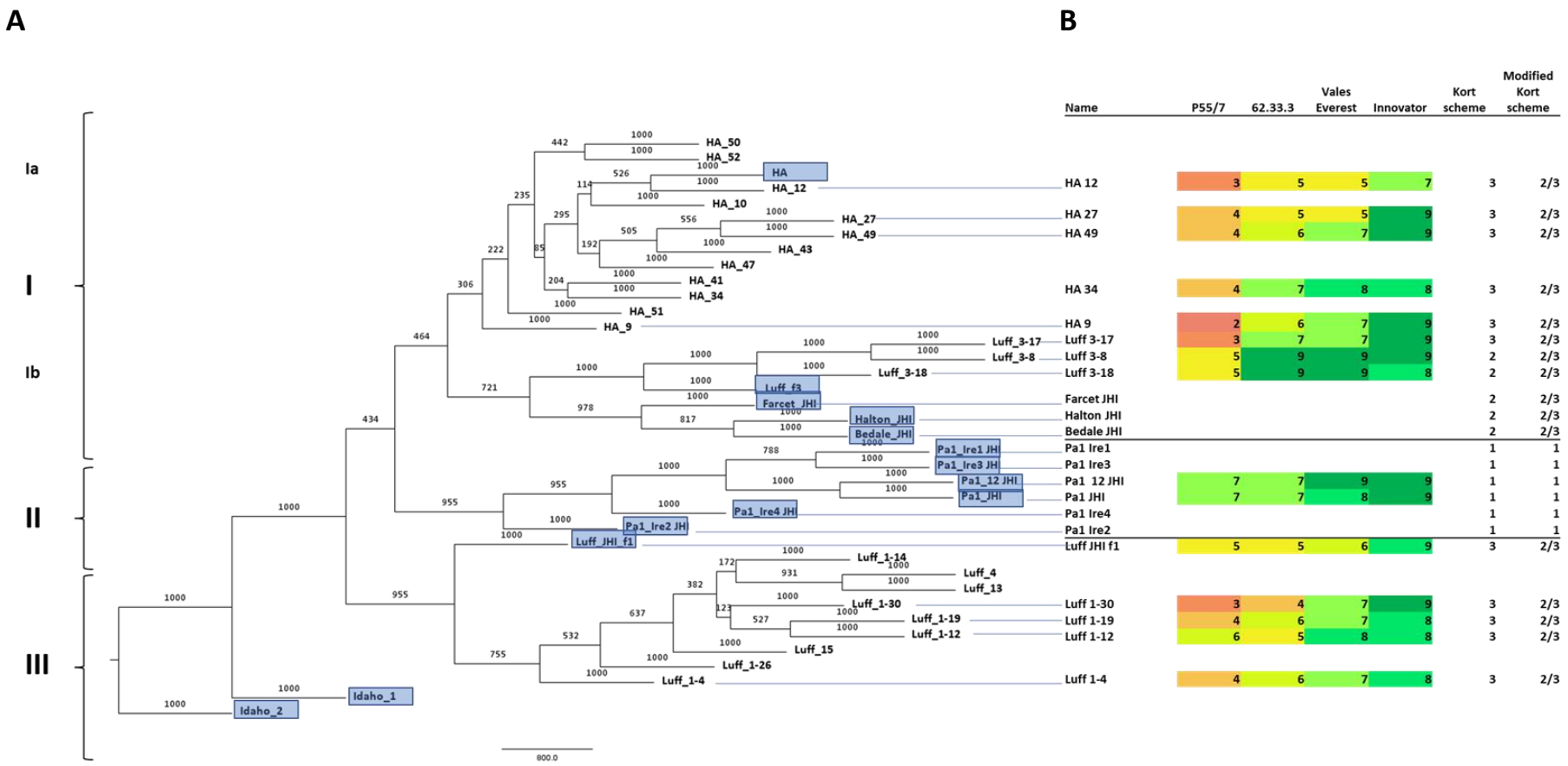


Figure 3-1 Phylogenetic tree and level of resistance of selected British *G. pallida* populations

Panel A shows a rectangular phylogenetic tree with population Idaho 1 used as outgroup. Populations are highlighted in blue; "single cyst" lines are not highlighted. The numbers at the branches are the bootstrap frequency for the bifurcation at the nodes. Panel B shows the resistance scores determined in Chapter 2 of four potato genotypes with different levels of resistance, and historical pathotypes of some of the populations/"single cyst" lines, the resistant score can range from 1 to 9, with 9 being the highest level of resistance. The blue lines connect the same the

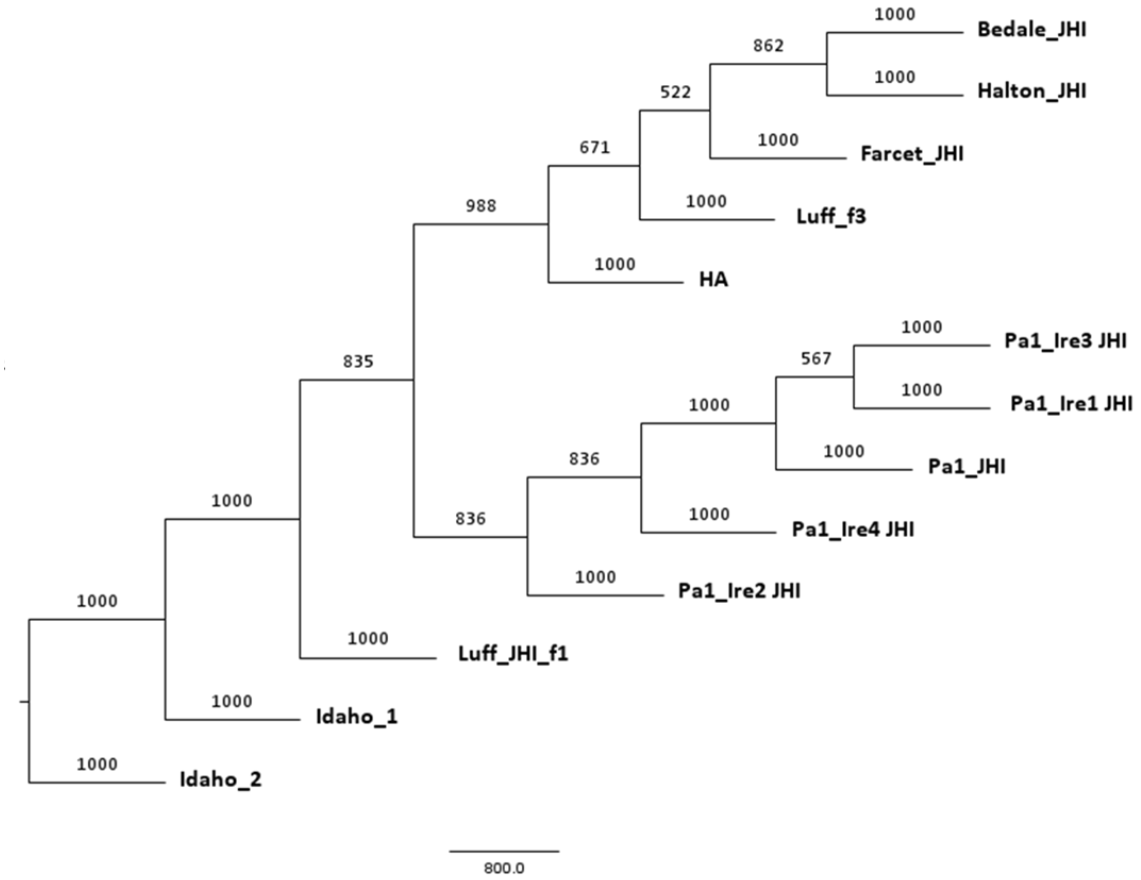


Figure 3-2 Phylogenetic tree with *G. pallida* populations only
Rectangular phylogenetic tree with only populations and no "single cyst" lines, therefore each population is represented only once, with the bootstrap values provided at the branches of the tree.

3.4.3 Using the frequency difference of alleles in different *G. pallida* populations to find candidate SNPs for molecular markers

3.4.3.1 Finding SNP candidates for defining phylogenetic groups

Next, I examined whether SNPs can be found that can differentiate between the different phylogenetic groups shown in Figure 3-1A and which, therefore, could be candidate molecular markers for these populations. Initially, 4 groups, Ia, Ib, II and III were screened with the different search criteria shown in Table 3-2. No candidate SNPs could be identified that differentiate subgroups Ia and Ib. These two subgroups were therefore combined into one group for further analysis. Table 3-2 shows the search criteria of each group and the number of SNP candidates that were found to meet these criteria. The frequency of allele 1 was set to be either ≤0.2 or ≥0.8 for the average for each group, and the allele frequency in one group had to differ from the

others. As group I can be subdivided into Ia and Ib, it was also screened for candidate SNPs to differentiate the subgroups. However, no SNP candidates could be found for the subgroups Ia and Ib. For group I, 2 SNP candidates could be found, both of them having less than 20% allele 1 frequency in the averaged allele frequencies of all HA, Luffness field 3, Halton JHI, Bedale JHI and Farcet JHI populations/"single cyst" lines. For group II, a total of 9 SNP candidates were detected, 5 with less than 20%, 4 with more that 80% allele 1 frequency of the average of all Pa1 populations/"single cyst" lines. For group III, 4 candidate SNPs were found, 3 with less than 20% and 1 with >80% allele 1 frequency of the averaged Luffness field 1 populations/"single cyst" lines.

Table 3-2 Search criteria for detecting potential molecular marker candidates
All 1505 SNPs were screened with the search criteria shown in the table, the number of SNPs which fulfil these criteria for each phylogenetic groups shown in the last column.

	Average allele 1 frequency of				
Group I	**Group Ia**	**Group Ib**	**Group II**	**Group III**	**No. of SNP candidates**
<20%	>80%	>80%	>80%	>80%	2
>80%	<20%	<20%	<20%	<20%	0
-	<20%	>80%	>80%	>80%	0
-	>80%	<20%	<20%	<20%	0
-	>80%	<20%	>80%	>80%	0
-	<20%	>80%	<20%	<20%	0
>80%	>80%	>80%	<20%	>80%	5
<20%	<20%	<20%	>80%	<20%	4
>80%	>80%	>80%	>80%	<20%	3
<20%	<20%	<20%	<20%	>80%	1

Looking at the frequencies of allele 1 in the individual populations/"single cyst" lines shown in Table 3-3 revealed a high variation between the lines, which is not desirable. When searching for SNP candidates that are indicative for group I and have an average allele 1 frequency of < 20%, two SNPs, TP54252 and TP54261, were identified. 6 of the 20 *G. pallida* populations/"single cyst" lines from group I (HA, HA 9, HA 10, Luffness 3-18, Luffness 3-8 and Bedale JHI) show a higher frequency than 20% for allele 1, up to 55%. The *G. pallida* populations/"single cyst" lines from groups II and III, which need to be > 80%, are more uniform, with only population Pa1 Ire2 having less than 80% of allele 1.

When searching for candidate SNPs indicative for group II (Pa1), Pa1 Ire2 did not meet the criteria for most of the identified SNP candidates. The phylogenetic tree in Figure

3-2 shows that group II could be divided into two subgroups Pa1 Ire2 and all other Pa1s like group I. This was not done, as all Pa1 populations have the same virulence phenotype.

Candidate SNPs that are indicative for group III (Luffness f1) showed individual “single cyst” lines. with a remarkably higher or lower allele 1 frequency than according to the search criteria. This allele frequencies are highlighted in pink in Table 3-3.

Table 3-3 Allele 1 frequencies for selected British *G. pallida* populations/"single cyst" lines

The frequencies labelled light green indicate the groups that were screened for. The frequencies labelled in pink show the values of the individual populations/"single cyst" lines which did not have the allele 1 frequency screened for (*i.e.*, either <20% or >80%).

	Indicative for group														
	I		II									III			
Name	TP 54252	TP 54261	TP 125062	TP 43715	TP 267135	TP 267313	TP 107533	TP 185033	TP 194356	TP 120467	TP 94607	TP 278125	TP 18094	TP 125097	TP 100957
Group I (a+b)	11%	15%	94%	91%	89%	99%	96%	2%	0%	0%	11%	84%	100%	82%	3%
Ia	10%	12%	94%	98%	91%	100%	100%	0%	0%	0%	9%	83%	100%	82%	1%
Ib	14%	21%	94%	80%	84%	96%	89%	5%	0%	1%	15%	85%	99%	84%	7%
Individual lines															
HA 9	32%	46%	100%	100%	100%	100%	100%	0%	0%	0%	0%	100%	100%	46%	0%
HA 10	41%	55%	100%	100%	100%	100%	100%	0%	0%	0%	0%	50%	100%	80%	0%
HA 12	1%	2%	100%	100%	70%	100%	100%	0%	0%	0%	0%	99%	100%	89%	0%
HA 27	10%	11%	100%	100%	100%	100%	100%	0%	0%	0%	0%	100%	100%	100%	0%
HA 34	0%	0%	100%	100%	70%	100%	100%	0%	0%	0%	0%	99%	100%	100%	0%
HA 41	0%	0%	100%	100%	78%	100%	100%	0%	0%	0%	0%	55%	100%	58%	0%
HA 43	0%	0%	100%	100%	100%	100%	100%	0%	0%	0%	0%	99%	100%	100%	0%
HA 47	0%	0%	73%	99%	100%	100%	100%	0%	0%	0%	3%	100%	100%	99%	0%
HA 49	14%	12%	100%	100%	100%	100%	100%	0%	0%	0%	0%	55%	100%	97%	0%
HA 50	0%	0%	68%	100%	100%	100%	100%	0%	2%	0%	22%	71%	100%	65%	0%
HA 51	0%	0%	100%	100%	100%	100%	100%	0%	0%	0%	90%	64%	100%	70%	15%
HA 52	0%	0%	100%	100%	65%	100%	100%	0%	0%	0%	0%	100%	100%	77%	1%
HA	27%	33%	82%	72%	100%	100%	100%	0%	0%	0%	0%	81%	100%	81%	3%
Luffness 3-8	25%	37%	100%	100%	100%	100%	34%	26%	0%	0%	0%	100%	100%	99%	0%
Luffness 3-17	3%	3%	96%	100%	96%	91%	100%	8%	0%	0%	13%	100%	100%	97%	0%
Luffness 3-18	28%	31%	100%	71%	100%	100%	100%	0%	0%	7%	0%	100%	100%	100%	0%
Luffness pop	14%	21%	100%	93%	27%	84%	100%	0%	0%	0%	4%	44%	94%	62%	4%
Bedale JHI	23%	33%	96%	53%	100%	100%	91%	0%	0%	0%	61%	86%	100%	84%	15%
Farcet JHI	4%	9%	82%	92%	89%	100%	100%	0%	0%	0%	27%	83%	99%	65%	7%
Halton JHI	3%	9%	85%	50%	77%	100%	100%	0%	0%	0%	0%	83%	100%	78%	22%

Table 3-3 continued

	Indicative for group														
	I		II									III			
Name	**TP 54252**	**TP 54261**	**TP 125062**	**TP 43715**	**TP 267135**	**TP 267313**	**TP 107533**	**TP 185033**	**TP 194356**	**TP 120467**	**TP 94607**	**TP 278125**	**TP 18094**	**TP 125097**	**TP 100957**
Group II	91%	94%	10%	11%	17%	17%	20%	80%	84%	85%	88%	86%	100%	84%	2%
Individual lines															
Pa1 12 JHI	100%	100%	4%	0%	6%	37%	0%	66%	92%	100%	100%	100%	100%	97%	0%
Pa1 JHI	89%	94%	6%	3%	25%	17%	7%	84%	77%	71%	100%	100%	100%	100%	0%
Pa1 Ire1	99%	99%	3%	0%	19%	6%	30%	92%	97%	88%	78%	85%	100%	63%	0%
Pa1 Ire2	62%	71%	27%	0%	40%	28%	36%	49%	57%	62%	96%	91%	100%	78%	12%
Pa1 Ire3	98%	99%	5%	1%	4%	10%	29%	90%	83%	90%	100%	99%	100%	95%	2%
Pa1 Ire4	100%	100%	15%	63%	6%	5%	17%	97%	100%	100%	52%	100%	100%	100%	0%
Group III	100%	100%	100%	100%	83%	100%	100%	6%	3%	3%	0%	6%	9%	20%	92%
Individual lines															
Luffness f1 4 JHI	100%	100%	100%	100%	94%	100%	100%	0%	0%	0%	0%	0%	0%	11%	100%
Luffness f1 13 JHI	100%	100%	100%	100%	87%	100%	100%	0%	3%	0%	0%	0%	2%	9%	100%
Luffness f1 15 JHI	95%	99%	100%	100%	100%	100%	100%	0%	0%	0%	0%	0%	0%	3%	100%
Luffness 1-4	100%	100%	100%	100%	97%	100%	100%	16%	0%	22%	0%	59%	0%	23%	43%
Luffness 1-12	100%	100%	97%	100%	71%	98%	100%	8%	0%	0%	0%	0%	0%	2%	100%
Luffness 1-19	100%	100%	100%	100%	94%	100%	100%	15%	0%	0%	0%	1%	0%	31%	100%
Luffness 1-26	100%	100%	100%	100%	78%	100%	100%	0%	0%	0%	0%	0%	1%	23%	100%
Luffness 1-30	100%	100%	100%	100%	86%	100%	100%	18%	0%	7%	0%	0%	66%	78%	77%
Luffness f1	100%	100%	100%	100%	41%	100%	100%	1%	23%	1%	0%	0%	14%	11%	100%

The results from the BLAST searches of the 64 bp sequences containing the SNP candidates are shown in Table 3-4. TP54252 and TP54261, where a frequency of allele 1 of less than 0.2 indicates group I, are very similar, with a deletion of 1x nucleotide A in TP54261 compared to TP54252 and the reference sequence (indicated in red in Table 3-4). They are in the coding region of the predicted genes GPLIN_001058600 and GPLIN_001408600. 5 of the 9 SNP candidates for group II are within protein coding regions, GPLIN_000027600, GPLIN_000711900, GPLIN_000597100 & GPLIN_001007700 (for the same SNP), GPLIN_000500900 and GPLIN_000521200. One SNP that is not in any known *G. pallida* coding region, is found to be in a coding region of *G. rostochiensis* (GROS_g13093). A SNP candidate for identifying group III, is within a *G. pallida* gene GPLIN_000903000, 2 further SNPs are within the corresponding *G. rostochiensis* genes, GROS_g00474 and GROS_g01605, however no corresponding *G. pallida* coding region is detected.

Table 3-4 BLAST search results of Pool-Seq sequences that are potentially useful as molecular marker

The bases in brackets indicate the two alleles which were identified in the Pool-Seq analysis.

		PJB-123-Lindley		PRJEB13504-Ro1	
SNP ID	**64 bp sequence containing candidate SNP [Allele1/allele2]**	**GpaI scaffold & position**	**Overlapping gene**	**Overlapping gene**	**Notes**
Low levels of allele 1 indicative for group I					
TP54252	TGCAG[G/A]TCAGCAAAATTGCATTCGTACAACGCCCAATGGTCGGTCATGAACAGGCACATTTGGC	477: 25072-25135 (+)	GPLIN_001058600 &	GROS_g12035	Very similar sequences
TP54261	TGCAG[G/A]TCAGCAAATTGCATTCGTACAACGCCCAATGGTCGGTCATGAACAGGCACATTTGGCC	1522: 2757-2820 (-)	GPLIN_001408600		TP54252 is in ref. seq.
Low levels of allele 1 indicative for group II					
TP125062	TGCAGCGACAATGCCTCGGCACCGCTCGTGTGGGCACAAAGCTCCTGGCT[G/A]ATCTCCACCACTT	4: 14128-14191 (+)	GPLIN_000027600	GROS_g04742	
TP43715	TGCAGAGAGCAGCCGTGTTCATCGGACAGCTCCA[G/A]AGACGACTTGGAGTCGCTGGCCCAACATC	18: 281707-281770 (-)		GROS_g13093	
TP267135	TGCAGTCCATCGTGCGCAAGTCGCACGAGCTCTACAC[G/C]GAATTGGACGAGCACGGAACGCTGGG	224: 114264-114327 (+)	GPLIN_000711900	inx	
TP267313	TGCAGTCCCC[C/A]CGCCCCCTTCAGGCTCTCTCCCCGTCCAACTGCCGACCCGCGCCCGCCTCTTC	305: 100379-11442 (-)			
TP107533	TGCAGCCAACCAAATATGTTTG[G/A]AATTCCATTTCTTACTGAAGCAATTAGAAAATCGTTTTTGC	780: 37818037881 (-)			
High levels of allele 1 indicative for group II					
TP185033	TGCAGGAAGTTCATTTCTTCCCCTTTCCCTCTTCACCTGCTCAGTGTAATTT[T/C]TGAGCGCCACA	980: 17891-17954 (+)		GROS_g11748	
TP194356	TGCAGGAGGAGATGAAGCATCCGCAACTGC[G/A]CGACGAGAAGAAGCATCGGCAGCTGCACGACGA	169:4857-4920 (+) & 427: 78142-78205 (+)	GPLIN_00597100 & GPLIN_001007700		More than one alignment in these regions
TP120467	TGCAGCCGTCCGTGCCGTTGTTGCGCGT[T/C]CGTGCCGTCGACATGGACAGCAGCTCCGTTTCTGT	128: 28847-28910 (-)	GPLIN_000500900	GROS_g09456 & GROS_g14115	
TP94607	TGCAGCAGCTGATCAGTCCCC[T/C]AGTGATGCGGACGTCACCCTGACCATGTCGTCGTCCTCGTCG	136: 147913-147976 (+) & 777: 2876-2935 (+)	GPLIN_000521200	GROS_g03545	
Low levels of allele 1 indicative for group III					
TP278125	TGCAGTGCAGGTGAAACGACCTCGATGAGAGGGAGTAGGAATGGCGGGG[T/G]GTGGACAATAATGA	186: 145266-145329 (-)			
TP18094	TGCAGAAG[T/C]CGCGCAGCCGCCGATGGGCTGGCGGAACAACCAGTTTGGCGATGACACTGAACAA	337: 100641-100704 (+)	GPLIN_000903000	GROS_g02065	
TP125097	TGCAGCGACACCATTTTTGCCAACGGCAGAAAGTCAGCGGCTGGTGACGCAAGCTGAACAACC[G/A]	56: 26498-26561 (-)		GROS_g00474	1xT deletion at pos 19
High levels of allele 1 indicative for group III					
TP100957	TGCAGCATAAATCGGATATG[T/C]CGAAGTTCGTCCCCAACGGCGATTTGGTCCATCTTCACGCCCT	7093: 513-576 (-)		GROS_g-1605	

3.4.3.2 Sanger sequencing a subset of the SNP candidates for a molecular marker

Three candidate SNPs, TP120067, TP125062 and TP194356 were selected to be Sanger sequenced to determinate whether these differences in the frequency of allele 1 can be reproduced by low depth (Sanger) sequencing. Primers were designed as described in 3.3.6 and are shown in Table 3-5.

Table 3-5 PCR primer sequences used for three candidate SNPs

Name	Sequence 5'-3'	Size bp]
TP120467for	AGATGGTAGTGAGGCGGATG	216
TP120467rev	ATCTTGTCGGTGCCAGAACT	
TP125062for	GTCGGCCAGCTTTGTGTAG	191
TP125062rev	GAAGTGTTTGGAGGAAAAGCG	
TP194356for	AAATCAACATACACGCCAGGA	314
TP194356rev	CTTCATCTTGTGCGCAGTT	

One of the three SNP candidates tested, TP194356, shows a difference in allele frequencies in Sanger sequencing depending on the phylogenetic group (Table 3-6). Group II (Pa1) shows allele G only or a mixture of G and A, whereas groups I and III show allele A only, with the exemption of Luffness 3-17, which is a mixture of A and G in the sequenced populations/"single cyst" lines. The other two SNP candidates share the same allele in all tested *G. pallida* populations the reference sequence is the same for TP120467, whereas for TP125062 the reference base shows the alternative allele.

Table 3-6 Sequencing of the potential candidate alleles of selected *G. pallida* populations.
R means a mixture of G and A, "-" means not successfully sequenced.

		Allele ID		
Group	**Population/line**	**TP120467**	**TP125062**	**TP194356**
	Reference	C	A	A
I	**HA 12**	C	G	-
	HA 27	C	G	A
	Luffness 3-17	C	G	R
	Luffness 3-18	C	G	A
II	**Pa1 12 JHI**	-	-	R
	Pa1 JHI	C	G	R
	Pa1_Ire2	C	G	G
	Pa1_Ire3	C	G	-
	Pa1_Ire4	C	-	G
III	**Luffness 1-4**	C	G	A
	Luffness 1-30	C	G	A
	Luffness field 1 JHI	C	G	A

3.5 Discussion

In this chapter, a phylogenetic tree based on 1505 SNPs between different British *G. pallida* populations and "single cyst" lines derived from these populations was generated to determine their phylogenetic relationships to each other. Furthermore, it was investigated whether the differences in the allele frequencies of these 1505 loci can be used to develop molecular markers for the different groups which can be used in high throughput screens.

The finding that population Luffness field 1 separates from the other populations/"single cyst" lines in this analysis confirms the findings of Hockland et al. (2012), who compared European and North American *G. pallida* populations with South American ones. In their study, the European *G. pallida* populations fell into three groups: Pa1, Luffness (field1) and a group containing the populations Bedale, Halton and Farcet. These groups were all genetically different from the majority of South American populations analysed. Group II, which consists only of one pathotype, Pa1, appears to be one introduction although the bootstrap-values for the bifurcation are low with a value of 434 (of 1000). Group III may represent another introduction and only consists of the Luffness field 1 population from both the recently collected field cyst and the historical JHI collection. Group I comprises different populations from the UK and shows different levels of virulence on selected potato genotypes suggesting a more diverse genetic background within the group. Group I can be split in two subgroups Ia (HA) and Ib (all other PCN populations). Ia shows similarities in virulence against potato genotypes to population Luffness field 1, also the multiplication rate was similar to Luffness, whereas phylogenetically is seems to be closer to the third introduction. Pa1 seems to looks like one pathotype, however Pa2 and Pa3 (Kort et al., 1977) are not always reliably distinguishable and can occur within one population, therefore Phillips and Trudgill (1998) introduced the pathotype Pa2/3 instead. The data from this study suggest that the virulence from group I and group III could have different genetic origins even if the phenotypic result (pathotype) is the same. Population Luffness f1 is phylogenetically different from group I and II, as shown by Hockland et al. (2012). Population HA was never investigated before. It is distinct from the other populations from group I, the virulence on the tested potato genotypes is

like Luffness field 1 (Chapter 2). Testing this population for virulence on more potato genotypes with different sources of resistance, e.g. as described in (Canto Saenz and Mayer de Scurrah, 1977) could possibly indicate a new introduction (see also Chapter 6).

The screening of the 1505 loci with two different alleles for "absence" (<0.2) and "presence" (>0.8) of allele 1 in phylogenetic group at the same time the "presence" or "absence" of allele 1 in the other phenotype, resulted in 15 candidate SNPs, 2 for group I, 9 for group II and 4 for group III. Three of these candidates, all indicative for group II, were Sanger sequenced, and one picked up a difference in allele frequency. The fact that the differences were not picked up in the other two loci does not mean that frequency differences do not exist. For example, the PCR reaction with the chosen primers and amplification conditions might favour one allele over the other. By changing the conditions of the PCR and/or primers, detection of the other allele could be possible. With the chosen conditions and primers, one candidate SNP, TP194356, shows the potential to be developed into a high throughput pathotype marker to distinct group II (Pa1) from the others. The next step would be to test this locus with as many different *G. pallida* populations as possible, especially with ones that were not used in the Pool-Seq analysis and then, in case TP194356 still proves to be useful as diagnostic marker to reliably detect group II (Pa1) *G. pallida* populations, a KASP^{TM} assay (Section 4.3.9) that can discriminate between the different alleles, could be designed to allow for routine high throughput screening of unknown PCN populations to detect Pa1. This marker could be of interest for *e.g.*, SASA, where many bio-assays are performed each year to determine the pathotypes of PCN populations in Scotland. However, having more than one marker would be desirable. Furthermore, it would be interesting to investigate whether Pa1 shows a mixture of A and G (R) at the SNP location, or if they segregate into A and G at a single nematode level. It also would be interesting to Sanger sequence the remaining candidate SNPs indicative for groups I, II and III on different *G. pallida* populations, to determine if there is another molecular marker candidate.

With the availability of a better assembled genome for *G. pallida* (Thorpe et al., unpublished, paper in preparation), the locus could be analysed to see if there are effector genes nearby and if they differ in the phylogenetic groups, as well as if the

candidate SNPs are close to each other. It is extremely unlikely that the SNPs detected here are the cause of different virulence levels. Furthermore, B. Mimee's lab are exploring different methods of analysing the Pool-Seq data; for example they recently used the pipeline stacks (https://catchenlab.life.illinois.edu/stacks/) to trim and create stacks of sequences, followed by the software populations (built-in stacks) to generate population genetics files which were then filtered with a custom script (*e.g.*, different depths of coverage (5/20/50) and no missing data) provided 3945 loci with different allele frequency in the original raw vcf-file. In this analysis, a new assembled *G. pallida* sequence Thorpe et al. (2021) was used as reference genome. Shortly, these new set of loci with different allele frequency will be used to filter again for SNP candidates that could be used as pathotype markers.

4 Characterisation and mapping a *G. pallida* resistance derived from *S. spegazzinii* accession 7195

4.1 Introduction

4.1.1 Wild potato species are a source of resistance to *G. pallida*

Solanum tuberosum ssp. *tuberosum*, the most commonly cultivated potato species, does not show significant resistance to PCN. Many related wild potato species and landraces show a diversity of desirable traits, including resistance to pathogens such as PCN or blight, resilience to abiotic stress, such as drought or high temperature, and tuber quality traits such as flesh colour and dry matter content. These traits can, in many cases, be introgressed into commercial potato cultivars via hybridisation.

Many wild potato species are native to Mexico and the Andean Highlands (Bethke et al., 2017).Genebanks all over the world maintain germplasm from wild potato species, landraces and potato cultivars; they also distribute material to potato breeders and other scientists. A list of organisations maintaining germplasm can be found in Machida-Hirano (2015). The UK potato genebank is the Commonwealth Potato Collection (CPC), originally founded in 1938/39 as the Empire Potato Collection. The CPC is held in trust by The James Hutton Institute with support from the Scottish Government (ICS, 2020). This collection was stocked with samples of berries or tubers from potato plants collected from natural habitats and local markets in Central and South America and is continuously expanded by the addition of new accessions from other collections. In the 1960s, the collection was converted to a true seed collection and currently consists of over 1500 accessions from over 80 wild and cultivated potato species. To date, 151 wild potato species have been described (CIP, 2020), with more than 50 of these showing resistance to *G. rostochiensis* and/or *G. pallida*.

4.1.2 Chromosomal loci conferring resistance to PCN in potato

An important strategy for the management of PCN is the breeding and use of potato cultivars that show resistance to one or more PCN species. The first resistance (*H1*) to *G. rostochiensis* pathotypes Ro1 and Ro4 was identified in *S. tuberosum* ssp. *andigena* accession CPC 2802 (Ellenby, 1952) after screening more than 1200 accessions of the

CPC. Subsequentially, wild potato species from different potato collections were screened for resistance to *G. rostochiensis* (*e.g.,* Hermsen and Verdenius (1971)), and later for resistance to *G. pallida*, to identify additional potential sources of resistance for breeding (Dellaert and Hoekstra, 1987; Chavez et al., 1988; Jackson et al., 1988; Turner, 1989; Rousselle-Bourgeois and Mugniery, 1995; Castelli et al., 2003; Bachmann-Pfabe et al., 2019). So far, 25 resistance loci have been described in potato and the closely related tomato, three of which have been isolated and cloned. The potato gene *Gpa2* (van der Vossen et al., 2000) and *Hero* from the *G. pallida* resistant tomato variety *Lycopersicum esculentum* LA 1792 (Ernst et al., 2002), encode CC-NB-LRR proteins with a coiled-coil (CC) domain at the N-terminus. The third, *Gro1-4* (Paal et al., 2004), was isolated from the wild tomato species *Solanum pimpinellifolium* and encodes a NB-LRR protein with a Toll-interleukin receptor (TIR) domain at the N-terminus. Most of the other resistances are so-called quantitative trait loci (QTL), which are regions in the genome controlling a specific phenotypic trait in a quantitative manner. QTLs carry allelic variants which contribute to the phenotype, but which do not confer complete resistance. All identified QTLs for PCN resistance are located within resistance (*R*) gene clusters, encoding proteins belonging to the NB-LRR family (Section 1.4.1.2), suggesting that such QTLs are also due to the action of NB-LRR genes. Figure 4-1 shows the clustering of known nematode *R*-gene loci within predicted hotspots of NB-LRR resistance genes, in potato clone DM, which was developed as the first potato reference genome.

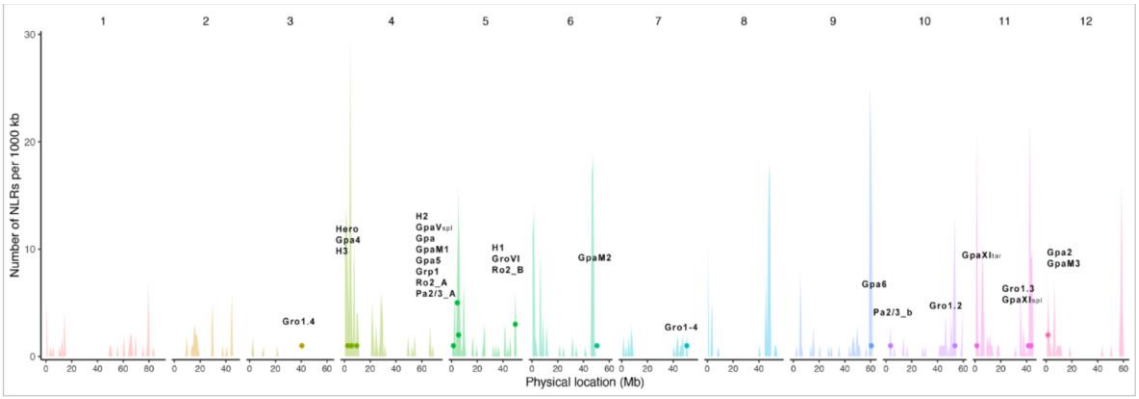


Figure 4-1 PCN resistance loci align with known *R*-gene clusters.
The unequal distribution and position of predicted NB-LRR genes in the genome of DM is schematically illustrated, with spikes indicating clusters of resistance genes. The approximate relative location in the reference genome DM (PGSC v4.03) of known nematode resistance genes and QTLs are displayed as dots. The histogram spikes indicate numbers of predicted NB-LRR genes per 1000 kb across the potato genome. Reprinted from Gartner et al., 2021. Permission to reprint/adapt by a Creative Commons Attribution International License CC BY 4.0.

The fact that several potato wild species show QTLs for PCN resistance that map to one region on chromosome V, could be explained by them having a common ancestor in which the resistance first evolved (Finkers-Tomczak et al., 2009).

4.1.3 Mapping of resistance loci on the potato chromosomes

Genetic mapping of QTLs is based on detecting molecular markers that are genetically linked to the resistance phenotype. Linkage maps show the relative genetic distances between markers and QTLs on chromosomes. Based on the segregation of genes during meiosis via recombination events, the genetic distances between markers/QTLs can be determined. The closer two genes or markers are, the less likely it is that recombination occurs between them. If there is no recombination event between genetic loci, they are referred to as completely linked and the haplotype of the linked DNA can be established.

Molecular markers used before the advance of next generation sequencing (NGS) included restriction fragment length polymorphism (RFLP), random amplified polymorphic DNA (RAPD), simple sequence repeats (SSRs, also known as microsatellites) and amplified fragment length polymorphism (AFLP) (Collard et al., 2005). Following the publication of the first potato reference genome in 2011 (The Potato Genome Sequencing Consortium, 2011), the development of next generation

sequencing and the decreasing costs of sequencing, have meant that new methods for linking phenotypes to specific loci have emerged such as GBS (Elshire et al., 2011) and genome-wide association studies (GWAS) (reviewed in Visscher et al. (2012)) for the discovery of QTLs. This has led to the development of more advanced approaches to marker-trait analysis, which have been applied to potato (*e.g.*, Sharma et al. (2018)).

In addition, advances in potato genomics have led to the development of tools for mapping resistance genes and to the development of molecular markers. Examples are targeted enrichment sequencing assays, such as whole exome capture (Giolai et al., 2016), and generic mapping enrichment sequencing (GenSeq) that targets single-copy, conserved genes dispersed throughout the potato genome (Chen et al., 2018). In addition, resistance gene enrichment sequencing (RenSeq) (Jupe et al., 2013) has been used to enrich NB-LRR resistance genes and to make the mapping of new resistances more efficient. More recently, receptor-like protein (RLP) and receptor-like kinase (RLK) enrichment sequencing (Lin et al., 2020) workflows were developed for potato, as the resistance loci often occur in those specific gene familes. Diagnostic resistance gene enrichment sequencing (dRenSeq) was developed to determine if known resistance loci are present in potato wild species or breeding material (Armstrong et al., 2019).

Although genomics has revolutionised gene mappingin potato, the use of the first published potato genome, the doubled monoploid *S. tuberosum* group *Phureja* clone DM1-3 516 R44 (DM), as reference genome has some limitations. For example, wild potato species are not very close relatives of DM, and many show significant differences in sequences and in the order of genes on chromosomes. This is especially true for rapidly evolving genes such as *R*-genes, where the reference is from a background that is susceptible to many pests and diseases, including both PCN species,*G. pallida* and *G. rostochiensis*. More recently, the potato genome of the diploid inbred clone M6 derived from the wild potato species *S. chacoense*, which is used in breeding programs, was sequenced (Leisner et al., 2018) and a high-quality haplotype-resolved assembly of the autotetraploid potato cultivar Otava was reported by Sun et al. (2022).

A newer approach for mapping and isolating resistance genes uses K-mer studies (association genetics) in combination with RenSeq (AgRenSeq) or GWAS studies. K-mers

are sub-sequences of a certain length (K) contained within a biological sequence, facilitating the identification of sequences, such as *R*-genes, that are significantly different from the reference without the use of a reference genome. An illustration of the workflow of the K-mer analysis is shown in Figure 4-2. The K-mers from the sequencing reads of the two sets of samples to be compared are counted to detect K-mers with significantly different counts in the two sets. Overlapping K-mers are then assembled to derive a sequence for each associated locus. The sequences may correspond to a single nucleotide polymorphism (SNP) (in Figure 4-2 underlined), in which case corresponding sequence may be detected in the other group. Using AgRenSeq, four resistance genes in wheat have recently been isolated (Arora et al., 2019). In this work, the Illumina reads were assembled *de novo* and then association was established based on the K-mers. Highly repetitive sequences, as in NLRs, pose a challenge, which might be overcome by using PacBio reads.

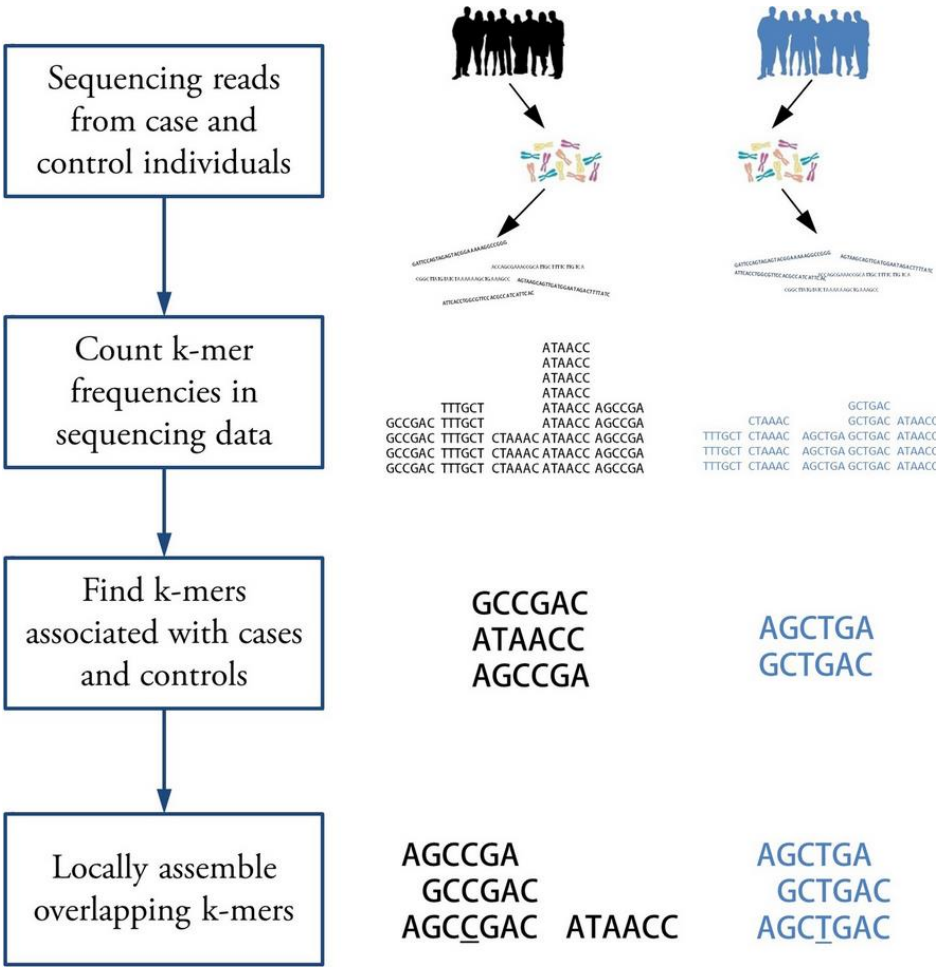


Figure 4-2 Workflow of the association mapping using K-mers instead of a reference genome
The K-mers from the sequencing reads of the two sets of samples to be compared are counted to detect K-mers with significantly different counts in the two sets. Overlapping K-mers are then assembled to derive a sequence for each associated locus. The sequences may correspond to a SNP (underlined) in which case the corresponding sequence may be detected in the other group. This diagram was taken from Rahman et al. (2018). Permission to reprint/adapt by a Creative Commons Attribution International License CC BY 4.0.

4.2 Scope of this chapter

The aim of this chapter is to determine the chromosomal location of a novel locus that confers resistance *to G. pallida.* The resistance is derived from the diploid potato species *S. spegazzinii* accession 7195. The long-term aim of this project is to isolate the gene underlying the resistance locus, and to better understand the molecular mechanisms of disease resistance.

4.3 Material and methods

4.3.1 Potato lines

***S. spegazzinii* CPC 7195** shows natural resistance to *G. pallida* (Kreike et al., 1993; Kreike et al., 1994). The diploid wild potato species was collected in 1966 from the Argentinian state la Riocha, los Coralles, Sierra Famatima, at an altitude of 1950 m. The tubers are round and very small (< 1 cm). True seeds were germinated, grown to produce tubers, and a single plant (#10), was chosen for the subsequent crossing after a canister test to determine the level of resistance.

Mayan Gold (*S. tuberosum* group Phureja DB337(37)) is a diploid potato cultivar that is susceptible to both *G. pallida* and *G. rostochiensis* and produces long, yellow-fleshed tubers. It was developed at the Scottish Crop Research Institute (now The James Hutton Institute, Invergowrie) and was the first Phureja variety released in Europe.

03.F1.3a(35) is a single progeny plant (#35) from a cross of *S. spegazzinii* accession 7195(10) with *S. tuberosum* group *Phureja* DB337(37), which was chosen as the PCN-resistant parent for a backcross named 13.A.02. This cross was performed in 2003.

***S. tuberosum* cv. Desirée** is a tetraploid commercial potato cultivar. It is susceptible to PCN and used as a positive control for susceptibility in resistance assessment experiments.

Potato clones of cross 13.A.02, which is a backcross of 03.F1.3a(35) with *S. tuberosum* group *Phureja* DB337(37), were selected. The cross was performed in 2013 and 993 progeny clonal backcross 1 (BX1) lines, called 13.A.02 (1) to 13.A.02 (993) were received (Figure 4-3).

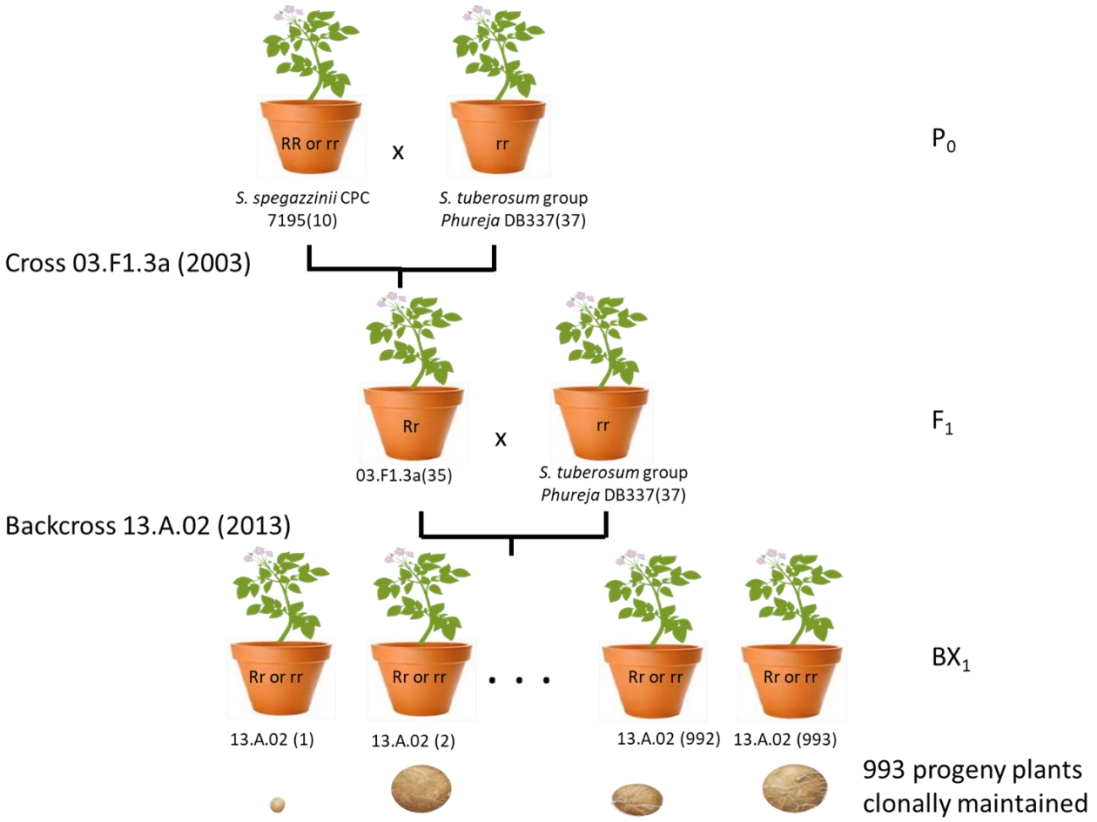


Figure 4-3 Crossing scheme to map the resistance to *G. pallida* derived from *S. spegazzinii*
The diploid potato species *S. spegazzinii* CPC 7195 and *S. tuberosum* group *Phureja* DB337(37) were crossed to ensure that the resistance is heterozygous; one resistant progeny, 03.F.3a(35), was selected for a backcross with DB337(37), whose progeny are investigated in this project. R and r indicate the resistance and non-resistance alleles, respectively.

4.3.2 PCN lines

G. pallida populations Lindley JHI, Luffness JHI f1 and Pa1 JHI (Table 2-4) and the *G. rostochiensis* pathotype Ro1, all from the JHI collection, were used for this work.

4.3.3 Assessing the resistance of potato to PCN

4.3.3.1 Root trainer bio-assay

Root trainer PCN screening assays were performed to determine if the clonal population derived from a potato tuber is susceptible or resistant to PCN, with resistant and susceptible lines serving as controls (Gartner et al., 2021). Prior to infection, cysts were inspected under a low power microscope. Empty and small cysts were removed, and full cysts were then collected in glass vials, one for each sample. A routine hatching test, described in 2.3.4, was performed to ascertain that the nematodes in the cysts were viable. Tubers were transferred from the cold store to RT

about two weeks before planting to encourage sprouting. Four-cell root trainers (Haxnicks) were filled with non-intercept compost (compost without insecticide). Only two non-adjacent cells were used in each root trainer and the adjoining root trainers in a box were separated by a plastic sheet to prevent the stolons from establishing in other pots. Each root trainer cell was infected with 15 PCN cysts from one population by pouring the cysts from the glass vial into a single small indent in the soil generated by a pencil. A single sprout surrounded by "a melon-scoop sized" sample of tuber material was planted on top of the cyst-containing indent. The experiment was set up in randomised blocks with four replicates each. The plants were then allowed to grow for eight weeks in a greenhouse. Subsequently, the root trainers were carefully opened and the number of female nematodes on the uncovered surface (Figure 4-9) determined and recorded for each plant. Scores were taken when at least three of the replicates could be counted.

4.3.3.2 Pot bio-assay

The pot bio-assay for the assessment of PCN virulence on the potato genotypes was performed as described in section 2.3.5.

4.3.4 Extraction of genomic DNA (gDNA)

4.3.4.1 Qiagen DNeasy Plant Kit

DNA extraction was performed using commercial kits, following the manufacturer's protocols. Leaf material was used for extraction.

When small amounts of DNA were needed, the Qiagen mini kit (cat. no. 69104 and 69106) was used: 100 mg leaf material (four small young leaflets) were snap frozen in liquid nitrogen in a 2 ml tube with a hole in the lid. The leaves were lysed in a Qiagen Tissue Lyzer II. To do this, the tube stands were precooled at -80°C for an hour, a 4 mm stainless steel ball bearing (Simplybearings, 4 mm diameter grade 100 AISI 316 stainless steel) was added to each well, the leaves were disrupted for 1 min at 20 Hz, then refrozen in liquid nitrogen and disrupted for another minute at 20 Hz.

For larger amounts of DNA, the Qiagen maxi kit (cat. no. 68163) was used. In total, 1 g of leaves (snap frozen in liquid nitrogen in a Falcon tube) were disrupted by mortar and pestle, cooled with liquid nitrogen.

4.3.4.2 Semi-automated DNA extraction

100 mg leaf material for each sample were put into the wells of a Corning® 96-well deep well plate (Sigma cat. no. CLS3961) sealed with a self-adhesive lid and frozen at -80˚C. For the DNA extraction with QIAamp 96 DNA QIAcube HT (Qiagen cat. no. 51336) and the QIAcube HT Plasticware (Qiagen cat no. 950067) kits, the samples where lysed as follows. One 4 mm stainless steel ball bearing (Simplybearings, 4 mm diameter grade 100 AISI 316 stainless steel) was added to each well, followed by the addition of 20 µl extraction buffer (1000 µl RNAse A (20 mg/ml) (ThermoFisher cat. no. 12091-021), 2.2 ml Proteinase K, 19.8 ml ATL Buffer (provided in the kit). Plates were sealed with a plastic sealing mat (ThermoFisher cat. no. AB-0675) and samples were disrupted using a Qiagen Tissue Lyzer II using the same conditions described in Section 4.2.4.1.
Plates were briefly centrifuged at 3,000 rpm and placed at 4°C for 60 min to reduce the number of bubbles. Thereafter, plates were incubated at 37°C for 30 min, followed by a 60 min incubation at 65°C in a water bath. Next, plates were centrifuged at 5,000 rpm for 10 min. The supernatant was transferred into the QIAcube HT S block, carefully avoiding disturbance to the debris that accumulated at the bottom of the wells. Thereafter, a QIAGEN QIAcube robotic workstation was used. The station was set up following standard procedures provided by the manufacturer. Before automated DNA extraction, robotic buffer reservoirs were filled with 64.6ml Buffer AW1, 64.6 ml Buffer AW2, 62.6 ml 90% Ethanol, 38.6 ml Buffer ACB and 22.2 ml Buffer AE. In addition, two boxes of 200 µl filter tips were mounted in the workstation along with the bin for used tips and a 96-well elution plate. Finally, the lysate plate, after removing the lid was mounted. The QIAcube was run with the with the following extraction program with the corresponding Qiagen HT software (stored as “QIAamp 96 DNA QIAcubeHT V1-short”):
1) Loading 350 µl ACB into 96 well plate; 2) mixing wells in 96 well plate; 3) incubating for 2 min 30 sec (steps 1-3 are repeated once); 4) loading of 550 µl lysate from 96 well plate into the plate; 5) vacuum on (35 kPa) for 5 min; 6) vacuum off (after the execution of this step it was necessary to check if the liquid in each well had been removed. If so, the extraction program was continued, otherwise, the vacuum step was repeated); 7) loading of 600 µl AW1 into vacuum plate; 8) vacuum on (35 kPa) for

2min; 9) vacuum off (after the execution of this step it was again necessary to check if the liquid in each well had been removed. If so, the extraction program was continued, otherwise, the vacuum step was repeated); 10) loading of 600 µl AW2 into vacuum plate; 11) vacuum on (35kPa) for 1 min; 12) vacuum off (after the execution of this step it was necessary to check if the liquid in each well had been removed. If so, the extraction program was continued, otherwise, the vacuum step was repeated); 13) loading of 600 µl 96% ethanol into vacuum plate; 14) vacuum on (35 kPa) for 30 sec; 15) vacuum off; 16) vacuum on (55 kPa) for 1 min; 17) vacuum on (35 kPa) for 2 min; 18) vacuum off (after the execution of this step it was necessary to check if the liquid in each well had been removed. If so, the extraction program was continued, otherwise, the vacuum step was repeated); 19) loading of 200 µl AE into vacuum plate.

4.3.5 Determination of the DNA concentration

4.3.5.1 Qubit-4 fluorometer

DNA concentrations were determined with a Qubit-4 fluorometer from ThermoFisher Scientific, using the Qubit dsDNA HS Assay kit (cat. no. Q32854) according to the manufacturer's protocol. For this assay, thin walled 500 µl Qubit tubes (cat. no. Q32856) were used. The assay is accurate for an initial DNA concentration range from 10 to 100 ng/ml.

4.3.5.2 Picogreen assay

The Picogreen procedure allows the measurement of DNA concentrations ranging from 50 pg/ml to 2 µg/ml. The assay is performed in a 96 well plate and requires a standard curve for each assay. The Quant-iT™ PicoGreen™ dsDNA Assay Kit (Invitrogen P/N: P11496), an Ascent fluorimeter, and the corresponding Fluoroskan software were used according to the manufacturer's protocol.

4.3.6 DNA purification procedures

4.3.6.1 Purification of gDNA with magnetic beads

For DNA purification, an equal volume of AMPure XP beads from Beckman Coulter (cat. no. A63880) was added to DNA samples and the mixture was incubated for 5 min with gentle shaking. Eppendorf tubes were then put on a magnetic stand to separate the beads from the remaining solution, which was discarded. Freshly prepared 80%

ethanol was then added to the tubes, which were still in the magnetic stand. After incubation for 30 sec the ethanol was discarded. Washing with ethanol was repeated twice. Beads were then air dried for three minutes, unless otherwise stated, with an open lid whilst tubes were placed in the magnetic stand. For gDNA elution, the required volume plus 15% of elution buffer (10 mM TE) was added, mixed by pipetting up and down, the tubes placed on the magnetic stand, and after separating the beads, the released gDNA was transferred into a fresh tube. All steps were done at room temperature. The beads, which are stored at 4°C, were warmed to room temperature before use.

4.3.6.2 Purification of PCR products for sequencing

The ExoSAP-IT™ (ThermoFisher cat. No. 78200) kit was used to enzymatically remove unused primer and dNTPs from PCR reactions, which would otherwise interfere with DNA sequencing reactions. The kit was used according to the manufacturer's protocol.

4.3.6.3 Purification of PCR products for GBS

The Qiagen QIAquick PCR purification kit (Qiagen cat. No. 28106) was used according to the manufacturer's recommendations.

4.3.7 Gene enrichment and sequencing assays (RenSeq and GenSeq)

The gene enrichment and sequencing assays RenSeq and GenSeq enrich for NB-LRR *R*-genes and low copy number genes throughout the chromosome, respectively, and are used to find informative SNPs linked to the trait of interest, in this case, resistance to *G. pallida* in the diploid wild potato species *S. spegazzinii* accession 7195. Figure 4-4 illustrates the general workflow of this method.

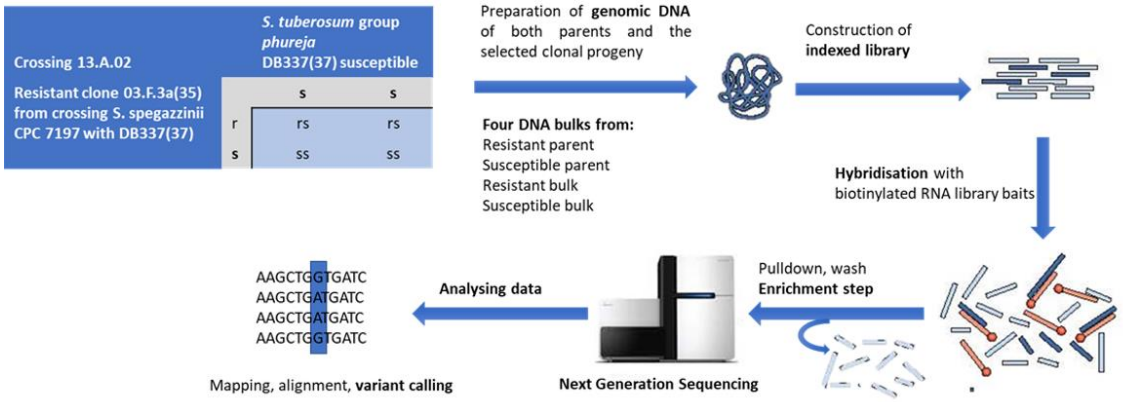


Figure 4-4 Workflow of enrichment sequencing
gDNA from all individuals to be tested is extracted (some gDNA samples are pooled), to obtain four different DNA samples (resistant bulk, susceptible bulk, resistant parent and susceptible parent), the DNA is fragmented to 500 bp fragments and each library is indexed. The library is hybridized with the RNA baits of the targeted. This figure is adapted from Bamshad et al. (2011). Permission to reprint/adapt by RightsLink®, licence no. 5416960432123.

Gene enrichment sequencing involves the preparation of gDNA and bulking the individual samples so that four indexed libraries can be generated. These include (1) the parent having the trait of interest and (2) the parent without the trait and (3) bulked gDNA samples of 20 trait bearing progeny and (4) a similar bulk of progeny lacking the target trait. DNA libraries are barcoded (indexed). The next step is the enrichment for target sequences by hybridisation (capturing) with a RNA library corresponding to target genes, and the enrichment of hybrid molecules facilitated by magnetic beads. Captured DNA molecules are amplified by PCR using barcoded primers and subjected to next generation sequencing. For *Solanum R*-gene enrichment (RenSeq), a bait library described by Jupe et al. (2013) was used. To identify SNPs from generic mapping (GenSeq), a bait library targeting single- or low-copy genes was used (Chen et al., 2018).

The individual steps are described below. The manufacturer's protocols were followed in each case. Figure 4-5 shows a summary of the procedures for capturing indexed enriched DNA libraries for NGS.

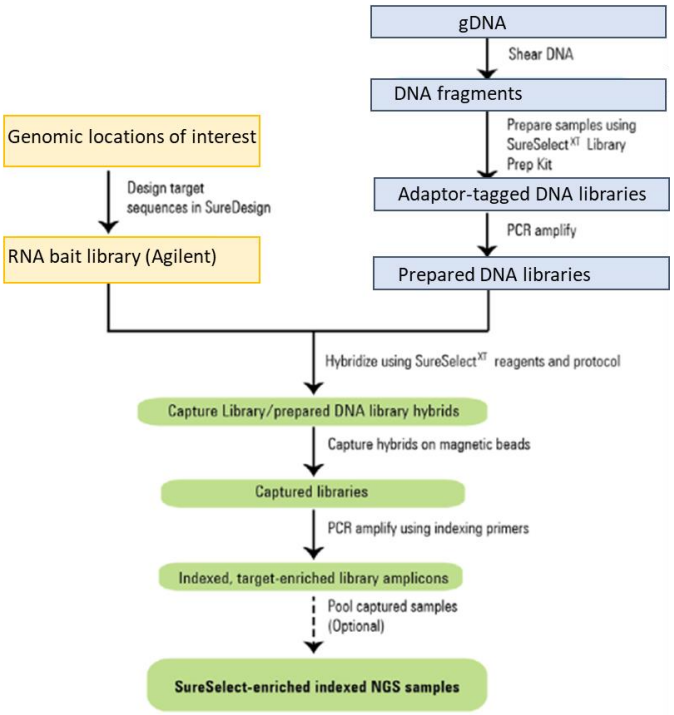


Figure 4-5 Workflow for the preparation of indexed enriched sequencing samples
The diagram was adapted from the SureSelect^{XT} Target Enrichment System for the Illumina Platform kit protocol (Agilent, 2020). The method is described in detail in the main text. © (Agilent Technologies, Inc. 2020. Reproduced with Permission, Courtesy of Agilent Technologies, Inc.)

4.3.7.1 Extraction of gDNA, bulking and fragmentation

gDNA was extracted from individual potato leaves using the Qiagen DNeasy Plant Mini Kit. gDNA concentrations were determined, and 1 µg gDNA was used for each library. When bulked libraries were prepared, 50 ng of gDNA from each of the 20 lines was pooled to generate 1 µg of gDNA. A Covaris Sonicator was used to fragment gDNA into the target length of approximately 500 bp needed for library generation. Conditions for sonication of 50 µl samples were as follows: 50 W 20% duty cycle, 200 cycles per burst, for 60 seconds. The correct fragment size was verified by the JHI Genome Technology Facility using a high-sensitivity DNA electrophoresis assay employing the Agilent Bioanalyzer 2100. The remaining fragmented gDNA samples were purified as described in 4.3.5.1 using 55.5 µl for the library preparation.

4.3.7.2 Library preparation

The NEBNext Ultra DNA library Prep Kit (New England BioLabs, E7370S) was used for library generation according to the manufacturer's protocol using the fragmented

gDNA samples/pools. The index primer sequences used for library generation are shown in Table 4-1. Next generation sequencing was conducted in flow cells where up to 12 libraries were sequenced at the same time, requiring each library to be barcoded. Barcoding was achieved by choosing compatible primers, so that each primer contains a unique 6 bp sequence (highlighted in bold in Table 4-1) serving as an index.

Table 4-1 List of index primers used for library generation.
Primers used for various libraries and corresponding barcoding indexes are shown. Unique 6-nucleotide sequences, highlighted in bold, are used to differentiate between libraries.

Library	Index no.	Index primer sequence
DB337(37)	11	5′-CAAGCAGAAGACGGCATACGAGAT**GTAGCC**GTGACTGGAGTTCAGACGTGTGCTCTTCCGATC-s-T-3′
03.F1.3a(35)	10	5′-CAAGCAGAAGACGGCATACGAGAT**AAGCTA**GTGACTGGAGTTCAGACGTGTGCTCTTCCGATC-s-T-3′
Susceptible. bulk 13.A.02	7	5′-CAAGCAGAAGACGGCATACGAGAT**GTAGCC**GTGACTGGAGTTCAGACGTGTGCTCTTCCGATC-s-T-3
Resistant. bulk 13.A.02	12	5′-CAAGCAGAAGACGGCATACGAGAT**TACAAG**GTGACTGGAGTTCAGACGTGTGCTCTTCCGATC-s-T-3′

4.3.7.3 Hybridisation, target enrichment, post-capture amplification and sequencing

gDNA libraries were hybridised with the respective RNA bait libraries using the Agilent SureSelect Target Enrichment System for Illumina Paired-End Sequencing Library. RNA baits were biotinylated RNA-based probes 120 nucleotides long, that target DNA sequence to be enriched. The generation of RenSeq and GenSeq bait libraries was described by Jupe et al. (2013) and Chen et al. (2018), respectively.

For RenSeq and GenSeq target capturing, a “SureSelect capture library mix for target enrichment” was prepared, following the manufacturer’s procedures for capturing <3.0 Mb DNA (Sure Select XT, Target Enrichment System for the Illumina Platform G7530-90000). The block solution was prepared with “Indexing Block #1 and #3” and, instead of Indexing Block #2, 1 µl soybean repetitive DNA was used. Target-enriched libraries were PCR amplified according to the kit instructions. Sequencing of enriched libraries was performed on a single flow cell by the Genome Technology Facility of the JHI.

4.3.7.4 Data analysis

The workflows and scripts for the bioinformatics data analysis were developed and adapted at the JHI. This study was supported by Joanne Tze-Yin Lim and Miles Armstrong, both at the time at the JHI, who provided an introduction for using the relevant scripts and the bioinformatics analysis. A more detailed description of the data analysis is provided in Van Weymers et al. (2016). Figure 4-6 illustrates the data analysis steps.

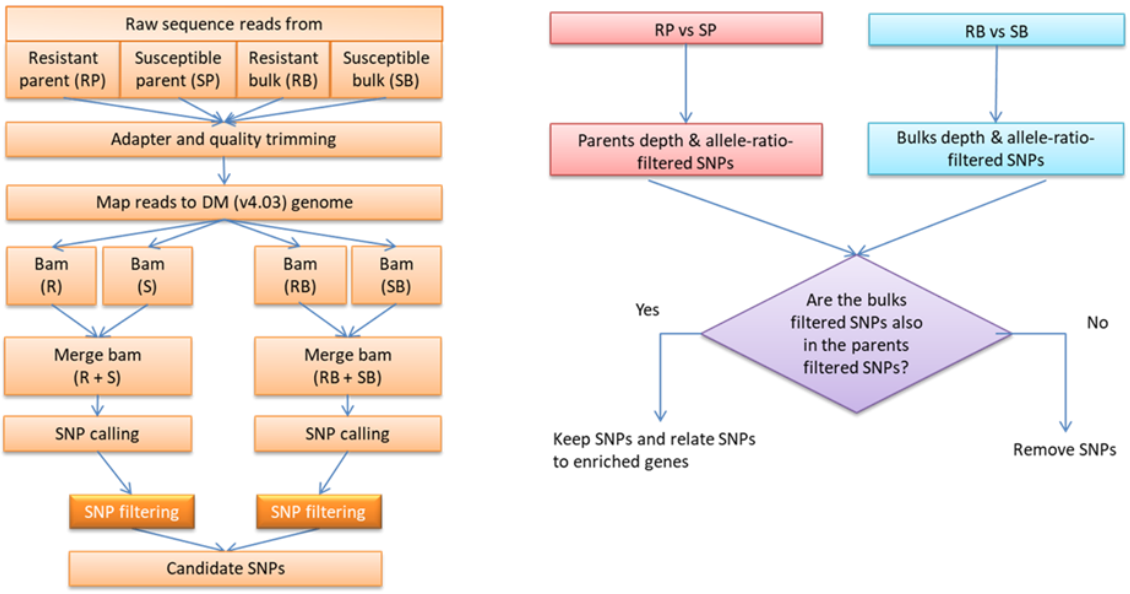


Figure 4-6 Workflow of the enrichment sequencing data analysis
This figure illustrates the steps of the data analysis (Joanne Tze-Yin Lim, pers. comm.). Raw sequence reads from the MiSeq machine are adapter and quality trimmed. Each set of sequence reads is mapped to the reference genome (DM in this case), the parents and the bulks are merged to call for SNPs, the SNPs are filtered and validated to parents and bulks. This way candidate SNPs as molecular markers for the target trait are obtained.

4.3.7.5 Read mapping

Adapters were trimmed with the software Fastq-mcf (v1.04.676). Trimmed reads were then mapped to the DM reference genome (V4.03) using Bowtie2 (v2.0.6, default mode for multi-mapping) program. The resulting BAM files for both the parents and the bulks were merged and indexed using the software package SAMtools v0.1.18, with SAMtool mpileup. The pileup files were generated for both bulks and parents and the software VarCan v2.3.7 was used for variant calling.

4.3.7.6 SNP filtering and identification of informative SNPs

Sequences were only used when the read depth was ≥50. The sequences were mapped to DM to determine the position on the chromosome. When the allele in the parents or bulks was different from that in DM, it was called the alternative allele. The filtering criteria to determine SNPs that indicate linkage to the resistance locus for informative SNPs were 40-60% for the resistant bulk and 0-10% or 90-100% for the susceptible bulk, depending which reference (resistant and susceptible, respectively) was used. These SNPs were compared to SNPs identified between the heterozygous resistant parent and the homozygous susceptible parent. Only SNPs that were present in the bulks and parents were kept and called informative SNPs. These informative SNPs were subsequently used to generate KASP markers (see below).

4.3.8 GBS

Another enrichment method for identifying informative SNPs is GB*S.* In this method gDNA fragments obtained by a restriction enzyme digest are enriched instead of genes/regions of genes. In this project, the *Pst*I and *Mse*I digested and indexed DNA samples from individuals used for the susceptible and resistant bulks, and these, along with similar samples from the parental lines were subject to Illumina sequencing and analysed to identify informative SNPs (data analysis undertaken by Sanjeev Kumar Sharma, JHI).

4.3.8.1 GBS library construction

The gDNAs of the susceptible DB337(37) and resistant 03.F1.3a(35) parents and the 20 resistant and susceptible progeny lines used for gene enrichment sequencing were used for GBS analysis. DNA concentrations were measured using the Picogreen assay and samples were normalised to 10 ng/µl. For the digestion with *Pst*I and *Mse*I, 5µl of a master-mix consisting of 82.5 µl 10x NEB Buffer 4, 11 µl *Pst*I, 22 µl *Mse*I and 159.5 µl H_2O were added to wells of a 96 well microtiter plate, then 10 µl DNA was added to each well. The plate was sealed and incubated at 37°C for 3 hours, followed by 80°C for 20 mins, before holding the temperature at 8°C. Individual fragments were barcoded in a reaction with 1.0 µl 10x NEB Ligation Buffer, 2.5 µl ATP*,* and 0.75µl T4 DNA Ligase and 2.0 µl Common *Mse*I Y Adapter and 1.75 µl H2O added per reaction to the 15 µl of

the *Pst*I/*Mse*I gDNA digestion. 2 µl of individual bar-coded *Pst*I Adapter were added to each sample. The plate was sealed and incubated at 22°C for 2 h, then 65°C for 20 min and held at 8°C. Then, 5 µl from each sample were pooled giving a total of 240 µl pooled library. The library was purified using the Qiagen QIAquick PCR purification kit and eluted into 65 µl H_2O. In the next step, the library was amplified in a PCR reaction in four replicates with 25.0 µl 2x Master mix (NEB), 2.5µl each of Primer A (10 µM specific to barcoded adapter side) and Primer B (10 µM, specific to the common adapter side), 12.5 µl H_2O and 7.5 µl of the pooled and ligated library, under the following conditions in a thermocycler: 1 cycle at 98°C for 30 sec, 12 cycles at 98°C for 10 sec and 72°C for 20 sec, 1 cycle 72°C for 5 min, then put on hold at 8°C. The PCR products were purified using a Qiagen QIAquick PCR purification kit and eluted into 40 µl Elution Buffer (Section 4.3.4). The quality and quantity of the purified library was checked using an Agilent Bioanalyzer 2100 by the Genome Technology Facility of the JHI.

4.3.8.2 GBS analysis

The GBS analysis was performed by Sanjeev Kumar Sharma. BAM files containing the SNP data were provided and only used to look for informative SNPs for the resistance to *G. pallida*. The reads were mapped against the reference genome of DM.

4.3.9 KASP assay

KASP is a method that uses the different alleles of a SNP of interest as allele-specific forward primers and a common reverse primer for both alleles to amplify allele-specific sequences. The allele-specific primers possess a unique sequence, corresponding to a universal fluorescence resonant energy transfer (FRET) cassette using the two different fluorescent dyes HEX and FAM for the different allele-specific primers. The KASP master-mix, provided by the company LGC Biosearch Technologies, is a buffer containing these FRET cassettes, in addition to a passive reference dye, Taq polymerase and dNTPs. During the PCR reactions, only the alleles-specific primers, which have a corresponding allele in the sequence of the sample, can bind and elongate. In this step, the tail sequence with the corresponding fluorescent cassette is attached to the newly synthesised strand, which enables the dye-labelled oligo to bind to new complementary sequence and therefore is no longer quenched and emits

fluorescence (Figure 4-7). This means that if a SNP is homozygous, only one of the two possible fluorescent signals will be generated, if the genotype is heterozygous, a mixed fluorescent signal will be generated.

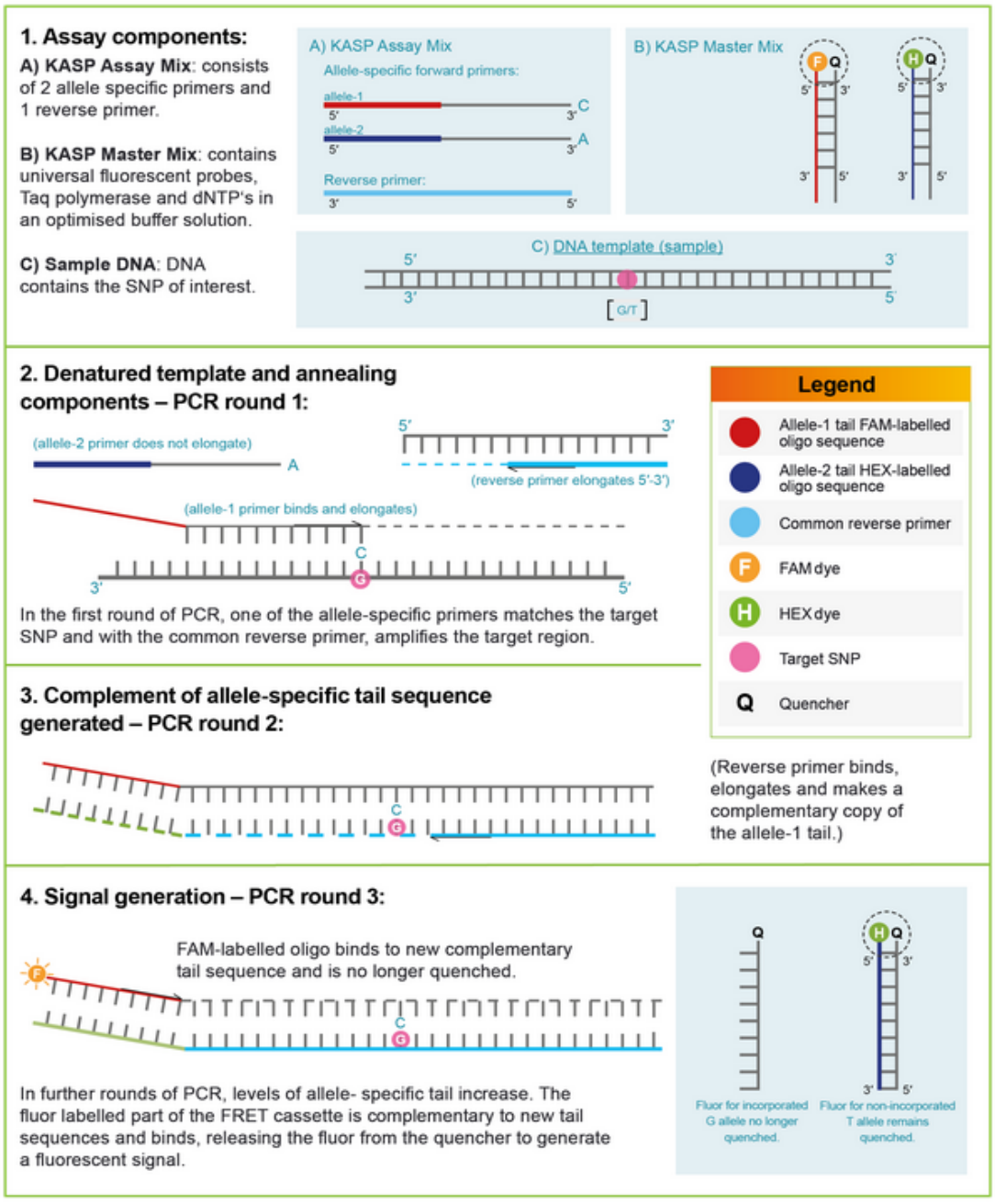


Figure 4-7 Mechanism of KASP assays for SNP detection
This diagram is reprinted from LGC Biosearch Technologies (2022) and illustrates how a KASP assay can discriminate between two alleles. Reproduced with Permission, Courtesy of LGC Biosearch Technologies.

Informative SNPs were used to generate KASP™-markers. The company LGC Genomics limited (now LGC Biosearch Technologies) designed and produced the primers from the submitted sequence of the informative SNP +/-50 bp additional sequence (in

addition to the SNP of interest). To enhance the probability that the primers anneal only to the target site, the +/-50 bp regions around each SNP were used in a BLAST search against pseudomolecules of the DM reference genome (database pseudomolecules v4.03 and 4.04) on the SpudDB[11] website of the University of Michigan using default settings. When no off-target BLAST hits were found in the DM genome (defined as >95% sequence identity over at least 28 bp), the informative SNPs were used for KASP assay synthesis.

The PCR amplifications were performed as follows: DNA was extracted from young leaf material from individual plants, DNA concentration was measured by either Picogreen or Qubit (if only a few samples were needed), and diluted to a concentration of 20 ng/µl. DNA was mixed with the KASP reagent (provided by LGC) and primer mix (provided by LGC) and run on a StepOne Plus (ThermoFisher Scientific) thermocycler using the following conditions: 1 cycle at 20°C for 2 min, 94°C for 15 min, 10 cycles at 94°C for 20 sec at 62°C for 1 min (decreasing by 0.7°C per cycle), 32 cycles at 94°C for 20 sec at 55°C for 1 min, and 1 cycle at 20°C for 2 min. In some cases, a recycling step was performed in order to tighten clusters: 3 cycles at 94°C for 20 sec at 57°C for 60 sec and at 37°C for 1 min. The corresponding software StepOne v2.3 was used to analyse the data.

4.3.10 Sanger sequencing

Sanger sequencing was performed as described in Section 2.3.7.5.

4.4 Results

4.4.1 *S. spegazzinii* derived PCN resistance protects against various PCN populations

The level of PCN resistance in *S. spegazzinii* to three UK *G. pallida* populations, "Lindley" Pa2/3, "Luffness" Pa2/3 and Pa1, which correspond to the three introductions to the UK and that differ in their virulence against other resistance sources, was assessed. In addition, resistance to *G. rostochiensis* Ro1 was assessed in the parental lines 03.F.1.3a(35) and DB337(37) by pot bio-assays (Section 2.3.5). The potato cultivar Desirée served as a positive control for PCN susceptibility. The original P_0 clone *S. spegazzinii* CPC 7195(10) had been lost before the start of this project. The

[11] http://spuddb.uga.edu/blast.shtml

resistant F1 parent 03.F.1.3a(35) showed a reduced number of cysts across all four PCN populations tested, ranging from 4 to 23 cysts per pot at 12 weeks post infection (Figure 4-8, green bars) and was used as a control for resistance. In contrast, the susceptible parent DB337(37) showed cyst numbers that are comparable to those observed for Desirée, ranging from 100 to 332 cysts (Figure 4-8, blue and yellow bars, respectively). The differences between the resistant parent and the two susceptible lines were statistically significant, as determined using Fisher's exact test.

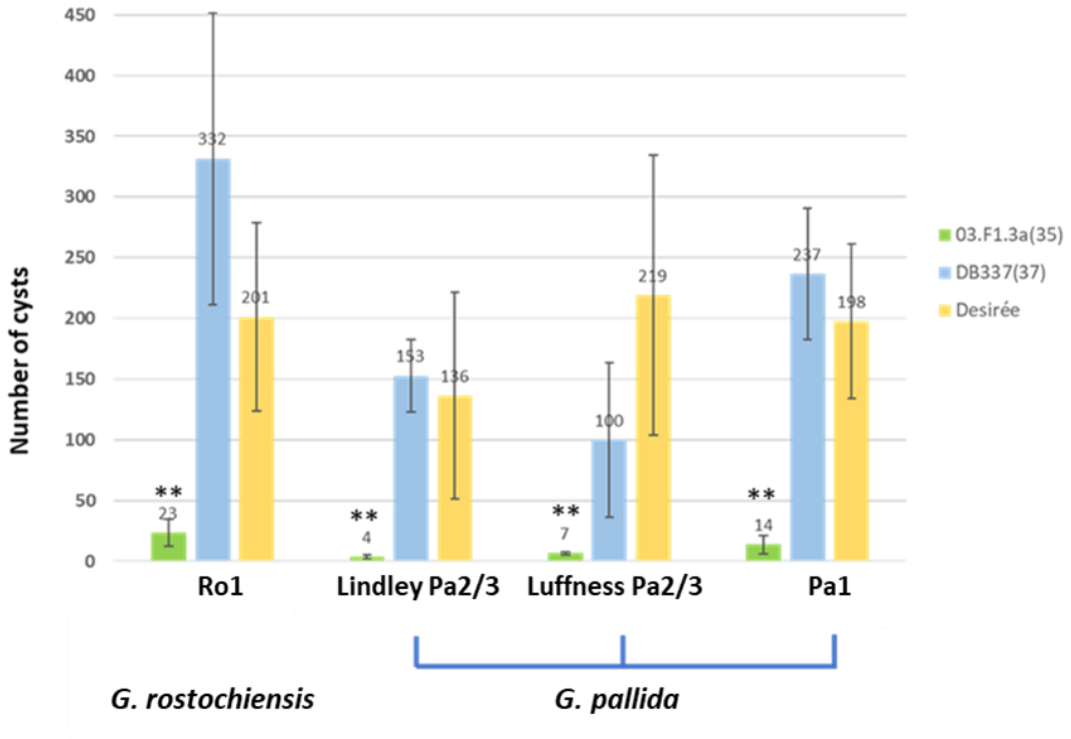


Figure 4-8 Resistance to different PCN populations in 03.F1.3a(35), DB337(37)and Desirée
Using a pot test, the level of resistance for different PCN populations was determined for the parental lines 03.F1.3a(35) and DB337(37), with Desirée serving as a susceptible control. The number of cysts is highly reduced in the resistant parent for all four PCN populations tested. The cyst numbers for the susceptible parent DB337(37) is comparable to the numbers obtained for Desirée. ** indicates that number of nematodes are highly significantly ($p<0.01$) lower than on Desirée and DB337(37).

To obtain the reproduction factors for each PCN population on each potato genotype, the final cyst numbers were divided by the initial cyst number, which was 15 for this experiment (Table 4-2). Full resistance, which is indicated by a reproduction factor <1, was observed in 03.F.1.3a(35) for the *G. pallida* populations Lindley, Luffness and Pa1. *G. rostochiensis* scored slightly higher in 03.F.1.3a(35) with a reproduction factor of 1.6, which means that cyst numbers were increased but to a far lesser extent

compared to the susceptible potato lines. As expected, DB337(37) and Desirée were very susceptible to all four PCN populations with reproduction factors ranging from 6.6 to 22. These results also demonstrate that mapping the resistance locus, or resistance loci (if resistance is caused by multiple loci) using progeny from resistant 03.F.1.3a(35) and susceptible DB337(37) is feasible with a segregating population.

Table 4-2 Reproduction factors of PCN populations
The first number indicates the reproduction factor, followed by the standard error of the mean.

	PCN population			
	G. rostochiensis		***G. pallida***	
Potato genotype	**A Ro1**	**Lindley Pa2/3**	**Luffness Pa2/3**	**Pa1**
Desirée	13.4±5.2	9.1±5.7	14.6±7.7	13.2±4.2
03.F1.3a(35)	1.6±0.8	0.2±0.1	0.4±0.1	0.9±0.5
DB337(37)	22.1±8	10.2±2	6.6±4.2	15.8±3.6

4.4.2 Segregation pattern of the resistance to *G. pallida* in 13.A.02

In order to map the resistance locus, progeny clones from backcross 13.A.02 that resulted from the cross of resistant 03.F.1.3a(35) and susceptible DB337(37) were used (Figure 4-3). A random subset of 200 of the 993 original BX1 clones were picked and the segregation pattern of the resistance and susceptibility to *G. pallida* "Lindley" Pa2/3 was determined using root trainer assays (Section 4.3.3.1). In Figure 4-9 panel A, examples of BX1 progeny growing in root trainers are shown; panel B shows an open root trainer with female nematodes on the surface roots. The area of the blue square is enlarged to show the female nematodes.

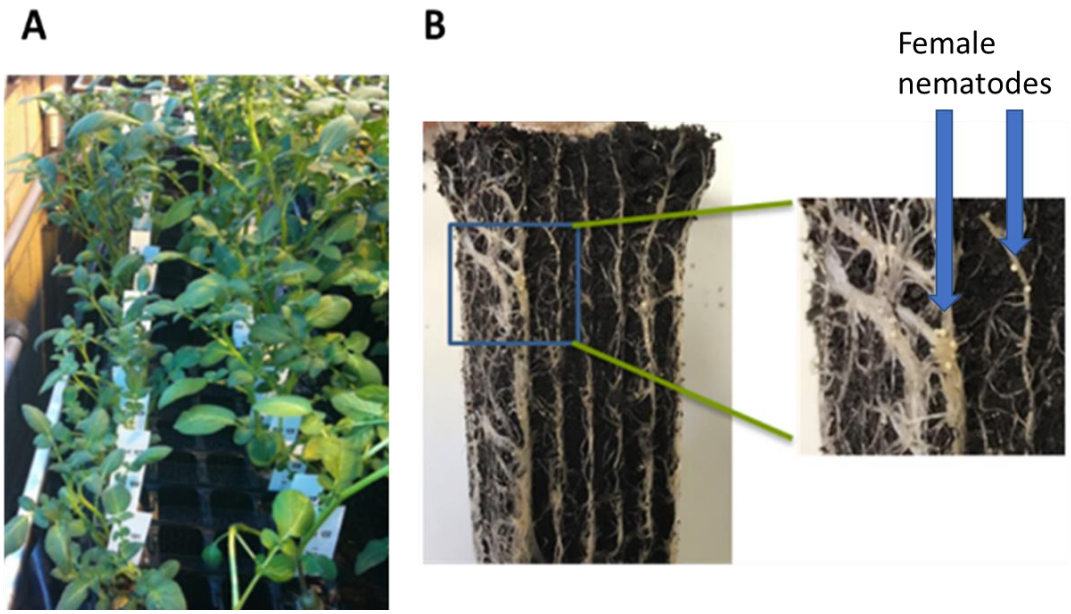


Figure 4-9 Illustration of a root trainer assay
Panel A shows clonal progenies of the cross 13.A.02 growing in root trainers. Panel B shows an example of an open root trainer with female nematodes residing on the surface roots. The area within the blue square is enlarged to show female nematodes (highlighted by blue arrows).

In 158 of 200 cases, backcross progeny clones could be propagated in three or four replicates and individually assessed for resistance. The segregation patterns of *G. pallida* Lindley Pa2/3 resistance is shown in Figure 4-8 together with the resistance scores of the parental lines. The resistant 03.F.1.3a(35) line showed an average of 1 female nematode per root trainer, while the susceptible DB337(37) scored an average of 122 female nematodes.

The following criteria were defined: full resistance was set as having less than 6 female nematodes visible on the outside roots of the plants; plants with 6 to 25 nematodes were defined as having reduced susceptibility and plants with > 25 nematodes as susceptible. All in all, out of the 158 clones tested in replicates, 51 clones were reproducibly classified as susceptible (>25 female nematodes), 61 as resistant (≤5 female nematodes) and 46 as reduced susceptibility (>5, <26 female nematodes) (Figure 4-10).

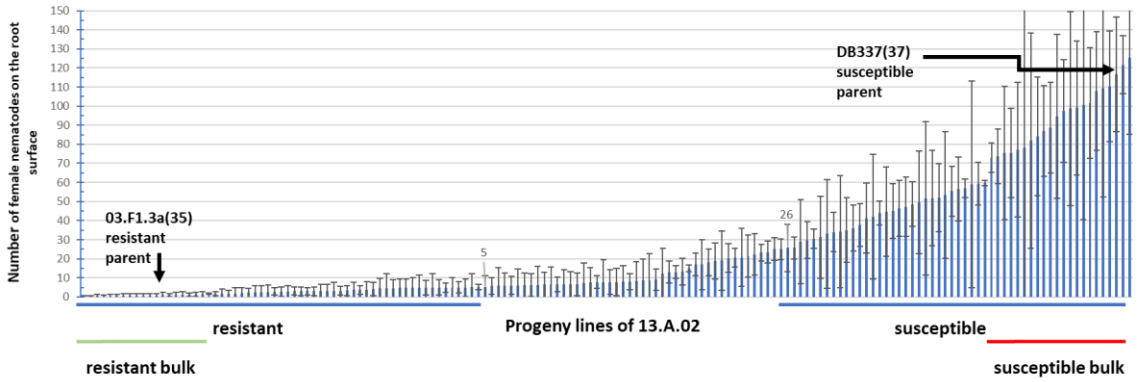


Figure 4-10 Determination of *G. pallida* resistant and susceptible progeny clones of 13.A.02
This diagram shows the average number of female nematodes in the 158 initially tested progeny clones of 13.A.02 in a root trainer assay. 62 clones showed full resistance with less than 6 female nematodes on the outside roots, 26 were moderately resistant, 20 showed reduced susceptibility and 52 were susceptible. The resistant and susceptible parents are indicated with black arrows.

To determine if the resistance is likely to be conferred by one or more resistance genes, five Mendelian segregation patterns were considered, as outlined in Figure 4-11. The parental line 03.F1.3a(35) is known to be heterozygous as shown in Figure 4-3. Therefore, the resistance must be inherited in a dominant manner. Assuming that resistance is conferred by one or two independently segregating loci, five outcomes are considered. 1) If only one gene (or more if they are located on one haplotype) confers the resistance, the ratio of resistant to susceptible plants is expected to be 1:1. 2) If two independently segregating genes are conferring the resistance, and the two genes have an additive effect on the resistance, the ratio is expected to be 1:2:1 (both resistances, one resistance (less effective), no resistance). 3) If they do not have an additive effect and contribute approximately the same levels of resistance, the expected ratio of resistant to susceptible plants would be 3:1. 4) If one of the two resistances is much stronger than the other, the expected ratio would be 2:1:1. Finally, 5) if both resistant genes are needed to confer resistance, then the expected ratio would be 1:3.

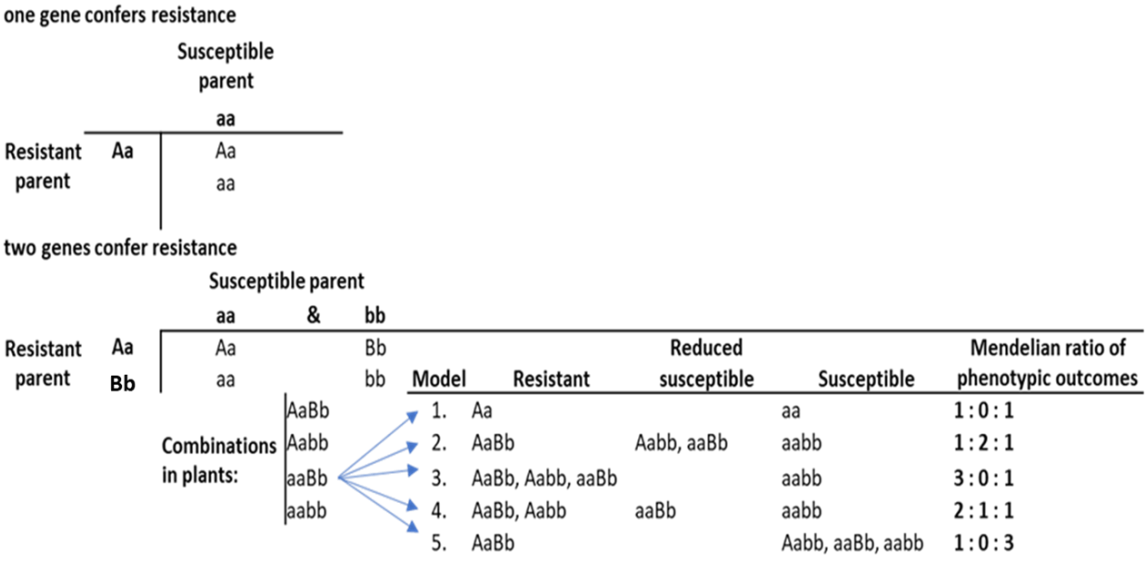


Figure 4-11 Expected ratios of phenotypes with one or two genes conferring resistance
Scheme illustrating potential patterns of the Mendelian inheritance of resistance genes. A, B indicate dominant alleles; a, b indicate recessive alleles. Model 1 involves only one resistance gene, models 2 to 5 have two independent resistances with different weighting of the genes (see main text). Resistant means < 6 female nematodes. Reduced susceptible means between 6 and 25 female nematodes, and susceptible means >25 female nematodes visible on the outside roots of a root trainer.

Chi-square (χ^2) tests were performed for each outcome, comparing the expected outcome for cases 1 to 5 with the observed outcome, to determine if the observed phenotype ratios were different from the theoretical ratios, which would mean the model could be excluded. If a range of different phenotypes were expected, the reduced susceptible clones were counted, but if only two phenotypes were expected, the reduced susceptible clones were not considered in the calculation. Table 4-3 shows the p-values, calculated from χ^2 values. In short, two models can explain the observed phenotype numbers: firstly, model number 1, where only one gene confers the resistance, when the clones with a female nematode score of >5 and <26 are not considered, and, secondly, model number 4, which assumes two independent resistance genes, with one having a much larger effect than the other.

Table 4-3 p-values from χ^2 tests to determine models of genes conferring resistance
A p-value of <0.05 indicates that the model and the observed data are significantly different, a p-value of >0.05 means the model and the observed data are not significantly different,

Model No.	No. of genes	Ratio of phenotypes resistant:(reduced susceptible):susceptible			Compatible with observed data
		Theoretically expected	Observed	p-value	
1	1	1:1	1.2:1	0.69	yes
2	2	1:2:1	1.2:0.9:1	0.0006	no
3	2	3:1	1.2:1	0.0104	no
4	2	2:1:1	1.2: 0.9:1	0.12	yes
5	2	1:3	1.2: 1	<0.00001	no

indicating that the model can explain the observed data.

To identify the single resistance genes predicted by Model 1 or the largest effect gene for the resistance in Model 4, the extreme phenotypes with the 20 most and 20 least resistant clonal lines were selected and used as bulks for the enrichment sequencing. These lines, together with the number of female nematodes scored in the root trainer assay are listed in Table 4-4.

Table 4-4 13.A.02 progeny and parents divided into groups for RenSeq and GenSeq analysis
The table shows the clone IDs that were used BSA, the average nematode number of 3 or 4 independent replicates per clone and the standard deviation.

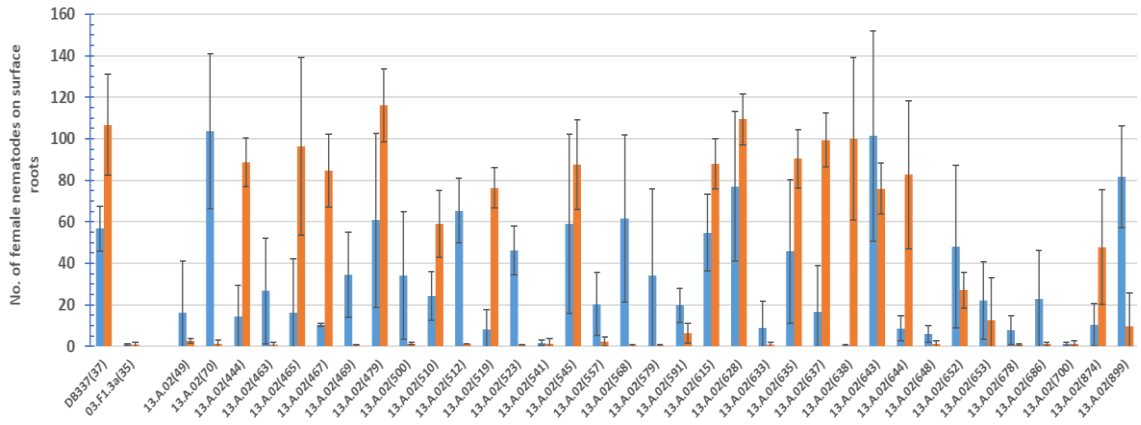
	Susceptible plants			Resistant plants		
	Clone ID	Average no. female nematodes	Standard deviation	Clone ID	Average no. female nematodes	Standard deviation
Bulk	13.A.02(58)	84	31.1	13.A.02(49)	2	1.2
	13.A.02(444)	77	35.3	13.A.02(70)	1	1.7
	13.A.02(448)	74	14.4	13.A.02(446)	1	0.8
	13.A.02(465)	109	56.9	13.A.02(463)	0	0.4
	13.A.02(467)	75	34.9	13.A.02(469)	1	0.8
	13.A.02(471)	99	51.0	13.A.02(495)	1	0.8
	13.A.02(479)	125	40.0	13.A.02(500)	1	0.7
	13.A.02(510)	78	81.3	13.A.02(512)	1	0.9
	13.A.02(519)	110	29.0	13.A.02(516)	1	0.9
	13.A.02(524)	75	23.3	13.A.02(523)	1	0.5
	13.A.02(545)	95	43.1	13.A.02(568)	1	0.8
	13.A.02(566)	87	23.8	13.A.02(579)	0	0.4
	13.A.02(608)	82	56.4	13.A.02(586)	1	1.2
	13.A.02(615)	99	35.1	13.A.02(619)	1	0.9
	13.A.02(628)	108	31.2	13.A.02(625)	2	0.8
	13.A.02(635)	97	26.9	13.A.02(633)	1	0.8
	13.A.02(637)	102	29.0	13.A.02(648)	1	1.2
	13.A.02(638)	101	60.2	13.A.02(678)	1	0.9
	13.A.02(643)	89	23.7	13.A.02(686)	1	0.8
	13.A.02(644)	117	30.1	13.A.02(700)	1	1.1
Parent						
	DB337(37)	122	15.2	03.F1.3a(35)	1	1.2

4.4.3 The resistances to *G. rostochiensis* and *G. pallida* do not co-segregate

The next aim was to determine whether the resistance to *G. pallida* Lindley Pa2/3 and to *G. rostochiensis* Ro1 are conferred by the same resistance locus. It was reasoned that the same plants that are either resistant or susceptible to *G. pallida* Lindley Pa2/3 are also resistant or susceptible, respectively to *G. rostochiensis* if resistances are conferred by the same locus. Root trainer assays were performed on 35 clonal lines that were classified as fully resistant or susceptible to *G. pallida,* to compare PCN resistance/susceptibility of *G. pallida* with *G. rostochiensis,* including the parental potato lines. The number of female nematodes developing on the surface roots were counted. This analysis of confirmed the previous phenotypic assessment of resistance

against *G. pallida* Lindley Pa2/3 and demonstrated that the *G. pallida* Lindley Pa2/3 and the *G. rostochiensis* resistance loci do not co-segregate (Figure 4-12).

In summary, these data are consistent with Lindley Pa2/3 and the *G. rostochiensis* Ro1 resistance being caused by different loci. It is likely that the locus conferring resistance to *G. pallida* Lindley Pa2/3 also confers resistance to *G. pallida* Luffness Pa2/3 and



G. pallida Pa1, given that these lines are all derived from the same species.

Figure 4-12 The resistances to *G. rostochiensis* and *G. pallida* do not co-segregate
Root trainer assays for assessing the segregation pattern for *G. rostochiensis* and *G. pallida* in the 13.A.02 line were performed, and the results compared. The blue bars indicate *G. rostochiensis* and the orange bars *G. pallida* infection levels. The numbers of females are plotted for each potato line tested; the parents serve as controls. The infections assays were repeated four times per clone.

4.4.4 Mapping the resistance to *G. pallida* derived from *S. spegazzinii* CPC 7195

4.4.4.1 Identification of informative SNPs using RenSeq

In order to find SNPs linked to the *S. spegazzinii* CPC 7195 resistance to *G. pallida*, gDNA libraries of individually indexed parents, bulked susceptible and bulked resistant samples were first subjected to RenSeq-based enrichment, which specifically targets NB-LRR genes (Jupe et al., 2013), as it seemed likely that the gene conferring resistance belongs to the NB-LRR family of R-genes. The sequences were analysed at a mismatch rate of 3, 5 and 10% to allow for differences between the reference genome which belongs to *S. tuberosum* group Phureja and the target species, *S. spegazzinii*. In Table 4-5 the total number of reads and the reads mapped to DM as reference are shown. The rate of mapped reads ranged, depending on the mismatch rate from 43 to 79%.

Table 4-5 Read numbers and on-target reads to the reference DM in the RenSeq experiment

Library	Paired end reads	On target mapping reads (%) to reference DM at mismatch rate 3%	5%	10%
PS	3784716	1892069 (49.9%)	2374294 (62.7%)	2958385 (78.17%)
PR	4230434	1816183 (42.9%)	2486173 (58.8%)	3299361 (77.99%)
BS	1400608	668834 (47.8%)	864709 (61.7%)	1105056 (78.89%)
BR	1072296	467944 (43.6%)	624529 (58.2%)	832852 (77.67%)

The filtering conditions for passing as SNPs were set at an allele frequency of 0.4 to 0.6 for resistant and at an allele frequency 0-0.1 or 0.9-1.0 for susceptible plants. The number of SNPs between the resistant and susceptible bulks, as well as the number of SNPs between the resistant and susceptible parent was determined. Then, the number of SNPs that conform to the expected ratio of 0.4 to 0.6 in the resistant bulk and the resistant parent, and of 0-0.1 or 0.9-1 for the susceptible bulk and susceptible parent was calculated. These represented the informative SNP candidates. Table 4-6 summarizes the number of SNPs determined in bulks, parents, and both, as well as the distribution on the chromosomes of the informative SNP candidates for 3, 5 and 10% mismatch rate.

Table 4-6 Different mismatch rates provided different number of SNPs in RenSeq

Mismatch rate	Number of SNPs (in transcripts)			Locations of informative SNPs
	Bulks	Parents	Bulks & parents	
3%	120	5256	4	4 on chr. XI
5%	162	10404	8	8 on chr. XI
10%	131	15257	10	7 on chr. XI, 1 each on chr. IV, IX and X

For illustration, the results of the RenSeq analysis at a 5% mismatch rate are shown in Figure 4-13. With these settings, 10,404 parental SNPs (Panel A) are present, 162 SNPs from the bulks passed the filtering for being heterozygous (40-60% of the alternative allele) for the resistant bulk and being homozygous for the susceptible bulk (Panel B), and eight SNPs (Panel C) were validated for having the expected frequency in both parents and bulks and for being intragenic.

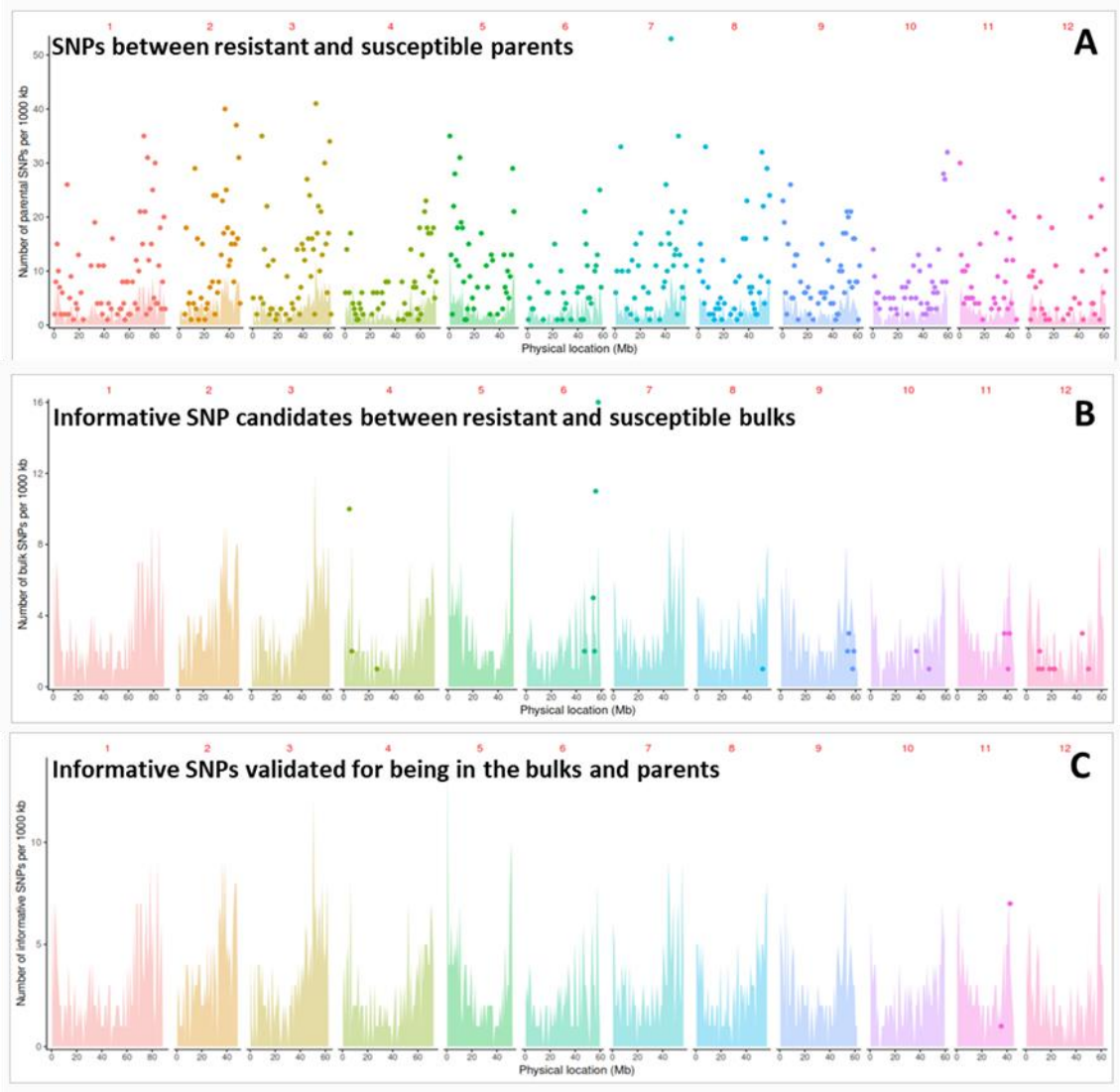


Figure 4-13 Distribution of SNPs identified by RenSeq at a 5% mismatch rate
Each set of coloured data represents a specific chromosome. Each coloured 'spike' represents the number of NB-LRR genes targeted by probes across the chromosome. Each dot represents SNPs and its placement on the y-axis determines the number of SNPs identified in a 1Mb bin. Panel A shows the SNPs identified between the two parental lines. Panel B shows the SNPs found in the two bulks with the expected ratio of the alternative allele, 0.5 ±0.1 for the resistant bulk and 0 or 1 ± 0.1 for the susceptible bulk. Panel C shows the informative SNPS identified and validated to be intragenic in the bulks.

The eight informative SNPs found on chromosome XI at a mismatch rate of 5%, are distributed in two genes, two in one gene and six in the other, spanning an interval of 7.16 Mb. At a rate of 3% mismatch, 4 informative SNPs were found, these were identical with 4 SNPs found at 5% mismatch. At 10% mismatch, one informative SNP candidate each was found on chromosome IV, IX and X. Seven were found on chromosome XI, 6 of which were in one of the genes that were identified with 3 and

5% mismatch rate, whereby 4 of the 6 SNPs were identical with the SNPs found at 5% mismatch. Table 4-7 lists the informative SNPs, their location on the chromosomes, their gene IDs and the number of SNPs in each gene.

Table 4-7 List of genes in which informative SNPs were found with RenSeq

Mismatch rate	Chr.	Start	Stop	Gene ID	No. of SNPs
3%	XI	35562292	35566031	RDC0001NLR0273	2
	XI	42728187	42733523	PGSC0003DMG400018572	2
5%	XI	35562292	35566031	RDC0001NLR0273	2
	XI	42728187	42733523	PGSC0003DMG400018572	6
10%	IV	7749465	7752286	RDC0001NLR0043	1
	IX	47147352	47150352	RDC0001NLR0214	1
	X	42008904	42010860	RDC0001NLR0236	1
	XI	42728187	42733523	PGSC0003DMG400018572	6
	XI	42817399	42822611	RDC0001NLR0289	1

4.4.4.2 KASP assays from the SNP candidates obtained by RenSeq

For six of the eight SNPs obtained in the RenSeq analysis (5% mismatch rate), KASP primer pairs were designed (Table 4-8; section A) in order to determine if the identified SNP can be used for discrimination and mapping of the resistant and susceptible plants.

Table 4-8 List of KASP primers generated for the mapping of the resistance locus

The assay ID shows the chromosome number followed by the position. Section A shows the KASP primers derived from RenSeq, section B shows the KASP primers derived from the GenSeq experiment with DM as reference genome. Section C shows the KASP primers derived from GBS analysis. Assays in bold indicate that these can discriminate between the parent's alleles

Assay ID	Allele		Primer sequence allele X	Primer sequence allele Y	Primer sequence common
	FAM	HEX			
A SNPs obtained by RenSeq					
ST4_03ch11_35563456	G	A	ATTCTTCCGTCACCTCTGTTATTCG	AAATTCTTCCGTCACCTCTGTTATTCA	TGAAATTCCTTTCGATTAGAGGGATGCAT
ST4_03ch11_35563531	**A**	**T**	**AGCTAGTACAATTGTCTCTTAGCAACT**	**AGCTAGTACAATTGTCTCTTAGCAACA**	**TGCTGGCAAGGAATCACAGTCCTT**
ST4_03ch11_42728387	**G**	**T**	**ATGCCTGCTTATTCTCTGCATCC**	**GATGCCTGCTTATTCTCTGCATCA**	**CTTTGCTTGGTCTTCAAGCTGTGCTA**
ST4_03ch11_42729956	G	A	ACAACGATAATGCCCTCAAGGATC	GACAACGATAATGCCCTCAAGGATT	GTGCAGCATTACATACTGCCAAGACTA
ST4_03ch11_42730229	G	T	CTTGAAAATGCCGCTACATCTGAG	CTCTTGAAAATGCCGCTACATCTGAT	CCACTAGCACTTGGAGGCTTTTCAA
ST4_03ch11_42730455	C	T	GAGTCGTAGTAGTGCCGACAAC	GGAGTCGTAGTAGTGCCGACAAT	TAGCTCAGCAAGTATGTCTCTTTCAGTTT
B SNPs obtained by GenSeq					
ST4_03ch06_53821580	**A**	**T**	**GCGAGTTTCCTCGTCTGCTTCT**	**GCGAGTTTCCTCGTCTGCTTCA**	**CCTTCGATACTCTCGAACAACACTTAATT**
ST4_03ch06_54218046	**T**	**C**	**CCATTGGAACATATGCTTCATACTGT**	**CCATTGGAACATATGCTTCATACTGC**	**CAACGCGTGATACATTTGATGGTAAAGAA**
ST4_03ch06_54661772	**G**	**A**	**CCACCCTGTGGATGAGAGGG**	**CCACCCTGTGGATGAGAGGA**	**CTATCTCCCTTCATATGAATCATGGTTCTT**
ST4_03ch06_55096874	C	T	AAACGATCCCAACCATTCCTTTGTC	GAAACGATCCCAACCATTCCTTTGTT	CGCGATGATGGGACACTGTCTAAAT
ST4_03ch06_55909792	**G**	**C**	**CCCTATTTTACTAGCTATCTCAAAACAG**	**CCCTATTTTACTAGCTATCTCAAAACAC**	**ACCTTCGGGCATTGGGAACACATT**
ST4_03ch06_57322514	**G**	**A**	**CCTTGACATGAGGATCTGTGTAC**	**AGCCTTGACATGAGGATCTGTGTAT**	**CATCTGCGTTTCTCTTTTGAGAATCCAAA**
ST4_03ch06_58781798	**G**	**A**	**AATGCAAATGCAAGAAACGAAGGTAG**	**GAATGCAAATGCAAGAAACGAAGGTAA**	**CCTGAGTGTCTCTTCCAAAGTGTRAAA**
C SNPs obtained by GBS					
ST4_03ch06_56153692	**C**	**T**	**GACCATTTGTTTGGTATGTTATAATTGCC**	**AGACCATTTGTTTGGTATGTTATAATTGCT**	**GATGGAGGAAGCTAARATTGGAATAAAGTA**
ST4_03ch06_56915767	**T**	**G**	**GAAGTGAATCAATACATTGCCAGAAT**	**GAAGTGAATCAATACATTGCCAGAAG**	**CACCTGGGCTCCCTCTGGAT**
ST4_03ch06_56987876	**T**	**G**	**AACCAACTTGACGTGAGCAGAAGA**	**CCAACTTGACGTGAGCAGAAGC**	**GAGGTTAACGAAGGCAAGTATATGTCAAA**

In a first step, KASP assays were evaluated on the resistant and susceptible parents. Only two KASP assays, ST4_03ch11_35563531 and ST4_03ch11_42730455, could discriminate between the different alleles in the parental genomes which are shown in Figure 4-14 Panels A and B. Panel C shows KASP assay ST4_03ch11_42730455, as an example for a KASP assay which cannot discriminate between the different parental alleles.

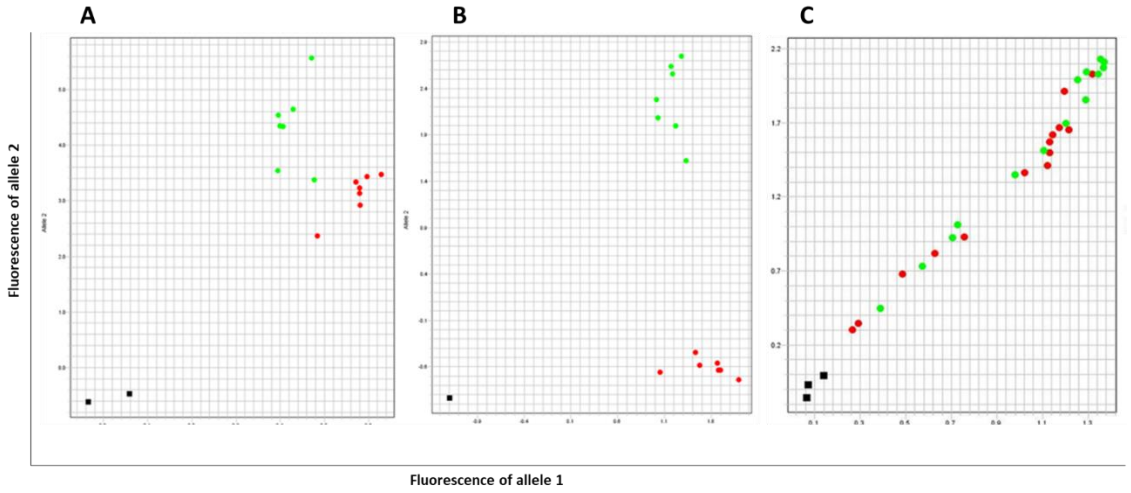


Figure 4-14 Allele discrimination plots from KASP assays
Panel A and B show KASP assays ST4_03ch11_35563531 and ST4_03ch11_42730455, in which clusters of resistant and susceptible parents are formed, panel C shows ST4_03ch11_42728387 as one example for a KASP assay which cannot discriminate between resistant and susceptible parents. Red dots represent the susceptible homozygous parent (susceptible), and green dots represent the resistant heterozygous parent in relation to the candidate SNP. The black squares represent the negative controls (no DNA added).

Next, KASP assays ST4_03ch11_35563531 and ST4_03ch11_42730455 were tested on the DNA of each individual plant of the resistant and suspectable bulks. The parents did not cluster the same way as in Figure 4-15, ST4_03ch11_35563531 clustered slightly better, ST4_03ch11_42730455 slightly worse. The progeny clones did not separate into separate clusters, in fact the results suggest that they all have the same alleles as the resistant parent (Figure 4-15), which cannot be explained by Mendelian genetics. this result indicates that these KASP assays are not robust. Consequently, none of the KASP markers derived from RenSeq provided results usable for mapping. Therefore, an alternative approach using the GenSeq procedure, to identify SNPs demarking PCN resistant and susceptible lines, was chosen.

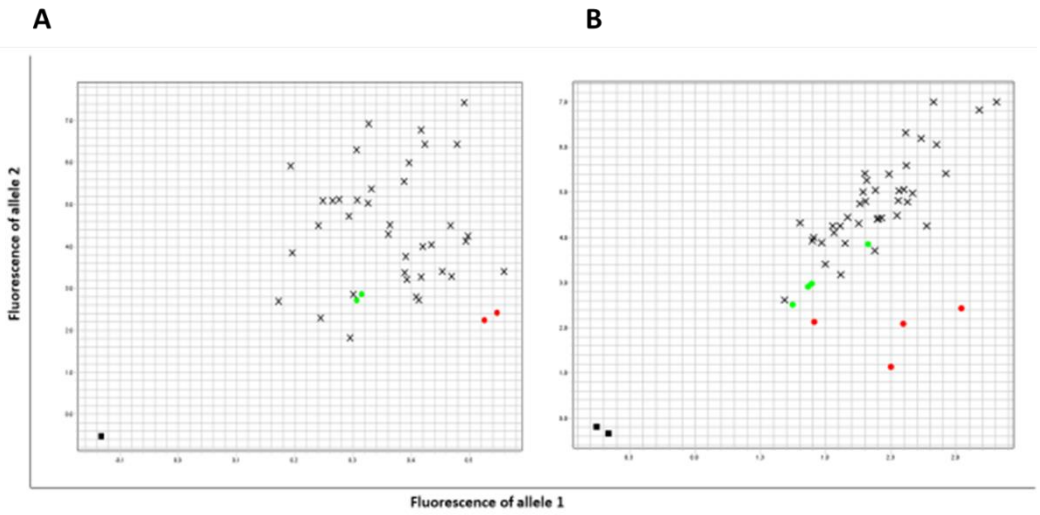


Figure 4-15 Allele discrimination plots of two KASP assays obtained from RenSeq
Allelic discrimination plots of the two RenSeq derived KASP assays with DM as reference. A is ST4_03ch11_35563531 and B is ST4_03ch11_42728387. Although the parents (green for the resistant heterozygous parent and red (for the susceptible, homozygous parent) can be discriminated, the clones from the two bulks (X) behave as if all progeny clones had the same alleles as the resistant parent.

4.4.4.3 Identification of informative SNPs using GenSeq

The gene enrichment method GenSeq is very similar to RenSeq and differs only in targeting single or low-copy number genes located throughout all potato chromosomes (Chen et al., 2018) instead of LB-NRR domains of *R*-genes. GenSeq was used as a tool to identify informative SNPs that can be used for mapping the resistance to *G. pallida*. The same indexed DNA libraries that were used for RenSeq were used for GenSeq enrichment sequencing. The GenSeq reads were also mapped to DM as the reference genome. Table 4-9 shows the total reads and mapped reads to the reference at different mismatch rates of the GenSeq experiment. The on-target rate ranged from 52 to 89%, depending on the mismatch rate.

Table 4-9 Read numbers and on-target reads to the reference DM in the GenSeq experiment

Library	Paired end reads	On target mapping reads (%) to reference DM at mismatch rate 2%	3%	5%	10%
PS	1084865	758946 (70.0)	831000 (76.6)	903072 (83.2)	9693835 (88,8)
PR	1246752	657153 (52.8)	776376 (62.7)	922222 (74.0)	1065983 (85.5)
BS	1508457	909928 (60.3)	1032420 (68.4	1172930 (77.8)	1306989 (86.4)
BR	2263405	1367635 (60.4)	1552759 (68.6)	1763464 (86.7)	1961416 (86.7)

In Table 4-10, the number of SNPs determined in bulks, parents and both are listed for different mismatch rates.

Table 4-10 Different mismatch rates provided different number of SNPs in GenSeq

	Number of SNPs (in transcripts)			
Mismatch rate	**Bulks**	**Parents**	**Bulks & parents**	**Location of informative SNPs**
2%	41	1794	15	12 on chr. VI and 2 on chr. XII
3%	48	3112	14	11 on chr. VI and 1 each on chr. IX, X, and XII
5%	77	4637	24	20 on chr. VI, 3 on chr. IX, and 1 on XII
10%	79	5717	22	20 on chr. VI and 1 each on chr. IX, and XII

As in RenSeq, the results of the GenSeq analysis at a 5% mismatch rate are illustrated in Figure 4-16 At this mismatch rate, 4637 parental SNPs (Panel A) are present, 77 SNPs from the bulks passed the filtering for being heterozygous (40-60%, of the alternative allele) for the resistant bulk and being homozygous (0-10% or 90-100%) for the susceptible bulk (Panel B), and 24 SNPs (Panel C) remained, when they were validated for having the expected frequency in both parents and bulks and being intragenic. In total, 20 informative SNPs were identified on chromosome VI, three on chromosome IX and one on chromosome XII. Their gene IDs and the number of SNPs in each gene are shown in Table 4-11.

Table 4-11 Informative SNP candidates detected from the GBS experiment

Three SNPs were used to create KASP assays: their primer sequences are shown in Table 4-8 section C. Sequencing primers were designed for the other SNPs.

Mismatch rate	Chr.	Start	Stop	Gene ID	No. of SNPs	SNP Position (Alleles)
2%	VI	53821292	53822172	PGSC0003DMG400027013	1	53821580 (A/T)
	VI	54660358	54663473	PGSC0003DMG400005895	1	54661772 (G/A)
	VI	55904773	55910691	PGSC0003DMG400007504	6	55909792 (G/C), 55910203 (T/C), 55910206 (A/C), 55910257 (C/G), 55910404 (C/G), 55910409 (G/C)
	VI	57309861	57313784	PGSC0003DMG400030365	1	57313272 (G/A)
	VI	57314827	57323565	PGSC0003DMG400030366	2	57322514 (G/A), 57322806 (A/T)
	VI	58776636	58782360	PGSC0003DMG400020145	1	58781798 (G/A)
	IX	54146053	54150042	PGSC0003DMG400032241	1	54146755 (G/A)
	XII	21536758	21537672	PGSC0003DMG400014943	1	21536868 (T/A)
	XII	49744644	49746244	PGSC0003DMG400028627	1	49746075 (C/T)
3%	VI	53821292	53822172	PGSC0003DMG400027013	1	53821580 (A/T)
	VI	54660358	54663473	PGSC0003DMG400005895	1	54661772 (G/A)
	VI	55904773	55910691	PGSC0003DMG400007504	6	55909792 (G/C), 55910203 (T/C), 55910206 (A/C), 55910257 (C/G), 55910404 (C/G), 55910409 (G/C)
	VI	57309861	57313784	PGSC0003DMG400030365	1	57313272 (G/A)
	VI	57314827	57323565	PGSC0003DMG400030366	2	57322514 (G/A), 57322806 (A/T)
	IX	54146053	54150042	PGSC0003DMG400032241	1	54146755 (G/A)
	X	28756497	28758184	PGSC0003DMG400017694	1	28757235 (T/C)
	XII	49744644	49746244	PGSC0003DMG400028627	1	49746075 (C/T)
5%	VI	46214746	46219803	PGSC0003DMG400026085	1	46215098 (A/G)
	VI	53821292	53822172	PGSC0003DMG400027013	1	53821580 (A/T)
	VI	54217175	54219255	PGSC0003DMG400005919	1	54218046 (T/C)
	VI	54660358	54663473	PGSC0003DMG400005895	1	54661772 (G/A)
	VI	55096366	55102143	PGSC0003DMG400007149	3	55096799 (C/T), 55096832 (A/G), 55096874 (C/T)

Table 4-11 continued

Mismatch rate	Chr.	Start	Stop	Gene ID	No. of SNPs	SNP Position (Alleles)
5%	VI	55904773	55910691	PGSC0003DMG400007504	6	55909792 (G/C), 55910203 (T/C), 55910206 (A/C), 55910257 (C/G), 55910404 (C/G), 55910409 (G/C)
	VI	57309861	57313784	PGSC0003DMG400030365	1	57313272 (G/A)
	VI	57314827	57323565	PGSC0003DMG400030366	6	57322514 (G/A), 57322806 (A/T), 57323327 (C/G), 57323385 (T/C), 57323393 (A/T), 57323514 (A/G)
	IX	53672502	53677067	PGSC0003DMG400030078	2	53673705 (A/C), 53673779 (G/C)
	IX	54146053	54150042	PGSC0003DMG400032241	1	54146755 (G/A)
	XII	49744644	49746244	PGSC0003DMG400028627	1	49746075 (C/T)
10%	VI	52509378	52510568	PGSC0003DMG400026990	1	52510349 (C/T)
	VI	53821292	53822172	PGSC0003DMG400027013	1	53821580 (A/T)
	VI	54217175	54219255	PGSC0003DMG400005919	1	54218046 (T/C)
	VI	54660358	54663473	PGSC0003DMG400005895	1	54661772 (G/A)
	VI	55096366	55102143	PGSC0003DMG400007149	4	55096794 (G/C), 55096799 (C/T), 55096832 (A/G), 55096874 (C/T)
	VI	55904773	55910691	PGSC0003DMG400007504	6	55909792 (G/C), 55910203 (T/C), 55910206 (A/C), 55910257 (C/G), 55910404 (C/G), 55910409 (G/C)
	VI	57309861	57313784	PGSC0003DMG400030365	1	57313272 (G/A)
	VI	57314827	57323565	PGSC0003DMG400030366	5	57322514 (G/A), 57322806 (A/T), 57323385 (T/C), 57323393 (A/T), 57323514 (A/G)
	IX	54146053	54150042	PGSC0003DMG400032241	1	54146755 (G/A)
	XII	49744644	49746244	PGSC0003DMG400028627	1	49746075 (C/T)

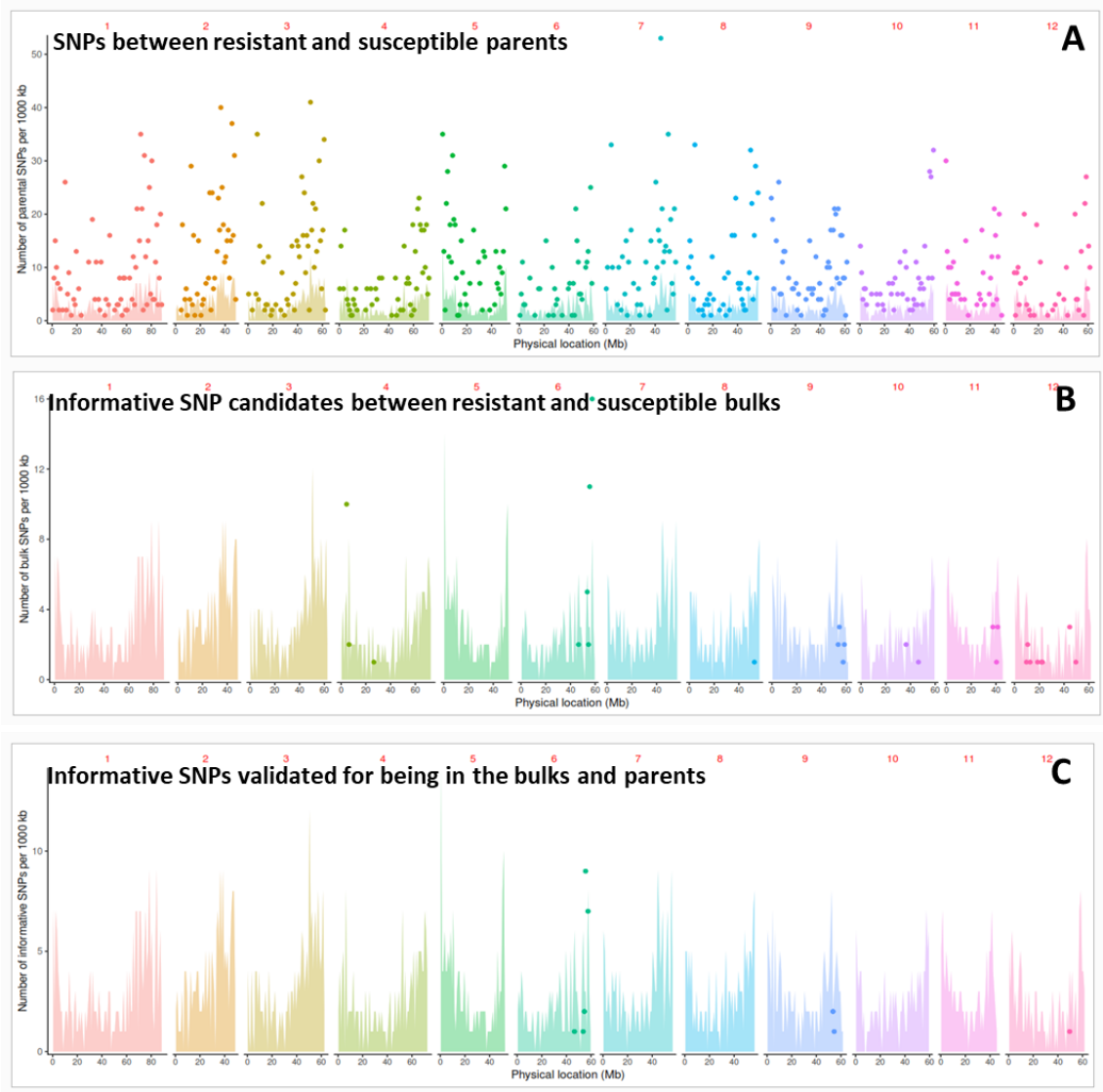


Figure 4-16 Distribution of SNPs identified by GenSeq at 5% mismatch rate
Each set of coloured data represents a specific chromosome. Coloured "spikes" represent the number of single and low copy number genes targeted by probes across the chromosome. Each dot represents SNPs and its placement on the y-axis determines the number of SNPs identified in a 1 Mb bin. Panel A shows the SNPs identified between the two parental lines. Panel B shows the SNPs found in the two bulks with the expected ratio of the alternative allele being 0.5±0.1 for the resistant bulk and 0 or 1±0.1 for the susceptible bulk. Panel C shows the informative SNPS identified and validated to be intragenic in the bulks and parents.

4.4.4.4 Identifying informative SNPs in the region of interest using GBS

GenSeq analysis did not provide any informative SNPs in the region on chromosome VI from 55,909,792 to 57,322,514. Therefore, GBS, which was performed with the individually labelled DNA of the resistant and susceptible clones used as bulks in GenSeq of the cross 13.A.02 and their parents, was used to identify other informative SNPs for the resistance to *G. pallida* in this region. The sequences were visualized in the software Tablet 1.19.09.03 (Milne et al. 2013) and screened for having two alleles in the ratio 0.4 to 0.6 in 03.F1.3a(35), then checked to determine whether this position DB337(37) only had one allele. If this was the case, then this SNP was examined in all clones. Table 4-12 shows the SNPs found to be linked to resistance/susceptibility. For the SNPs on chromosome VI positions 56,135,692, 56,915,767 and 56,987,876, KASP markers were designed and tested on recombinants (Figure 4-19). The SNPs on chromosome VI positions 56,718,837, 56,787,735, 56.796,582 and 56,914,572 were successfully Sanger sequenced, the PCR amplification of the two SNPs on chromosome VI positions 56,341,694 and 56,550,855 provided many amplification products of different lengths and were not sequenced.

Table 4-12 Informative SNP candidates detected from the GBS experiment

Three SNPs were used to create KASP assays: their primer sequences are shown in Table 4-8 section C. Sequencing primers were designed for the other SNPs.

Informative SNP position on chr. VI	Alleles in parents Resistant	susceptible	Primer name and sequence(5'->3') (or KASP)		Notes
56,135,692	C/T	T	KASP	Table 4-8 C	Graphical genotyping Figures 4-19 and 4-20
56,341,694	A/G	A	K22F	GGCACACTACCAAGAATCTG	PCR resulted in many bands, not analysed
			K22R	TTTGGGCACTTCTCCTCTG	
56,550,855	G/T	T	K23F	AGGATTGAGGCACAGTAGTAG	PCR resulted in many bands, not analysed
			K23R	GAAGAAAGACGCTGCTACC	
56,718,837	C/T	T	K24F	GTTTTCAGAGCGAGCACAC	Sanger sequenced Figure 4-20
			K24R	AGCTTCTCAACAGCTTGAAC	
56,787,735	A/T	A	K25F	GATCCTGAATATCTTGGTGCTC	Sanger sequenced Figure 4-20
			K25R	GGACTATCAGGTAATTTCTCCG	
56,796,582	C/T	C	K26F	GTTGCTCTTGTTTGCCTGTC	Sanger sequenced Figure 4-20
			K26R	AGCATTAGTCGCCTTCCTC	
56,914,572	A/G	G	K27F	GTATCACAAGGGGTCAAACATC	Sanger sequenced Figure 4-20
			K27R	GAAGACCATTCTTCACAGCTAC	
56,915,767	T/G	G	KASP	Table 4-8 C	Graphical genotyping Figures 4-19 and 4-20
56,987,876	T/G	G	KASP	Table 4-8 C	Graphical genotyping Figures 4-19 and 4-20

4.4.4.5 Converting informative SNPs into Kasp assays obtained by GenSeq and GBS

Initially, KASP primers were generated from the seven informative SNPs on chromosome VI obtained in the GenSeq experiment with DM as reference; these KASP primers are listed in Table 4-8 panel B. They were validated in the same way as the KASP markers from RenSeq described in Section 4.4.4.2 to determine if the parents can be discriminated in this KASP assay. Six of the seven assays could discriminate between the parents. Next, the six different KASP marker assays that clustered (ST4_03ch06_53821580, ST4_03ch06_54218046, ST4_03ch06_55096874, ST4_03ch06_55909792, ST4_03ch06_57322514 and ST4_03ch06_58781798) were initially performed on each individual plant of the resistant and susceptible bulks used for the enrichment sequencing. Figure 4-17 shows the results, presented as graphical genotyping. The clones labelled "–"in red have the same alleles as the susceptible parent, whereas the clones labelled "+"in green have the same alleles as the resistant parent.

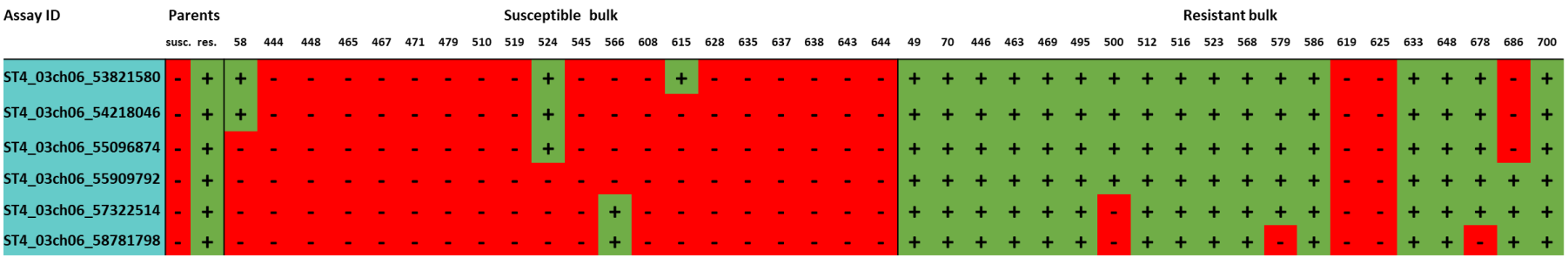


Figure 4-17 Graphical genotyping of the individuals of the bulks (KASP assays from GenSeq)

The KASP assays are sorted by chromosome position; ST4_03ch0653821580 is upstream of ST4_03ch0658781798. The clones labelled – in red have the allele of the susceptible parent in the SNP tested; the clones labelled + in green share the alleles from the resistant parent.

These results suggest that there is a link between being heterozygous and being resistant and between being homozygous and being susceptible, apart from the two individuals 619 and 625, which do not fit the general pattern. If there is a correlation, these two individuals would be expected to be susceptible. Consequently, clones 619 and 625 were retested for the level of resistance in a pot test, which is more sensitive than a root trainer assay, and found not to be resistant, but to have reduced susceptibility with an average cyst count of 60 (±9) and 50 (±34), respectively, with a value of 216 (±122) for the susceptible parent and 13 (±9) for the resistant parent. Thus, the GenSeq-derived KASP markers derived from chromosome VI are linked to the phenotypes of the parents and the bulks, suggesting that the resistance locus resides on chromosome VI.

Mapping a resistance relies on individuals with recombination events between marker and resistance gene, which leads to susceptible progeny plants with the “resistance pattern” and to resistant progeny plants with the “susceptibility pattern”. Such SNPs, typically somewhat removed from the actual gene locus are highly informative as these SNPs narrow down the location of the resistance. An example is susceptible clone 524 that shows the “resistance pattern” upstream from position 55,096,874, therefore the resistance locus must be downstream from this position. The more distant the marker from the resistance gene, the more likely it is that recombination events will occur. Of the 40 individual clones that were used for the enrichment sequencing, only eight were identified as having recombination events that are informative to define the locus for mapping, as shown in Figure 4-18. Clone 566 shows the “resistance pattern” downstream from position 57,322,514, therefore the resistance locus must be upstream. Resistant clone 500 shows the “susceptibility pattern” downstream from position 57,322,514, therefore the resistance locus must be upstream, and finally, clone 686 shows the “susceptibility pattern” upstream from position 55,096,874, therefore the resistance locus must be downstream.

	Parents		Susceptible bulk				Resistant bulk			
Assay ID	susc.	res.	58	524	566	615	500	579	678	686
ST4_03ch06_53821580	-	+	+	+	-	+	+	+	+	-
ST4_03ch06_54218046	-	+	+	+	-	-	+	+	+	-
ST4_03ch06_55096874	-	+	-	+	-	-	+	+	+	-
ST4_03ch06_55909792	-	+	-	-	-	-	+	+	+	+
ST4_03ch06_57322514	-	+	-	-	+	-	-	+	+	+
ST4_03ch06_58781798	-	+	-	-	+	-	-	-	-	+

Figure 4-18 Graphical genotyping showing recombinant clones from the bulks
Only the recombinants from Figure 4-17 are shown, as they are informative about the position of the resistance locus. The KASP assays are sorted by chromosome position: ST4_03ch0653821580 is upstream of ST4_03ch0658781798. The clones labelled – in red have the allele of the susceptible parent in the SNP tested, the clones labelled + in green share the alleles from the resistant parent.

In summary, the six KASP assays designed from the GenSeq candidate SNPs applied to the DNA of the 40 plants as resistant and susceptible identified eight recombinant plants, which narrowed the resistance locus to a region of 2.23 Mb on chromosome VI, with four plants remaining recombinant in that region.

To further define the position of the resistance locus, additional resistant and susceptible backcross clones with a recombination event and additional KASP markers between positions 55,096,874 and 57,322,514 on chromosome VI were needed. With the two KASP assays ST4_03ch06_55096874 and ST4_03ch06_57322514 that flank the resistance locus, all 993 progeny clones of 13.A.02 were screened for recombinants with DNA obtained from the original clones. Of the 884 clones that yielded robust KASP data in both assays 112 were recombinant. These recombinant clones were phenotyped with a root trainer assay, and the same plants used for the root trainer assay were tested again with the KASP assays, to both eliminate tubers that were accidentally mislabelled during the years of maintenance and to determine which plants scored with either fewer than 6 (resistant) or more than 25 female (susceptible) nematodes in the root trainer assay. 23 resistant clones and 29 susceptible clones remained for further fine mapping. These recombinants were tested with more KASP markers derived from GenSeq and GBS, ranging from position 53,821,580 to 58,781,798 on chromosome VI. Figure 4-19 shows the graphical genotyping results from the recombinant clones tested. Relevant for mapping are the clones with a

recombination event between the 2.23 Mb interval on chromosome VI from position 55,096,874 to 58,781,798. The results of the other assays are shown to test for consistency. The susceptible clones 188, 317, and 326 in Figure 4-19A show the "resistance pattern" upstream from position 56,135,692, the resistance locus must therefore be downstream. Similarly, susceptible clone 322 shows the "resistance pattern" downstream from position 56,915,767, therefore the resistance locus must be upstream. Figure 4-19B shows resistant clones 419 with the "susceptibility pattern" downstream from position 56,915,767, therefore the resistance locus must be upstream, and finally, clones 679 and 723 show the "susceptibility pattern" upstream from position 56,135,692, therefore the resistance locus must be downstream. Susceptible clone 566 from the bulk was lost, so it could not be tested with additional KASP assays.

In summary, by using KASP assays designed from SNPs obtained by GenSeq and GBS analysis to analyse the additional 52 recombinant BX1 progeny plants resulted in the delineation of the resistance locus to a region of 780 kb on chromosome VI between position 56,135,692 and 56,915,767, with three resistant and four susceptible recombinants remaining.

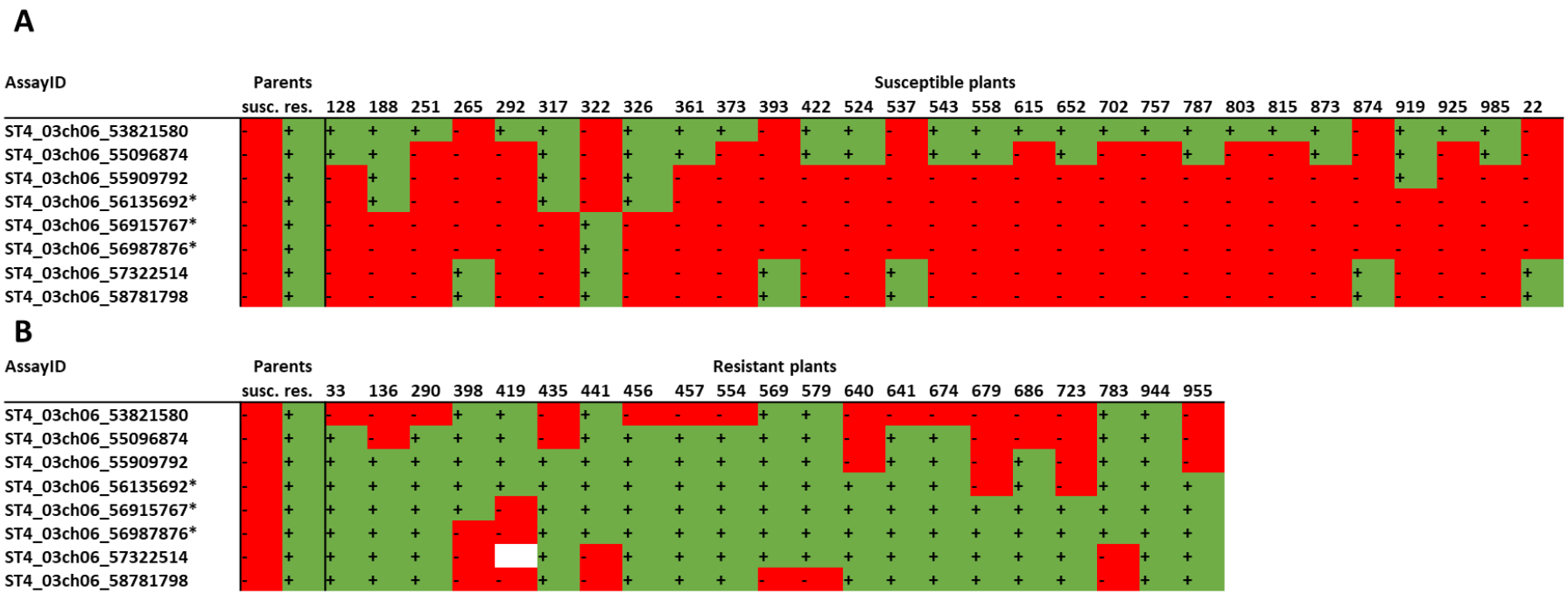


Figure 4-19 Graphical genotyping with a recombination event near the resistance locus

The clones shown represent the progeny of the cross 13.A.02 had a recombination event near the resistance and showed a clear phenotype. The clones labelled – in red have the allele of the susceptible parent in the SNP tested; the clones labelled + in green share the alleles from the resistant parent. The white cells indicates that the assay did not work. The KASP assay labelled with a * were obtained by SNPs obtained by GBS. Panel A shows the allele patterns from the parents and the susceptible bulk, Panel B from the parents and the resistant bulk.

In Figure 4-20, the SNPs detected by GBS that were either sequenced or converted into a KASP assay are shown in the graphical genotyping format. Panel A shows the results of all assays performed. The KASP assays provided interpretable results for all tested samples. Similarly, the Sanger sequencing of the SNPs at positions 56,718,837 and 56,914,572 could be interpreted for all samples, whereas for the SNPs at positions 56,787,735 and 56,796,582 Sanger sequencing yielded an inconclusive result for the DNA of clones 326, 679 and 723. Furthermore, the detected SNP pattern from clone 326 indicated three recombination events in a region of less than 800 kb, which is extremely unlikely. The three resistant recombinants all had the same SNP pattern as the resistant parent between positions 56,135,692 and 56,914,572, whereas the results are contradictory for the susceptible recombinants. The transition from "resistance pattern" to "susceptibility pattern" in clone 188 indicates that the resistance locus is downstream of position 56,796,582, but the graphical genotyping of clone 322 suggests the resistant locus is upstream of position 56,718,837. This could, for example, be a limitation of using the DM genome for the fine mapping. Figure 4-20B shows the interval in which the mapping shows no inconsistencies, which could narrow down the resistance locus to a region of ~779 kb on chromosome VI, with seven recombinant plants remaining.

A

		Parents		Susceptible plants				Resistant plants		
Assay type	Assay ID	susc.	res.	188	317	322	326	419	679	723
KASP	ST4_03ch06_53821580	-	+	+	+	-	+	+	-	-
KASP	ST4_03ch06_55096874	-	+	+	+	-	+	+	-	-
KASP	ST4_03ch06_55909792	-	+	+	+	-	+	+	-	-
KASP	ST4_03ch06_56135692	-	+	+	+	-	+	+	-	-
Sequencing	ch06_56718837	-	+	+	-	+	-	+		
Sequencing	ch06_56787735	-	+	+	-	+		+		
Sequencing	ch06_56796582	-	+	+	-	+	+	+	+	+
Sequencing	ch06_56914572	-	+	-	-	+	-	-	+	+
KASP	ST4_03ch06_56915767	-	+	-	-	+	-	-	+	+
KASP	ST4_03ch06_56987876	-	+	-	-	+	-	-	+	+

B

		Parents		Susceptible plants				Resistant plants		
Assay type	Assay ID	susc.	res.	188	317	322	326	419	679	723
KASP	ST4_03ch06_56135692	-	+	+	+	-	+	+	-	-
Sequencing	ch06_56914572	-	+	-	-	+	-	-	+	+

Figure 4-20 Further narrowing down the resistance by graphical genotyping
SNP candidates obtained from GBS and KASP assays were used. Panel A shows the results of all SNP analyses, obtained by GBS, that were performed, resulting in inconsistencies. Panel B shows the DNA region (mapped to DM) in mapping for the resistance does not show any inconsistencies. The clones labelled – in red have the allele of the susceptible parent in the SNP tested, the clones labelled + in green share the alleles from the resistant parent.

4.4.4.6 Re-evaluation and re-interpretation of the seven informative recombinants

Phenotyping of the remaining seven clones and their parents was repeated in a root trainer assay[12], in order check if the assignments for being susceptible and resistant were reproducible. Figure 4-21 shows the numbers of female nematodes in the four replicates, the average, the standard deviation and the % of female nematodes of the susceptible parent DMB337(37) in the root trainer assays from 2021 and 2019. The resistant parent 03.F.3a(35) provided unexpectedly high numbers of female nematodes in two of the four replicates in 2021. The number of nematodes on the susceptible parent was much higher in 2021 than in 2019. Clones 419, 679 and 723 are consistently resistant in both years tested. Looking at the four clones 188, 317, 322 and 326 shows a different picture. In 2021, 317, 322 and 326 have more resemblance to the resistant parent than to the susceptible parent, although the values are too high and atypical for 03.F.3a(35). They are best described as having reduced susceptibility, and therefore not to be used in the analysis. Clone 188 is categorized as susceptible in 2021, the same as in 2019.

[12] In 2019 this assay was performed by me, in 2021 by Vivian Blok, as I could not enter the UK due to Covid restrictions.

Table 4-13 Number of female nematodes in root trainer assays in 2021 and 2019

	2021							2019						
Potato clone	Rep1	Rep2	Rep3	Rep4	Average	Stdev	% DB337(37)	Rep1	Rep2	Rep3	Rep4	Average	Stdev	% DB337(37)
DB337(37)	164	124	115	176	145	30	100	62	20	55	57	49	19	100
13.A.02 (188)	128	100	83	23	84	44	58	40	15	64	51	43	21	88
13.A.02 (317)	36	19	43	64	41	19	28	35	32	30	37	34	3	69
13.A.02 (322)	45	22	45	13	31	16	22	40	35	19	87	45	29	93
13.A.02 (326)	20	33	27	20	25	6	17	33	31	3	55	31	21	63
13.A.02 (419)	7	34	23	0	16	15	11	6	4	8	5	6	2	12
13.A.02 (679)	0	6	10	7	6	4	4	2	1	0	2	1	1	3
13.A.02 (723)	2	5	12	17	9	7	6	3	2	0	1	2	1	3
03F3a(35)	1	37	63	12	28	28	20	1	0	7	2	3	3	5

When the three clones 317, 322 and 326, previously assigned as susceptible but which are most likely resistant or have reduced susceptibility, are removed from the graphical genotyping, the phenotypic and genotypic data are in full agreement. Furthermore, clones 679 and 723 can no longer be considered as recombinant plants, therefore they are not included in the graphical genotyping. One resistant (479) and one susceptible (188) recombinant clone are remaining, narrowing down the resistance locus on chromosome VI between positions 56,796,582 and 56,914,572, that is 118 kb (Figure 4-21). When looking at this region on the reference genome DM (v6.01) on SpudDB[13], 14 high confidence gene models were detected. Table 4-14 lists these genes.

		Parents		Progeny plants	
				susc.	res.
Assay Type	Assay ID	susc.	res.	188	419
KASP	ST4_03ch06_53821580	-	+	+	+
KASP	ST4_03ch06_55096874	-	+	+	+
KASP	ST4_03ch06_55909792	-	+	+	+
KASP	ST4_03ch06_56135692	-	+	+	+
Sequencing	ch06_56718837	-	+	+	+
Sequencing	ch06_56787735	-	+	+	+
Sequencing	ch06_56796582	-	+	+	+
Sequencing	ch06_56914572	-	+	-	-
KASP	ST4_03ch06_56915767	-	+	-	-
KASP	ST4_03ch06_56987876	-	+	-	-

Figure 4-21 Graphical genotyping after re-evaluation of recombinant progenies

Only two recombinant plants remain with the sequencing and KASP assays after re-evaluation of the phenotype. The clones labelled – in red have the allele of the susceptible parent in the SNP tested, the clones labelled + in green share the alleles from the resistant parent.

13 http://spuddb.uga.edu/jbrowse/index.html?data=data%2FDM_potato_v61&loc=chr06%3A56796628..56914618&tracks=DNA%2Cloci%2Chc_gene_models&highlight=

Table 4-14 List of genes in DM in the interval with the resistance to *G. pallid*a in *S. spegazzinii*
These are the high confidence genes detected in DM (v6.1).

Gene name	Description	Position on chr.VI
Soltu.DM.06G032060.1	Laccase/diphenol oxidase family protein	56797112..56800721
Soltu.DM.06G032070.1	Homeodomain-like transcriptional regulator	56816128..56818397
Soltu.DM.06G032080.1	Glycosyl hydrolase family protein	56820344..56824583
Soltu.DM.06G032090.1	Josephin protein-related	56828401..56829147
Soltu.DM.06G032100.1	Thylakoid soluble phosphoprotein TSP9 domain containing protein	56833232..56834013
Soltu.DM.06G032110.1	Cytochrome P450, family 86, subfamily A, polypeptide	56836408..56838552
Soltu.DM.06G032120.1	Zinc finger (C2H2 type) family protein	56849541..56851152
Soltu.DM.06G032130.1	Golgi nucleotide sugar transporter	56854375..56859689
Soltu.DM.06G032140.1	Myb-domain protein	56862756..56865014
Soltu.DM.06G032150.1&.2	Tetratricopeptide repeat (TPR)-like superfamily protein	56873096..56876786
Soltu.DM.06G032160.1	Cell division control, Cdc6	56895973..56902969
Soltu.DM.06G032170.1	Ribosomal protein L10 family protein	56903608..56905903
Soltu.DM.06G032180.1&.2	Quinone reductase family protein	56908586..56912059
Soltu.DM.06G032190.1	Pleckstrin homology domain-containing protein 1	56913478..56914487

4.5 Discussion

Resistances to both *G. pallida* and *G. rostochiensis* have been described in the wild potato species *S. spegazzinii* Bitter (*e.g.*, Kreike et al. (1994), Caromel et al. (2005)). The findings in this study confirm the resistance to both species in the accession CPC 7195. For *G. pallida*, the resistance covers the full range of the different populations introduced to Europe, with the number of female nematodes reduced by more than 95% for three populations representing the different introductions to the UK. This makes this resistance a desirable target for introgression into commercial potato cultivars. For *G. rostochiensis* Ro1, the reduction in female numbers is slightly less, although it is more than 90%. As the two resistances segregate independently, there is no single locus that confers both resistances. A KASP assay specific to the *H1* resistance (Vanessa Young, personal communication) and dRenSeq analysis using recently established candidates for *H1* (Miles

Armstrong, personal communication) showed that the *H1* resistance in not present in *S. spegazzinii* CPC 7195, but it remains to be determined if this accession contains the gene *Gro1* (Ramakrishnan et al., 2015).

To map the new resistances, the genome of the potato genotype DM was used as a reference. By using a doubled monoploid, the heterozygosity of tetraploid potato cultivars was removed. There are more than 39,000 genes in more than 840 MB distributed over 12 chromosomes. With the development of next generation sequencing, it became possible to sequence a large amount of DNA sequence, but the genome contains many regions that are of no interest when "fishing" for SNPs in order to map genes of interest. Therefore, reducing the genome complexity by enriching for DNA of interest, *e.g*., whole exome capture, GBS, RenSeq or GenSeq, is highly desirable, as it also increases the read depth of the sequences, resulting in higher confidence in the deduced sequence polymorphisms that are used for genetic studies. Most of the known resistances to cyst nematodes are NB-LRR genes, so a RenSeq analysis combined with a GenSeq approach to localizing the resistance to *G. pallida* derived from *S. spegazzinii* CPC 7195 was applied.

This project focused on the *G. pallida* resistance. Both the resistant and susceptible parent are diploid, with no resistance to PCN present in the susceptible parent DB337(37), Mayan Gold. Of the 158 tested progeny, 52 were susceptible and 62 resistant – the remaining 26 clones showed an intermediate level of resistance and were either categorized as reduced or excluded from the analysis. Indeed, this intermediate level of susceptibly could be a consequence of a weak resistance allele or a favourable combination of susceptibility alleles that somewhat restrict PCN establishment (for a review about susceptibility alleles see Koseoglou et al. (2022)) . The resistant parent 03.F.3a(35) is known to be heterozygous as shown in Figure 4-3; therefore, this resistance, in common with other resistances, must be inherited as a dominant trait. Comparing the theoretical expectations of applying Mendelian genetics, with either one or two genes conferring resistance, with the observed phenotypes of the BX1 clones confirmed that either a single dominant gene or one major and one minor gene are responsible for the resistance observed. The RenSeq analysis, which was undertaken first, strongly suggested that the resistant locus is on

chromosome XI, whereas the GenSeq analysis indicates chromosome VI. The RenSeq analysis, when DM was used as reference for both parents, did not lead to usable KASP assays. The *R*-gene derived KASP assays either could not distinguish between the parents, or, where they could distinguish, they did not result in an interpretable pattern in the progeny. One possible conclusion is that that these assays were not robust enough. The difficulty with *R*-gene based KASP assays is that there are many very similar sequences, and although they have been used successfully to map the *H2* resistance to chromosome V (Strachan et al., 2019) (using gene enrichment of *R*-genes for SNP identification and gene mapping), this approach was not successful here. However, the SNP candidates from GenSeq on chromosome VI were linked to *G. pallida* resistance and, therefore, the focus shifted to using GenSeq analysis to use for mapping the gene(s conferring *G. pallida* resistance.

How can this discrepancy between RenSeq and GenSeq be explained? *R*-genes are evolving rapidly, they relocate and/or duplicate constantly and different members of this gene family share similar sequences. It is possible that the *R*-gene conferring resistance to *G. pallida* is in the same region as determined by GenSeq, however the *R*-genes were translocated after both *Solanum* species separated, although this cannot be proven in this project. A mis-assembly of this *R*-gene region to the DM reference genome could also be the reason, as there are no known *R*-genes reported in that specific region. It is also possible that the *R*-gene responsible for resistance could be absent in DM but present in *S. spegazzinii* and another possibility is that the informative GenSeq results reflect a resistance that differs from a NB-LRR conferred resistance, and this is the reason that the RenSeq experiment did not yield *R*-gene derived SNPs that are linked to the *G. pallida* resistance.

The KASP assays derived from GenSeq data show a linkage of SNPs and plants with a resistant/susceptible phenotype. The fact that KASP assays derived from SNPs obtained by GBS on chromosome VI also show the linkage, supports the finding that the resistance is indeed located on chromosome VI. Furthermore, the two plants in the resistant bulk chosen for enrichment sequencing that had been identified by KASP markers as

susceptible had their phenotype re-confirmed, which also supports this result. As a result, the resistance locus could be mapped with high certainty to a region on chromosome VI that in DM spans 779kb between positions 56,135,692 and 56,915,767 using SNP candidates. Indeed, two independent approaches, GenSeq and GBS, contributed informative SNPs to this genetic mapping analysis. Attempts to further narrow down the resistance location initially provided inconsistent results with graphical genotyping of the remaining susceptible recombinant plants 13.A.02(188), (13.A.02(317), (13.A.02(322), and 13.A.02(326). Graphical genotyping indicated that the resistance conferring locus is between position 56796582 and 56914572 on chromosome VI, in plants 13.A.02(188) and 13.A.02(419), which is in agreement with clones 13.A.02(317), 13.A.02(679) and13.A.02(723). However, clone 13.A.02(322) would indicate that the resistance is located between 56135692 and 56718837 on chromosome VI, which is in agreement with clones 13.A.02(317), and 13.A.02(419). One recombinant plant, 13.A.02(326), had not one but three recombination events, which is extremely unlikely, and therefore it was not considered. The KASP assays should be repeated with DNA from fresh leaf material to determine if this triple recombination event is real. After re-phenotyping the seven remaining recombinant plants, three (13.A.02(317), 13.A.02(322) and 13.A.02(326)) were removed from the analysis, as their phenotype was not clear enough. As a consequence, no further inconsistency was observable and the resistance locus for *G. pallida* could be localised to 118 kb on chromosome VI based on the DM genome assembly. The actual distance between the flanking markers may be different in *S. spegazzinii*, for example when it contains *R*-genes that are not present in DM.

The phenotyping of these three plants should be repeated once more to determine which of the phenotypes are reproducible. In case the initial assigning of the phenotype was correct, and there is an inconsistency in the graphical genotyping, one explanation could be that a gene inversion event occurred in *S. spegazzinii* (or DM) after the two species diverged. It is not unusual that the order of genes changes in different species. Gene inversion was detected the first time in studies of different Drosophila species (Sturtevant,

1921) and has since emerged as a general phenomenon that is believed to, “reduce recombination between favourable combinations of alleles” (Huang and Rieseberg, 2020).

As an example, Potato and tomato genomes differentiate by five major paracentric inversions (Bonierbale et al., 1988; Huang and Rieseberg, 2020), and potato and pepper differ in at least 19 inversions and 6 chromosome translocations (Wu et al., 2009; Huang and Rieseberg, 2020). In the scope of this project, it is impossible to further narrow down the resistance locus. The generating and sequencing of a bacterial artificial chromosome (BAC) library or genome walking could be used to obtain more sequence information in this region. Alternative approaches could also use CRISPR-Cas9 targeted excision of genomic DNA followed by Nanopore-based sequencing (López-Girona et al., 2020).

Although it was not possible to clone the gene which confers the resistance to *G. pallida* in *S. spegazzinii*, it was possible to narrow down the genomic region to 118 kb in the reference sequence of DM v6.01. This region contains 14 high confidence gene models, but no *R*-genes in DM. When more sequence information of this region is available, more informative SNPs can be detected and possibly converted to a genetic marker for the resistance, thereby further narrowing down the region of interest. It could also be determined whether *S. spegazzinii* shows *R*-genes in this region that are not present in DM. *R*-gene independent resistance against the CN *H. glycines* has been described in soybean (Section 1.4.1.6).

For the potato industry, it is important to introgress this resistance into breeding material. It is not essential to clone the gene responsible for resistance– a tightly linked molecular marker would be extremely useful to introduce the resistance by plant breeding, which is the topic of the next chapter.

5 Introgression of the *G. pallida* resistance derived from *S. spegazzinii* accession 7195

5.1 Introduction

Cultivated potato varieties are usually clonally propagated with about 10% of tubers produced used as seed potatoes for the next planting season (Birch et al., 2012), thereby ensuring that required and desired traits are conserved in the potato variety. Unlike other staple crops such as barley, rice or wheat, potatoes are not grown from botanical "true" seeds harbouring novel genotypes. However, for the development of new cultivars with novel beneficial traits, such as resistances to diseases and pests, enhanced quality and nutritional value, higher yield or improved efficiency in the use of water and minerals to secure sustainability of potato production with climate change (Birch et al., 2012), sexual hybridisation needs to be performed to obtain "true" seeds. Traditional potato breeding began in the early 19th century with the hybridisation of different potato cultivars by artificial pollination (Bradshaw and Ramsay, 2005). To date, breeding potatoes is still predominantly done at the tetraploid level. However, the use of diploid potato wild species for the introgression of beneficial traits into cultivated tetraploid species is becoming more important, requiring interploidy (4x-2x) crosses, followed by backcrossing to the cultivated species and the selection of progeny plants that combine the new desired trait with the background of the cultivated species.

Breeding that only relies on use of phenotypic data for selection is very time consuming and complex (Barone, 2004). Some 35 years ago, a different approach towards breeding using marker-assisted selection (MAS) was developed (Section 5.1.2). Beckmann and Soller (1986) were amongst the first to describe the use of RFLP markers for the introgression of new traits into agricultural plants.

5.1.1 Breeding of PCN-resistant potatoes

Potato breeding in Europe started to become important after the potato famine that occurred between 1845 and 1852. Following this, new germplasm was brought from

South America in an attempt to combat late blight through introgression of resistance into European potato genotypes. Both species of PCN, *G. rostochiensis* and *G. pallida*, were probably inadvertently introduced at the same time in contaminated soil attached to tubers and subsequently became established in Europe (Evans et al., 1975). The genetic variation of both species in Europe reflects only a small proportion of the total pathogen diversity occurring in South America, and thus quarantine rules now restrict imports of potatoes from South America (Plantard et al., 2008). Damage to the potato crop because of PCN was first reported in Europe at the end of the 19th century (Evans et al., 1975).

Breeding for PCN resistance started in the late 1940s. Ellenby (1952) discovered resistance to *G. rostochiensis* pathotype Ro1 and Ro4 in *S. tuberosum* ssp. *andigena* CPC 1673; the resistance being named as *H1*. A cross between *S. tuberosum* ssp. *andigena* CPC 1673 and the variety Kerr's Pink with subsequent backcrossing and selection for other important commercial traits led to the release of the potato variety Maris Piper in 1966. After more than 50 years in use, the resistance to *G. rostochiensis* provided by H1 has not been overcome. *H1* is the major source of resistance to *G. rostochiensis* in current potato cultivars in Europe and Northern America (Przetakiewicz and Milczarek, 2017).

Breeding for resistance to *G. pallida* has proven to be more challenging. The genetic variability of *G. pallida* populations outside South America is higher compared to *G. rostochiensis* (Gartner et al., 2021), and most resistance sources to this species are complex. In order to achieve high levels of resistance to *G. pallida*, more than one resistance locus is needed. The resistance *H2* from the diploid wild species *S. multidissectum* was introgressed into the breeding clone P55/7 (Dunnett, 1961), but it was never widely deployed, as the resistance only confers a high level of resistance to *G. pallida* pathotype Pa1, but a moderate- to low-level of resistance to Pa2/3, which is the most common pathotype in Europe.

The diploid wild species *S. vernei* has accessions showing quantitative resistance to both PCN species and consequently has been an important source of resistance to *G. pallida* and *G. rostochiensis* for introgression into commercially important tetraploid potato cultivars. In 1957 and 1958, the chromosome complement of *S. vernei* in somatic cells was

doubled with Colchicine at the Scottish Plant Breeding Station in Pentlandfield, and the resulting tetraploid plants were crossed several times with tetraploid cultivars and breeding lines at the Scottish Crop Institute (now JHI) (Bradshaw and Ramsay, 2005). Over decades of breeding this led to the cultivars Morag and Glenna that were released in 1985 and 1987, respectively (Bradshaw, 2009). In other places, PCN resistances from *S. vernei* were also introgressed into tetraploid potato cultivars and provided new cultivars such as Innovator, Arsenal and Eurostar, which show high levels of resistance for *G. pallida* Pa1 and Pa2/3, and in the case of Eurostar also a high level of resistance to *G. rostochiensis* Ro1. The resistance *Grp1*, which is closely related to the resistance *GpaV* from *S. vernei*, provided potato cultivars (*e.g.*, Iledher and Aveka) with high levels of resistance to *G. pallida*; however, it was reported that the resistance *Grp1* has been overcome in Germany (Niere et al., 2014).

The *H3* resistance source to *G. pallida*, which was discovered in *S. tuberosum* ssp. *andigena* (CPC 2775 and CPC 2802) has been successfully used in breeding programmes in the UK, resulting in a breeding line, 12601ab1, which has been used in the pedigrees for generating new potato cultivars such as Vales Everest and Olympus, which show moderate levels of resistance to *G. pallida*.

5.1.2 The development of MAS in potato breeding programs

Breeding using MAS is based on the identification of DNA-based molecular markers tightly linked to the desired traits. In potato, resistance to threats arising from pests and disease are normally controlled by only a few dominant genes, unlike other economically important traits, which are genetically much more complex and usually display continuous variation (Gartner et al., 2021). A major advantage of MAS is that, in the initial breeding steps, the validation of progeny genotypes having the resistance can be done by molecular methods such as PCR on many plants in a short time. The time-consuming and labour-intensive resistance tests are only performed at a later stage on a much lower number of plants. Using diagnostic molecular markers thus accelerates the generation of new cultivars, but also allows for pyramiding different sources of resistance in one potato genotype. Gene pyramiding, also known as gene stacking, is the combination of

favourable genes into one genotype of an individual plant. Pyramiding of resistance from different sources is an approach to ensure the development of durable and broad-spectrum resistance to *G. pallida*.

Two markers, TG689 and 57R, diagnostic for the *H1* resistance have been validated for many different potato genotypes and are used routinely to assess presence/absence of the *H1* resistance, with > 90% agreement between the marker assay and the PCN-resistance phenotype (Schultz et al., 2012). The presence or absence of a 452 bp amplicon for the marker 57R proved to be the most effective diagnostic marker for the prediction of PCN resistance conferred by *H1* (Park et al., 2018). A diagnostic marker to identify the presence or absence of the *G. pallida* resistance derived from *S. vernei* is the so-called HC-marker which is routinely used to screen for resistance (Sattarzadeh et al., 2006). However, in certain breeding lineages, false positives can pose a problem. Markers for the *H3* resistance, which comprises two QTLs on chromosomes IV (*GpaIV*) and XI (Bryan et al., 2002; 2004) derived from *S. tuberosum* group *andigena* CPC2802 have been developed, which are reliable in predicting *GpaIV* in a breeding population (Moloney et al., 2010). For other PCN resistance loci (*e.g., Gro1-4* and *GroVI*), the molecular markers identified to date have not been robust and reliable enough to predict presence or absence of the resistance for breeding purposes.

KASP-assays (Chapter 4) are ideal candidates for SNP markers to facilitate MAS, as they allow for high throughput screening. However, it has to be established if they can be used in different genetic backgrounds.

5.1.3 Can the KASP assays from Chapter 4 be used as diagnostic markers in tetraploid potato genotypes

The KASP assays developed in Chapter 4 for mapping the *G. pallida* resistance derived from *S. spegazzinii* CPC 7195 on chromosome VI can be used as molecular markers for the absence/presence of the resistance in the genetic background of the backcross of the diploid susceptible parent DB337(37) and the diploid resistant parent 03.F.3a(35) (Chapter 4). KASP assays can discriminate between two alleles “a” and “b” in one position on the chromosome, however, it cannot be distinguished whether an allele originates from the

maternal or paternal parent. In the susceptible plant DB337(37), allele “a” is present at a frequency of 100%, whereas in the heterozygous resistant plant 03.F.3a(35) alleles “a” and “b” occur at a frequency of 50% each for informative SNP that are linked to the resistance. In different genetic backgrounds, the linkage of these KASP alleles to the resistance is not necessarily preserved and is dependent on the level of linkage disequilibrium between the marker allele and the resistance gene in wider potato populations. Further, we presumed that bi-allelic markers and susceptible plants could, by chance, share the SNP that was associated with the resistances in *S. spegazzinii*. One requirement for a suitable diagnostic marker candidate is that the allele frequency must be 100% “a” in any potato genotype without resistance deriving from *S. spegazzinii* CPC 7195 on chromosome VI. If allele “b” is present in such material, the presence of the ‘resistant’ allele “b” is not indicative for the resistance to *G. pallida* in a potato tested for having the resistance or not, as it might originate from the tetraploid potato without the *S. spegazzinii*-derived resistance.

As autotetraploid species such as potato have four copies of each chromosome, five possible genotypes for the alleles “a” and “b” are possible: “aaaa”, “aaab”, “aabb”, “abbb”, “bbbb”, with 0, 25, 50, 75 and 100% of allele “b”, respectively. Figure 5-1 shows a sketch of the theoretical clustering of the different possible genotypes in a tetraploid plant in a KASP assay. In practice, it might be difficult to distinguish between some clusters without controls with known allele “a” and “b” content. The clones with known allele frequency used as control in this project are DB337(37) with 100% allele “a” and 03.F3a(35) with 50% of each allele “a” and “b”, respectively. For the other allele combinations, there are no samples with known allele frequency.

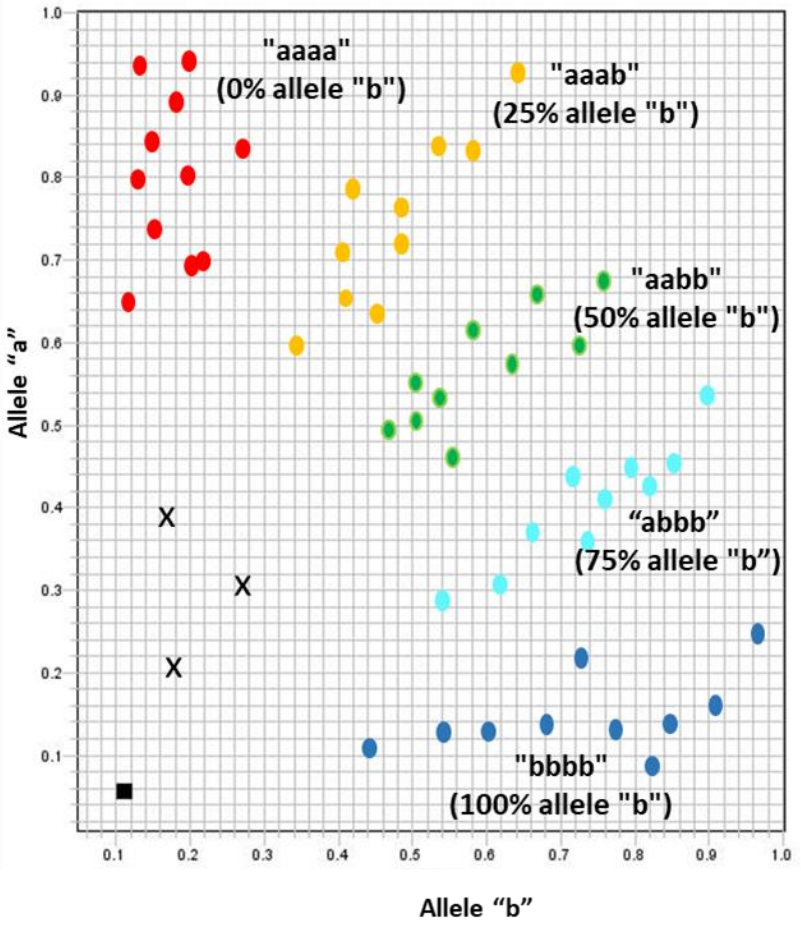


Figure 5-1 Sketch of an allelic discrimination plot for tetraploid organisms
In this theoretical allelic discrimination plot the clustering of the genotypes with different frequencies of alleles "a" and "b" is shown. The black square is the negative control, the "x" symbols represents improperly amplified samples, which cannot be assigned to a cluster. The red dots indicate the genotype with allele "a" only, no alternative allele "b", yellow dots indicate 25% allele "a" and 75% allele "b", green dots indicate 50% allele "a" and 50% allele "b", the crosses indicate 25% allele "a" and 75% allele "b", the blue dots indicate 100% allele "b".

5.1.4 Interploidy breeding

Diploid and tetraploid potato species are often crossed for the introgression of traits from diploid potato wild species into tetraploid potato genotypes by using the property of 2n gamete formation of the diploid species and the 'normal' haploid gamete production of the tetraploid species (Ortiz and Mihovilovich, 2020). The generation of 2n gametes occurs spontaneously at different rates for different plant species, at about 0.1-2%. However, choosing specific environmental conditions, such as high or low temperatures, can induce formation of 2n gametes at a higher rate (Younis et al., 2014).

During meiosis, gametes are generated; this involves a single round of DNA replication followed by two rounds of chromosome division, resulting in the chromosome set being reduced to half the number of the of the mother cell (2n →1n) (Brownfield and Kohler, 2011). 2n gametes are formed as a result of errors in meiosis. Figure 5-2 illustrates the two main mechanisms for the formation of 2n gametes in plants that are called first division restitution (FDR) and second division restitution (SDR). In FDR, pairing and split up of homologous chromosomes during meiosis-1 does not occur, while during the second division two sister chromatids of homologous chromosome are separated, leading to the formation of two 2n gametes, which have the same composition as the parent, except for cross-over events (*e.g.,* Brownfield and Kohler (2011); Younis et al. (2014)). In SDR, on the other hand, meiosis I occurs normally with homologous chromosomes pairing and separating, whereas in meiosis II, the sister chromatids are not separated, resulting in two 2n gametes that contain only one set of chromosomes, but with two copies (Brownfield and Kohler (2011); Younis et al. (2014). As a consequence, the heterozygosity in 2n gametes formed by FDR is higher (about 80%) than those resulting from SDR (about 40%) (Spooner et al., 2014).

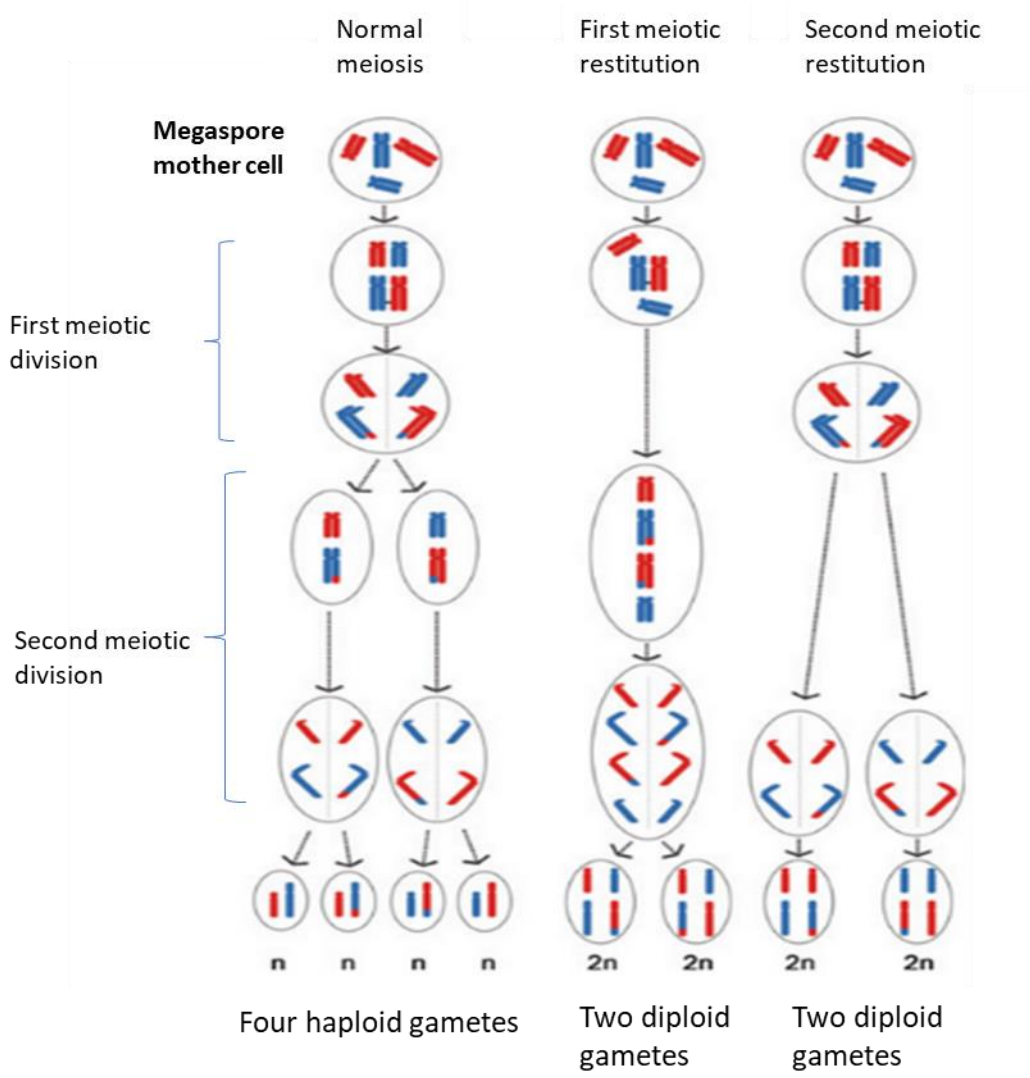


Figure 5-2 Schematic illustration of meiosis and defective meiosis mechanisms leading to 2n gametes
The shapes labelled in blue and red indicate the two sets of sister chromosomes; large and small shapes indicate different chromosomes. See main text for details. Adapted from Younis et al. 2014. Permission to reprint/adapt by RightsLink®, licence no. 5415760553286.

5.2 Scope of the chapter

The first part of this chapter evaluates five KASP assays of informative SNPs developed in Chapter 4 on a panel of tetraploid potato genotypes (without known *S. spegazzinii*-derived *G. pallida* resistance) to determine if they have the potential to be used more generally as diagnostic markers for the presence/absence of the *G. pallida* resistance derived from *S. spegazzinii*. In the second part of this chapter, tetraploid-diploid crosses are described. Several potato clones (progeny and parents of crosses), obtained from the breeding group

of James Hutton Limited (JHL), and one clone derived from crosses carried out as part of this project were evaluated for introgression of the *G. pallida* resistance.

5.3 Material and methods

5.3.1 Potato genotypes

The potato genotypes used to assess potential diagnostic markers and/or for interploidy crosses are listed in Table 5-1. Backcross population 13.A.02 is described in Figure 4-3; the population SPG09.A.03 was generated the same way, but it was done a few years earlier in 2009 and consisted then of 200 progeny plants. SPG09.A.03 was used by the JHL breeding group to cross individuals with the tetraploid potato genotypes Innovator, Performer (both *GpaV* from *S. vernei*) and Vales Everest (*H3* from *S. tuberosum* group *andigena*), with the aim of pyramiding different *G. pallida* resistance loci into one new potato genotype, and to assess agronomic traits. The tetraploid potato genotypes Alouette, Carolus, Desirée and Maris Piper are all susceptible to *G. pallida* according to the AHDB potato data base[14], which makes it easy to evaluate the resistance introgression by performing a root-trainer virulence bioassay. A tetraploid-diploid cross to introgress the resistance into the tetraploid potato genotype was also performed in this project.

[14] http://varieties.ahdb.org.uk/

Table 5-1 List of potato genotypes used in this chapter for assessment of KASP assays and/or interploidy cross

Potato ID	Description	Ploidy	Source
13.A.02(49) **13.A.02(463)**	See Figure 4-3. Two plants, 49 and 463, of backcross 13.A.02. *G. pallida* resistance derived from *S. spegazzinii* CPC 7195	diploid	This project
03.F.3a(35) **DB337(37)**	Resistant and susceptible parent, respectively, of 13.A.02 and 09.A.03.		JHI potato genetics group
SPG 09.A.03(6) **SPG 09.A.03(45)**	Two plants, 6and 45, of backcross SPG 09.A.02, this is the same cross as 13.A.02, except that it was performed some years earlier in 2009.		JHL potato breeding group
***S. tuberosum* cv. Alouette**	Resistant to *G. rostochiensis* (*H1*), susceptible to *G. pallida*	tetraploid	Potato stores from either JHI or JHL (Drummond Todd)
***S. tuberosum* cv. Carolus**	Very susceptible to *G. rostochiensis* and *G. pallida*		
***S. tuberosum* cv. Desirée**	Susceptible to *G. rostochiensis* and *G. pallida*		
***S. tuberosum* cv. Maris Piper**	Resistant to *G. rostochiensis (H1*), susceptible to *G. pallida*		
***S. tuberosum* cv. Innovator**	Susceptible to *G. rostochiensis*, good resistance to *G. pallida* (from *S. vernei*)		
***S. tuberosum* cv. Performer**	Moderately resistant to *G. rostochiensis*, high level of resistance and tolerance to *G. pallida*		
***S. tuberosum* cv. Vales Everest**	Moderate resistance to *G. rostochiensis* and *G. pallida* , resistance derived from different *S. vernei*.		
***S. tuberosum* Breeding clone 1** ***S. tuberosum* Breeding clone 2**	PCN breeding lines of JHL. Both lines show high level of resistance to *G. rostochiensis* and *G. pallida*		

Table 5-1 continued

Potato ID	Description	Ploidy	Source
15.JHL.120 A4	Performer x SPG 09.A.03 (6)	? Probably tetraploid	JHL potato breeding group Vanessa Young and Drummond Todd.
15.JHL.127 A4 **15.JHL.127 A8** **15.JHL.127 A13** **15.JHL.127 A15** **15.JHL.127 A21**	Vales Everest x SPG 09.A.03 (6)		
15.JHL.128 A1 **15.JHL.128 A21** **15.JHL.128 A3**	Vales Everest x SPG 09.A.03 (45)		
15.JHL.130 A1	Innovator x SPG 09.A.03 (6)		
15.JHL.131 A1	Innovator x SPG 09.A.03 (45)		

5.3.2 Tetraploid-diploid potato crosses

The protocol from Gaynor McKenzie, the curator of the CPC, was followed. All parents were grown in rich compost with Intercept (insecticide). The diploid potato plants 13.A.02(49) and 13.A.02(463) were used as paternal parents. They were checked for pollen in the anthers as soon as flowers opened by inserting the tip of a pollinator needle into the stomium of the anther and gently moving it to its top to see if pollen was released.

Pollination of the female parents was performed as described below. Importantly, the pollinator needle must be sterilized with 70% ethanol after handling each parent in a cross and between crosses to avoid cross-contamination.

1) A well-developed but not yet opened flower cluster (inflorescence) was chosen as the maternal parent in the tetraploid plants, the immature buds at the 1- or 2-day stage were removed.

2) These flower clusters were emasculated by removing the anthers by carefully pulling them off with the pollination needle, without damaging the stigma.

3) Older flowers, which had already opened on the maternal plant, were removed to prevent pollination of the freshly emasculated flowers.

4) The pollen from the diploid paternal parent was then collected by slicing open the anther cone lengthwise and through the side of one of the anthers. The pollen was visible as a whitish powder.

5) By holding the emasculated flower in one hand, the pollen was applied to the stigma surface using the pollinator needle. The whole stigma was covered with pollen.

6) The pollinated flowers were tagged with a note of the cross, female parent first, number of flowers pollinated and dated.

7) After a few days, flowers that were not fertilized fell off, and in the others a small berry was developing. A plastic bag with holes for air exchange was put on the remaining flowers to collect the berries and the berries were allowed to grow till they fell into the bag.

8) The berries were kept at 4˚C for 3 days, before they were opened and, if there were seeds present, the seeds were washed out with water onto a filter paper, dried overnight, put in a bag and stored at 4˚C for 3 months. Then the seeds were ready to be planted or to be stored at RT.

5.3.3 KASP assays

The KASP assays ST4_03ch06_53821580, ST4_03ch06_55096874, ST4_03ch06_55909792, ST4_03ch06_56915767 and ST4_03ch06_58781798 designed and described in Chapter 4 were used as described in Section 4.3.9.

5.3.4 Assessment of level of resistance to *G. pallida* in potato plants

The progeny plants of the potato crosses were assessed for their resistance with root trainer bio-assays as described in Section 4.3.3.1 with the parents used as controls.

5.4 Results

A closer look at the possible genetic outcomes of a tetraploid-diploid cross at a locus with two alleles allows the determination of which KASP assays can be useful as introgression markers. Table 5-2 shows possible combinations of two alleles, “a” and “b”, in a tetraploid organism with the specified gametes (relative frequencies in brackets). The table also shows unreduced gametes from the diploid parent with the SNPs “a” and “b” in the KASP assay positions and the possible genetic outcomes of the progeny plants, with homologous recombination not considered. Allele “b2” from the tetraploid parent is not linked to the resistance allele, whereas allele “b1”, from the diploid parent is linked to the *G. pallida* resistance allele derived from *S. spegazzinii*. The two mechanisms FDR and SDR provide 2n gametes with different allele distributions. It was shown that 2n pollen formation mainly occurs via FDR whereas ovule formation is via SDR (Hutten et al., 1994). The KASP assay can discriminate between the alleles “a” and “b”, but not between “b1” from the diploid potato and “b2” from the tetraploid parent, as they are the same allele in the KASP assay. To be potentially useful as an introgression marker, the percentage of allele “b” in the progeny has to be different from the tetraploid parent for all potential progeny clones. For FDR this is the case when the parents are either “aaaa” or “bbbb”, as

allele “b” and “a”, respectively, only can come from the diploid parent with the resistance. For SDR, introgression of the resistance in duplex (“bb”) can be identified by the KASP assays when the parental genotype is “aaaa” or “aaab”; for the other 2n gamete “aa”, this is the case when the parental genotype is “bbbb” or “abbb”, although this genotype is probably not of interest, as no resistance is introgressed. However, the introgression of 09.A.03(6) into the tetraploid potatoes exactly shows this 2n gamete genotype; so introgression can be evaluated by the KASP assays in this case.

In potato, tetraploid-diploid crosses are usually performed using the diploid potato as paternal parent, in which the 2n gametes are produced via the FDR mechanism. 2n ovules are mainly produced by the SDR mechanism.

Note, that this is a simplified scheme without taking homologous recombination into account, which occurs about once in a generation per chromosome. So, if the progeny plants have the same allele frequencies as the tetraploid parent it does not mean that the introgression did not work, and a recombination event might have happened.

Table 5-2 Genotypes of progeny and parents' gametes in a tetraploid-diploid cross

"a" and "b" indicate different alleles in one position on the chromosome, "1" and "2" indicate from which gamete allele "b" originates from: "1" deriving from the unreduced gamete, and "2" from the gamete of the tetraploid parent. Depending on the mechanism by which they were produced, unreduced gametes with a different level of heterozygosity can be formed.

		Unreduced gametes from diploid parent					
Tetraploid parent		**FDR (pollen)**		**SDR (ovule)**			
Genotype (% allele "b")	**Gametes**	**ab_1** (% allele "b")	**Usable as marker?**	**aa** (% allele "b")	**Usable as marker?**	**b_1b_1** (% allele "b")	**Usable as marker?**
"aaaa" (0)	"**aa**"	"$aaab_1$" (25)	**yes**	"aaaa" (0)	no	"aab_1b_1" (50)	**yes**
"$aaab_2$" (25)	"**aa**"	"$aaab_1$" (25)	no	"aaaa" (0)	no	"aab_1b_1" (50)	**yes**
	"**ab_2**"	"aab_1b_2" (50)		"$aaab_2$" (25)		"$ab_1b_1b_2$" (75)	
"aab_2b_2" (50)	"**aa**"	"$aaab_1$" (25)	no	"aaaa" (0)	no	"aab_1b_1" (50)	no
	"**ab_2**"	"aab_1b_2" (50)		"$aaab_2$" (25)		"$ab_1b_1b_2$" (75)	
	"**b_2b_2**"	"$ab_1b_2b_2$" (75)		"aab_2b_2"		"$b_1b_1b_2b_2$" (100)	
"$ab_2b_2b_2$" (75)	"**ab_2**"	"aab_1b_2" (50)	no	"$aaab_2$" (25)	**yes**	"$ab_1b_1b_2$" (75)	no
	"**b_2b_2**"	"$ab_1b_2b_2$" (75)		"aab_2b_2" (50)		"$b_1b_1b_2b_2$" (100)	
"$b_2b_2b_2b_2$" (100)	"**b_2b_2**"	"$ab_1b_2b_2$" (75)	**yes**	"aab_2b_2" (50)	**yes**	"$b_1b_1b_2b_2$" (100)	no

5.4.1 Analysis of selected KASP assays developed in Chapter 4 in tetraploid potato genotypes

Figure 5-3 panel A shows the relative position of the five potential KASP markers, ST4_03ch06_53821580, ST4_03ch06_55096874, ST4_03ch06_55909792, ST4_03ch06_56915767 and ST4_03ch06_58781798 to the *G. pallida* resistance on chromosome VI used to investigate their eligibility as diagnostic marker candidate. Potential markers for both sides of the resistance were chosen. Indeed, having at least one diagnostic marker on each flanking side of the resistance locus is desirable, as this is more likely to enable detection of resistant plants which underwent one homologous recombination event in the region of the resistance in a cross. These marker candidates were evaluated on a panel of nine diverse tetraploid potato genotypes, which are the *G. pallida*-susceptible *S. tuberosum* cultivars Alouette, Carolus, Desirée and Maris Piper, and the *G. pallida*-resistant *S. tuberosum* genotypes Innovator, Performer, Vales Everest, and breeding clones 1 & 2 with no known *S. spegazzinii*-derived *G. pallida* resistance.

Figure 5-3 panel B provides a list of the tested potato genotypes with the obtained genotypes of the SNPs in the KASP assays. The samples with 100% allele “a” are highlighted in bold as well as the diploid “controls” DB337(37) and 03.F.3a(35), with the genotype given in brackets, if different from “aaaa”. Only KASP assay ST4_03ch06_53821580, with a distance of 2.3 to 3.1 Mb from the resistance, shows the genotype “aaaa” in all tested potato varieties. The KASP assays ST4_03ch06_56915767 and ST4_03ch06_58781798 show at least 50% allele “b” in all tested potato genotypes, therefore they are not usable as KASP markers for the resistance gene being introgressed. KASP assays ST4_03ch06_55096874 and ST4_03ch06_55909792 show a more differentiated picture show some potato genotypes without allele “b” and some with 25 or 50% allele “b”.

A

B

	Genotype 'aaaa' (if no, the genotype is in brackets)				
Potato	**ST4_03ch06_53821580**	**ST4_03ch06_55096874**	**ST4_03ch06_55909792**	**ST4_03ch06_56915767**	**ST4_03ch06_58781798**
Alouette	**Yes**	**yes**	no ("aabb")	no ("bbbb")	no ("abbb" or "bbbb")
Breeding clone 1	**Yes**	no ("aabb")	no ("aabb")	no ("abbb")	no ("abbb" or "bbbb")
Breeding clone 2	**Yes**	**yes**	**yes**	no ("bbbb")	no ("abbb" or "bbbb")
Carolus	**Yes**	**yes**	**yes**	no ("aabb")	no ("aabb")
Desiree	**Yes**	**yes**	no ("aabb")	no ("abbb")	no ("abbb" or "bbbb")
Maris Piper	**Yes**	no ("aabb")	**yes**	no ("bbbb")	no ("abbb" or "bbbb")
Innovator	**Yes**	**yes**	no ("aaab" or "aabb")	no ("abbb")	no ("abbb" or "bbbb")
Performer	**Yes**	no ("aaab")	**yes**	no ("bbbb")	no ("abbb" or "bbbb")
Vales Everest	**Yes**	**yes**	**yes**	no ("abbb")	no ("abbb" or "bbbb")
DB337(37)	**Yes**	**yes**	**yes**	**yes**	**yes**
03.F.3a(35)	no ("ab")	no ("ab")	no ("ab")	no ("ab")	no ("ab")

Figure 5-3 Positions of diagnostic marker candidates and evaluation on tetraploid potatoes
Panel A shows the positions of the KASP assays used, mapped to DM, relative to the resistance locus. Panel B shows the allele distribution of selected tetraploid potato genotypes and the diploid potato clones 03.F.3a(35) and DB337(37) as controls for 0 and 50% allele frequency "b", obtained by KASP analysis.

5.4.1 Introgression of the *S. spegazzinii*-derived resistance to *G. pallida* into different tetraploid potato genotypes and use of KASP assays as molecular markers

The JHL breeding group routinely performs introgressions of new resistance loci into different potato genotypes at an early stage, even before there are diagnostic markers available for the new resistance to be tested. Therefore, a set of tetraploid-diploid crosses with progeny clones was available and tested with the KASP assays for introgression of the resistance locus. The tetraploid cultivars used by JHL all had at least one source of *G. pallida* resistance. In addition, interploidy crosses of susceptible tetraploid potato cultivars with two resistant diploid clones from this study were performed[15]. All interploidy crosses performed and/or tested with KASP assays are illustrated in Figure 5-4. Most of the crosses performed in this study did not provide any progeny. About 400 flower buds were pollinated per cross, except considerably less (about 50) for each Maris Piper cross, as it was not flowering well. Alouette gave 11 berries, Carolus 4, Desirée 7 and Maris Piper 0. Extraction of seeds revealed that most of the berries were empty which is consistent with sterility, only Alouette x 13.A.02(463) provided 1 berry with 8 seeds and 1 berry with 1 seed, and Desirée x 13.A.02(49) provided 1 berry with 3 seeds and one with 1 seed. Alouette gave 11 berries, Carolus 4, Desirée 7 and Maris Piper 0. Extraction of seeds revealed that most of the berries had no viable seeds, only Alouette x 13.A.02(463) provided 1 berry with 8 seeds and 1 berry with 1 seed, and Desirée x 13.A.02(49) provided 1 berry with 3 seeds and one with 1 seed. The 8 seeds from 1 berry of the cross Alouette x 13.A.02(463) and 3 seeds from 1 berry of the cross Desirée x 13.A.02(49) were planted. Of 4 seedlings of Alouette x 13.A.02(463), 2 survived and produced tubers and none of Desirée x 13.A.02(49) germinated.

[15] All crosses with the diploid resistant potato clones 09.A.03(6) and 09.A,03(45) were conducted by JHL, alle crosses with the potato clones 13.A.02(49) and 13.A.02(463) used as paternal parents were performed by me.

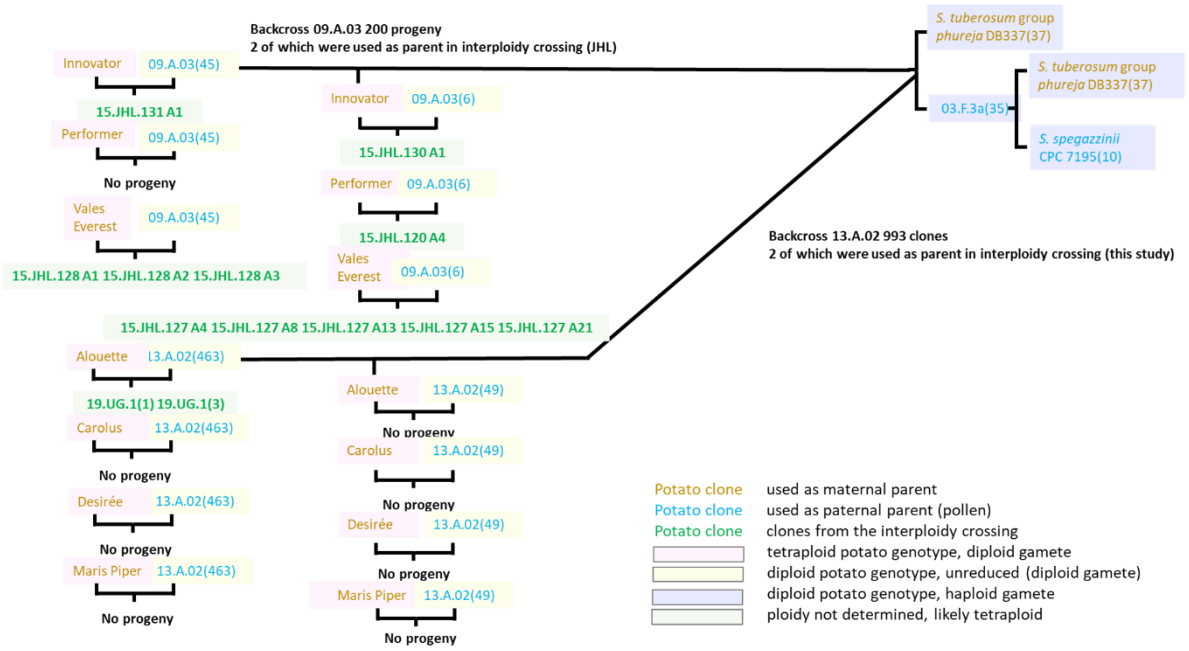


Figure 5-4 Pedigree of the crosses for introgression of a *G. pallida* resistance from *S. spegazzinii*
The number of progeny produced with inter-ploidy crossing was very low, with many crosses producing empty berries. The use of different colours/shading is described in the figure.

JHL provided plant material (tubers and genomic DNA) of five different tetraploid-diploid crosses to pyramid different *G. pallida* resistance sources and their respective parents. Two clones of the backcross 09.A.03 were chosen as the diploid parents. This cross has the same parents as in backcross 13.A.02, with the difference that it was performed a few years earlier in 2009. The two clones selected as parents for the tetraploid diploid crosses, 09.A.03(6) and 09.A.03(45) were tested for PCN resistance/susceptibility in a canister bioassay and showed a very low number of *G. pallida* female nematodes compared to the susceptible parent (Vanessa Young, personal communication). Innovator, Performer and Vales Everest were chosen as the tetraploid parents, as their *G. pallida* resistance loci differ from that of *S. spegazzinii*. For these crosses, having molecular markers is essential for the evaluation of the introgression, as it might be difficult to see a potential further additive effect of the *S. spegazzinii* resistance in bioassays. To be able to demonstrate introgression of the resistance into a tetraploid potato with a bioassay such as a root-trainer test, the susceptible cultivars Alouette, Carolus, Desirée and Maris Piper were crossed with two resistant progeny clones (13.A.02(49) and 13.A.02(463)) of the backcross 13.A.02 from this study. In all crosses pollen of the diploid parent was used. Studies have

shown that 2n gametes from pollen are almost completely formed by FDR (*e.g.,* Hutten et al., 1994).

The above described KASP assays are good markers for the presence/absence of the *S. spegazzinii*-derived *G. pallida* resistance in the *S. spegazzinii*, DB337(37) background (Chapter 4). The clones 09.A.03(6), 09.A.03(45), 13.A.02(49) and 13.A.02(463) were assessed by KASP assay analysis to determine whether they have the "b" allele, which is linked to the resistance to *G. pallida*, and whether a homologous recombination event had occurred during the cross. Figure 5-5 panel A shows the graphical genotyping of those plants. SPG 09.A.03(6) shows the genotype which would be expected for a susceptible plant, which means that the clone most likely does not have the *S. spegazzinii*-derived resistance on chromosome VI. In addition, this clone is not resistant (as defined above) but has reduced susceptibility. The clones SPG 09.A.03(45), 13.A.02(49) and 13.A.02(463) show the same genotype as the resistant plant 03.F.3a(35). A root-trainer PCN bioassay (Figure 5-5 panel B) revealed that 09.A.03(45), 13.A.02(49), 13.A.02(463) show a similar level of resistance to *G. pallida* as 03.F.3a(35), whereas 09.A.03(6) showed higher nematode numbers and is categorized as reduced susceptible and not resistant.

A

KASP assay	09.A.03(6)	09.A.03(45)	13.A.02(49)	13.A.02(463)	Parents resistant 03.F.3a(35)	Parents susceptible DB337(37)
ST4_03ch06_53821580	-	+	+	+	+	-
ST4_03ch06_55096874	-	+	+	+	+	-
ST4_03ch06_55909792	-	+	+	+	+	-
ST4_03ch06_56915767	-	+	+	+	+	-
ST4_03ch06_58781798	-	+	+	+	+	-

B

Root-trainer PCN assay						
No. of female nematodes average (each replicate)	15 (19, 1, 28, 10)	6 (5, 0, 11, 8)	2* (1, 3, 0, 4)	0.3 (0, 0, 1, 0)	2 (1, 0, 7, 0)	44 (41, 20, 55, 57)
% of female nematodes based on DB337(37)	34.1	13.6	1.7*	0.7	4.5	100

Figure 5-5 Assessment of genotype and resistance to *G. pallida* for the diploid parent plants
Panel A shows the graphical genotyping of the clones used for the tetraploid diploid cross. The clones labelled "–" in red have the allele of the susceptible parent in the SNP tested ("aa"), the clones labelled "+" in green share the alleles from the resistant parent ("ab"). Panel B shows the number of female nematodes in a root-trainer test with four replicates, the first number is the average, the numbers in brackets indicate the number of female nematodes in each replicate. * these numbers are from a different PCN root trainer test, as there were no progeny clones, no test was performed at the time.

Next, the results of the KASP assays, performed on all progeny plants of the tetraploid-diploid crosses were analysed to determine if they can be used to predict the successful introgression of the resistance locus. The KASP assays labelled in light green in Figure 5-6 indicate the assays that potentially can be used to evaluate the introgression. The allele frequencies labelled in darker green show the progeny where the KASP assay indicate introgression. All progeny plants from the JHI crosses have the DNA from the diploid parent incorporated. Clone 15.JHL.128.A1 appears to have a recombination event between ST4_03ch06_55096874 and the resistance locus. If this would be a setting, where only the marker is looked at and not the phenotype (*e.g.,* in early selection of clones in MAS), these false negative potato clone would not have been detected. However no false positive would be expected when the paternal parent has the genotype "ab".

The cross performed in this project, Alouette x 13.A.02(463), shows the same allele frequencies as the tetraploid susceptible parent. This means that either the tetraploid x diploid cross has not worked (*i.e.,* it is a self of Alouette x Alouette) or in both progeny

plants 18.UG.1(1) and 18.UG.1(3) a recombination event occurred between the resistance gene and the more upstream or downstream of the most distant marker.

Maternal x paternal parent	Innovator	x	09.A.03(45)	Vales Everest		x		09.A.03(45)
Progeny plants		15.JHL.131 A1			15.JHL.128 A1	15.JHL.128 A2	15.JHL.128 A3	
KASP assay ID	Frequency of allele "b" in percent			Frequency of allele "b" in percent				
ST4_03ch06_53821580	0%	25%	50%	0%	25%	25%	25%	50%
ST4_03ch06_55096874	0%	25%	50%	0%	50%	25%	25%	50%
ST4_03ch06_55909792	25-50%	25%	50%	25-50%	25%	50%	50%	50%
ST4_03ch06_56915767	75%	50%	50%	75%	25%	50%	50%	50%
ST4_03ch06_58781798	75%	50-75%	50%	75-100%	50%	75-100%	50%	50%

Maternal x paternal parent	Performer	x	09.A.03(6)	Innovator	x	09.A.03(6)	Vales Everest			x			SPG 09.A.03(6)
Progeny plants		15.JHL.120 A4			15.JHL.130 A1			15.JHL.127 A4	15.JHL.127 A8	15.JHL.127 A13	15.JHL.127 A15	15.JHL.127 A21	
KASP assay ID	Frequency of allele "b" in percent			Frequency of allele "b" in percent			Frequency of allele "b" in percent						
ST4_03ch06_53821580	0%	0%	0%	0%	0%	0%	0%	0%	0%	0%	0%	0%	0%
ST4_03ch06_55096874	25%	25%	0%	0%	0%	0%	0%	0%	0%	0%	0%	0%	0%
ST4_03ch06_55909792	0%	0%	0%	25-50%	0-25%	0%	25-50%	0%	0%	0%	0%	0%	0%
ST4_03ch06_56915767	100%	50%	0%	75%	25%	0%	75%	50%	25%	50%	25%	25%	0%
ST4_03ch06_58781798	75%	50%	0%	75%	0-25%	0%	75-100%	50%	50%	50%	50%	50%	0%

Maternal x paternal parent	Alouette	x		13.A.02(463)
Progeny plants		19.UG.1(1)	19.UG.1(3)	
KASP assay ID	Frequency of allele "b" in percent			
ST4_03ch06_53821580	0%	0%	0%	50%
ST4_03ch06_55096874	0%	0%	0%	50%
ST4_03ch06_55909792	50%	50%	50%	50%
ST4_03ch06_56915767	100%	100%	100%	50%
ST4_03ch06_58781798	75-100%	75-100%	75-100%	50%

Figure 5-6 Using SNP frequencies to determine if introgression of the resistance locus has occurred

This figure shows the frequencies of allele "b" for all progeny and parental plants from all crosses with five different KASP assays. The frequencies for the assays which can be used for evaluating the success of introgression are highlighted in light green. The allele frequencies highlighted in light green indicate that these can be used as introgression markers, allele frequencies in darker green indicate the introgression of the DNA region on chromosome VI. The allele frequencies in yellow indicate either a recombination event or that no introgression occurred.

Next, the level of resistance of the progeny plants of all crosses was determined with a root-trainer bioassay. The results are shown in Figure 5-7. The crosses of Innovator with 09.A.03(6) and with 09.A.03(45) do not show an additive effect of the *S. spegazzinii*-derived resistance on their single progeny plant, on the contrary, the number of female nematodes on Innovator is significantly lower than in 09.A.03(45) (Table 5-3). The three progeny plants from the cross of Vales Everest with 09.A.03(45) also do not show an additive effect, two of the five progeny plants from the cross of Vales Everest with 09.A.03(6) and the one progeny form the cross of Performer with 09.A.03(6) show fewer female nematodes than either of the parents, however only 15.JHL.127 A8 and A15 show statistical significance/high significance. Both progeny plants 18.UG1(1) and 18.UG.1(3) show highly significant and significant reduction of female nematodes compared to the maternal parent Alouette, which is susceptible to *G. pallida* (Table 5-3).

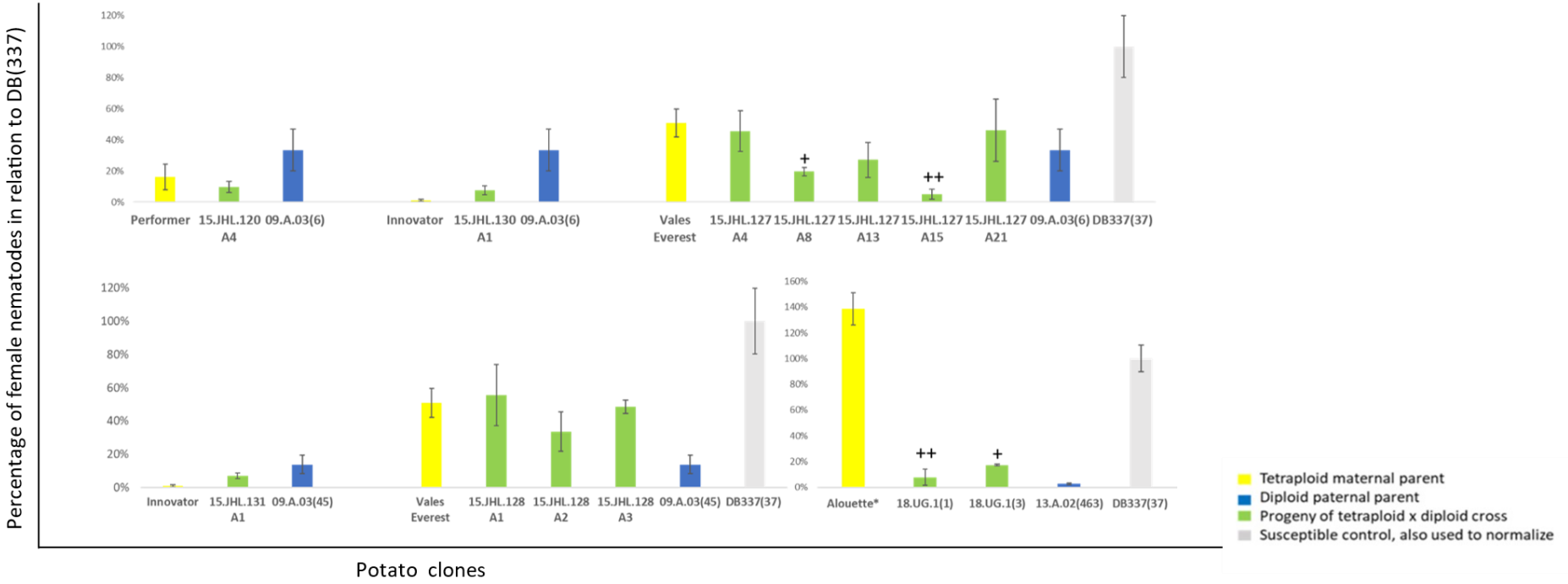


Figure 5-7 Assessment of resistance on progeny and parent plants of tetraploid-diploid crosses

In a root trainer bioassay, the resistance of the progeny of tetraploid-diploid crosses was evaluated in relation to the susceptible potato genotype DB337(37). The bars in yellow show the maternal (tetraploid) parents, the blue bars show the paternal (diploid) parents, and the progeny is highlighted in green. DB337(37) is used as the susceptible control.* Alouette scores come from a different root trainer assay than the scores from the progeny. "+" indicates significant and "++" highly significant lower number of female nematodes per clone compared to the maternal parent.

Table 5-3 One-way ANOVA analysis between maternal parent and progeny plants

This table shows the result of a one-way ANOVA test of the maternal parent with one progeny of a tetraploid-diploid cross. The aim was to determine if the progeny shows a significantly lower number of female nematodes in a root bio-assay. P-values between 0.05 and 0.01 mean significant difference and p-values < 0.01 are highly significant differences. + means significantly lower numbers of female nematodes on the progeny plants, ++ means very significantly lower numbers on the progeny plant, and * means significantly lower number on the maternal parent. The degree of freedom was 1 in all cases and the number of samples (counts) was 4 in all clones tested.

Cross	Groups	Count	No. of female nematodes			df	p-value
			Sum	Average	Variance		
Performer x 09.A.03(6)	Performer	4	28	7	51.3		
	15.JHL.120 A4	4	17	4.25	9.6	1	0.5074
Innovator x 09.A.03(6)	Innovator	4	2	0.5	0.3		
	15.JHL.130 A1	4	13	3.25	6.3	1	0.0758
Vales Everest x 09.A.03(6)	Vales Everest	4	88	22	58.0		
	15.JHL.127 A4	4	79	19.75	128.3	1	0.7528
	15.JHL.127 A8	4	34	8.5	5.7	1	0.0148 +
	15.JHL.127 A13	4	47	11.75	92.9	1	0.1462
	15.JHL.127 A15	4	9	2.25	8.3	1	0.0028 ++
	15.JHL.127 A21	4	80	20	302.0	1	0.8400
Innovator x 09.A.03(45)	Innovator	4	2	0.5	0.3		
	15.JHL.131 A1	4	12	3	2.0	1	0.0170 x
Vales Everest x 09.A.03(45)	Vales Everest	4	88	22	58.0		
	15.JHL.128 A1	4	96	24	254.0	1	0.8284
	15.JHL.128 A2	4	58	14.5	105.0	1	0.2845
	15.JHL.128 A3	4	84	21	12.0	1	0.8190
Alouette x 13.A.02(463)	Alouette	4	240	60	118.0		
	18.UG.1(1)	4	34	8.5	89.7	1	0.0004 ++
	18.UG.1(3)	4	100	25	318.0	1	0.0154 +

5.5 Discussion

In this chapter it was investigated whether the KASP assays developed in Chapter 4 are suitable to potentially be used as diagnostic markers for the detection of the *S. spegazzinii*-derived *G. pallida* resistance in different genetic backgrounds of tetraploid potato genotypes. Of the five tested KASP assays, only one, ST4_03ch06_53821580 (3 to 3.5 Mb away from the resistance locus), showed 100% frequency of allele "a" in all tested tetraploid potato genotypes which is a requirement for a potential diagnostic marker when a potato cultivar is tested for the presence of the resistance. More potato genotypes, both with known presence and absence of the *S. spegazzinii*-derived resistance on chromosome VI need to be tested and evaluated to establish whether ST4_03ch06_53821580 can generally be used as reliable diagnostic marker. Even if it proves to be useful as a diagnostic marker, it would be preferable to have at least two markers, one upstream and one downstream of the resistance to detect potential homologous recombinants.

Crosses of tetraploid susceptible potato genotypes with the diploid *G. pallida*-resistant *S. spegazzinii*-derived potatoes were performed in this study to facilitate the assessment of the reliability of the markers with a root-trainer virulence test. From a commercial point of view, the introgression of the *G. pallida* resistance into tetraploid potato genotypes which possess a *G. pallida* resistance from a different source is potentially more interesting. In this case, the evaluation of the introgression relies on molecular markers, as it might be difficult to assess additive effects of the two resistances phenotypically.

For the crosses with the tetraploid potato cultivars Performer, Innovator and Vales Everest the KASP assay tested showed that the two KASP assays ST4_03ch06_56915767 and ST4_03ch06_58781798 that are both downstream of the resistance (in respect to the reference genome DM) are suitable as markers in this specific genetic background. However, as mentioned before, the absence of an allele pattern that is different than that of the maternal parent does not indicate that the introgression has not occurred.

The KASP assays in cross 18.UG.1 between Alouette and 13.A.02(463) with its two progeny plants show the same genotype as the tetraploid maternal parent Alouette,

which can be explained if no interploidy cross took place or if homologous recombination occurred. A root-trainer assay showed that the introgression of the resistance was successful, with a reduction of female nematodes on the progeny of the cross by 14-fold compared to Alouette.

The interploidy crosses, performed by the JHL breeding group, were successful in the sense that they introgressed DNA from the diploid parent. However, one of the diploid parents, SPG 09.A.03(6), does not possess the *G. pallida* resistance on chromosome VI between positions 56,135,692 and 56,914,572, described in Chapter 4. Root-trainer virulence tests showed that this clone is not as resistant to *G. pallida* as *S. spegazzinii*; however, it shows reduced susceptibility, which could indicate the presence of another resistance locus, as were described for example in Kreike et al. (1994).

Interestingly, when comparing the number of females that could reproduce on the potato plants, it was the reduced susceptible diploid *S. spegazzinii*-derived parent SPG 09.A.03(6) without the resistance described in Chapter 4 that showed an additive effect of resistance on two progeny clones of a cross with the partial resistant potato Vales Everest and possibly in the clone from a cross with the potato cultivar Performer. The progeny plants of crosses with SPG 09.A.03(45) show no additive effects when two resistance loci are combined in one potato genotype. This means either that the two sources of resistance affect the same resistance pathway or that the difference in nematode numbers is too small to detect with the number and/or type of bioassays performed. However, more plants need to be tested to determine if there is an additive effect or not.

In summary, one of the KASP assays, developed for the mapping of the resistance gene in *S. spegazzinii*, ST4_03ch06_53821580, remains a candidate for a diagnostic marker for this resistance. The *G. pallida* resistance from *S. spegazzinii* on chromosome VI was successfully introgressed into the three tetraploid potato genotypes Innovator and Vales Everest and Alouette. Possibly, also a different resistance to *G. pallida* from *S. spegazzinii* CPC 7195 was introgressed into potato cultivars Performer, Innovator and Vales Everest by using clone 09.A.03(6) as paternal parent which appears to not have the resistance on chromosome VI.

6 General discussion

6.1 Global food security

The main challenges to provide global food security are the increasing world population and climate change. The UN created a Sustainable Development Goals program and Goal 2 is about “creating a world free of hunger by 2030” (Zero Hunger) (United Nations, 2017). Not only the food quantity but also the quality need to be increased to achieve this goal. Breeders have several different approaches to improve crops (Ahmad et al., 2021).

- **Conventional breeding**: plants with different desired traits are crossed and the progeny plants are tested for having the combined traits of interest and backcrossed. This is a laborious process that takes 7 to 10 years. MAS has accelerated the generation of crops with improved traits in conventional breeding. In the early stage of finding potential breeding clones, plants not having the marker can be eliminated without doing elaborative phenotyping. Ideally, at least 2 markers flanking the region of the desirable trait are used, to determine if a homologue recombination has occurred. When only one marker is available, plants having a homologous recombination event will be eliminated early. Using MAS, many different traits can be screened for easily, furthermore, *e.g.,* different sources of resistance to the same disease/pathogen can be pyramided. This method can be used when the gene(s) for the improved traits are unknown. When breeding resistant plant varieties, finding new sources of resistance is important for the generation of new breeding clones for the management of pathogens. Therefore, methods which allow for determining if resistance loci are new with a high probability have been developed. Sequencing of enriched DNA of the investigated plant cultivars allows for rapid mapping of the traits of interest with BSA. A further development is diagnostic RenSeq (dRenSeq) which enables to identify the presence of known functional R-genes in a plant cultivar, and therefore facilitates the determination whether a resistance phenotype is conferred by a yet unknown *R*-gene. (Chen et al., 2018; Armstrong et al., 2019; Beketova et al., 2021).

- **Mutagenesis breeding**: chemicals or beam irradiation induce mutations in the crop of interest are used to improve traits and to increase the genetic variability of traits in food crops (Chaudhary et al., 2019). This method is considered as producing genetically modified organisms (GMO) in Europe when the mutagenesis was applied after 2001 (Purnhagen and Wesseler, 2021). This method is challenged by of problems, like an uncontrolled number of mutations in the genome, loss of function of desirable traits, the selection process needs to be strict, and therefore the whole process is slow.
- **Transgenic breeding:** When a genetically modified crop variety is generated, a sequence of DNA conferring the desired trait is cloned into a carrier plasmid, delivered into the plant and screened for plants with the new characteristics. Genetically modified crop production has been controversial, especially because of the fear that DNA foreign to the organism may be present in the final crop variety. in the EU and UK GMO cannot be applied commercially, it only can be used for research. (Zaidi et al., 2020).
- **Genome-editing technology**: From the mid-1990s, gene editing technology started to develop, with meganucleases (Rouet et al., 1994), zinc-finger nucleases (Kim et al., 1996), transcription activator-like effector nucleases (TALEN) (Miller et al., 2011) and clustered regularly interspaced short palindromic repeats (CRISPR)/CRISPR-associated protein 9 (Cas9) (Charpentier and Doudna, 2013). Genome editing takes advantage of site-specific nucleases. With this technology, changes in the DNA sequence can be performed precisely and without leaving non host DNA in the manipulated plant and gene knock-in and knock-out, stacking of traits, targeted mutagenesis, modulation of translation can be performed. Furthermore, using gene editing methods in crop breeding is much faster than conventional breeding (reviewed in (Zaidi et al., 2020)). However, the crops resulting from gene editing technologies are considered to be GMOs in Europe and the UK, despite the differences to transgenic breeding. After leaving the EU, the UK considers permitting the use of genome editing technologies (UK Government, 2022)

6.2 My work

Potato is the most important non-cereal food crop worldwide (CIP, 2020). In the last 50 years, potato production has increased by about 25% to approximately 370 million tons per year in 2018 (FAO, 2020). While production in Europe decreased during that time, production in Asia has increased significantly since the 1990s, with China globally being both the biggest potato producer and consumer (FAO, 2020). Yields vary widely, from less than 5 t/ha in Southern Africa to almost 50 t/ha in the USA and Europe (FAO, 2020). Integrated pest and pathogen control strategies are needed to ensure food security and sustainability. This is particularly relevant in terms of plant-parasitic nematodes because of the poor efficacy and restrictions placed on the use of fumigant and non-fumigant nematicides in many countries as a result of their detrimental environmental and health effects.

Potato Cyst Nematodes, notably the two main species *G. pallida* and *G. rostochiensis*, are economically important potato pests that damage the crop by the causing poor growth, wilting during periods of water stress and early senescence. They are responsible for an estimated overall crop loss of 9% per year (Turner and Subbotin, 2013). These nematodes can lead to more than 80% yield loss in highly infested areas (SASA, 2022).

This work focuses on the PCN species *G. pallida* and is split into two sections. The first part investigates different British *G. pallida* populations (Chapters 2 and 3). The second part studies *G. pallida* resistance from the wild potato species, *S. spegazzinii* accession 7195, and maps and introgresses this resistance into tetraploid potato cultivars (Chapters 4 and 5).

6.2.1 Characterisation of British *G. pallida* populations

Understanding the behaviour of PCN field populations is important for potato breeders and growers to select appropriate pest management methods including the planting of resistant potato cultivars. An understanding of the genetic composition and complexity of these populations is also needed to indicate the direction of future research. A survey of PCN in English and Welsh fields by Minnis et al. (2002) concluded that approximately 8% of assessed fields were infested with *G. rostochiensis*, 67% with *G. pallida* and 25% contained both species. A more recent survey by Dybal (2018) showed

a shift in favour of *G. pallida* with 89% of infested fields having only this species. Eves-van den Akker et al. (2015b) performed a study on the DNA extracted from Scottish *G. pallida* field populations. They showed by analysing a combination of five SNPs in the mitochondrial *cytb* gene that complex mixtures of *G. pallida* could be present in fields, with some individual fields containing all three of the known introductions from South America to the UK. Later, Dybal (2018) looked at the composition of 93 English *G. pallida* populations and found only two of the three introductions using the same method. In the work presented in this study, British *G. pallida* populations were investigated for their composition and level of virulence on selected potato genotypes with different sources of resistance.

6.2.1.1 Are different mitotypes of G. pallida populations useful as virulence markers?

Recently collected *G. pallida* populations from England, Northern Ireland and Scotland, as well as maintained populations from the JHI collection of cysts taken some 50 years ago from England and Scotland, were characterised and compared to each other in respect to their mitotypes, virulence on six potato genotypes and cyst size after growing on those potato genotypes. The JHI has maintained *G. pallida* populations Lindley, Luffness and Pa1, which correspond to three distinct introductions. These populations show significant differences in virulence against various resistance sources and have been classified as different pathotypes (Kort et al., 1977). Previous work by Gartner, Mróz and Blok (personal communication) has shown that, over time, the *G. pallida* population in fields has become more complex (Table 2-3). This observation raised the question whether fields in the UK had suffered multiple introductions of *G. pallida,* leading to mixtures of pathotypes according to the correlation found in collection *G. pallida* populations? One approach to answer this question was to generate “single cyst” lines derived from selected British *G. pallida* populations by picking one individual cyst and multiplying it, thereby going through a genetic bottleneck for one generation. In order to get enough cysts to perform all experiments, 10 cysts of the “single cyst” lines were pooled and multiplied on the susceptible potato cultivar Desirée.

Firstly, a different mitochondrial marker, the TRFLP marker s222 (Grujic, 2010), was introduced to test if it can replace the polymorphic marker *cytb*. The advantage of the

TRFLP-assays is that they are semi high throughput and can be setup in 96-well plates. Furthermore, the outputs of the analysis are coloured chromatograms, which can be easily analysed visually. Therefore, TRFLP s222 would be useful for routine testing of *G. pallida* populations at low cost *e.g.* in institutions like SASA in Edinburgh, where each year thousands of DNA samples from PCN are extracted and analysed. Both mitochondrial markers, *cytb* and s222, show similar results; therefore, s222 can be used as a surrogate marker for *cytb* as a mitochondrial introduction marker.

In the three historical *G. pallida* populations Lindley JHI, Luffness JHI f1 and Pa1 JHI one mitotype dominates with at least 85% occurrence at the individual cyst level of mitotype A, B and C, respectively. The more recently collected field populations Luffness f1 and HA show all three mitotypes A, B and C at the individual cyst level. However, no new TRFLP pattern or different fragment lengths could be detected. When mitotyping single cyst lines, only one mitotype per single cyst line is expected, as mitochondrial DNA is maternally inherited, and all nematodes in one cyst share the same mother. Paternal leakage has been reported in *G. pallida*, although it is a rare event (Hoolahan et al., 2011). All "single cyst" lines except the HA lines showed one mitotype (at least 90% frequency). However, the "single cyst" lines derived from Pa1 show mitotype A and not C, which is the characteristic mitotype for the Pa1 JHI populations. Five of the seven tested HA "single cyst" lines show a mixture of two or three mitotypes with equal frequency in the TRFLP and *cytb* assays and the highest number of discrepancies between the two methods were seen in this cell line. It was not possible within the scope of this work to identify the reason for this unexpected behaviour of HA-derived "single cyst" lines. Firstly, the mitotyping with *cytb* and s222 would need to be repeated on a cyst level to make sure that this result is not an artefact. Further, mitotyping the individual nematodes in a cyst might be useful.

The assessment of virulence in pot-based bio-assays of the "single cyst" lines showed that Pa1 JHI derived lines had the same pathotype as the original Pa1 population. However, the mitotypes of the "single cyst" lines did not match the one determined for the population. In the case of population Lindley JHI, the mitotypes were the same in both "single cyst" lines. However, the virulence of the assessed potato genotypes looked more like the virulence of population Luffness JHI f1. "Single cyst" lines derived from Luffness f1 shared the same virulence pattern (Pa3) and mitotype (B) as Luffness

JHI field 1. “Single cyst” lines derived from populations Luffness f3 and AB had mitotype A and pathotype Pa2. “Single cyst” lines derived from population HA showed both pathotypes Pa2 and Pa3.

The conclusion from these results is that the mitotype of cysts of a population is not usable as a reliable pathotype marker. However, mitotype B was found to correlate with pathotype Pa3. Mitotype A is not indicative for any pathotype, and for mitotype C there were too few samples to draw any reliable conclusions. The different *G. pallida* introductions with distinct mitotypes and pathotypes were geographically isolated in South America, therefore no hybridisation could occur. However, once they were all brought into the UK, there is no reason why these populations should not mate.

When determining the virulence of the “single cyst” lines, cyst diameters were measured as a proxy for egg numbers, and these data were used to determine if there is a correlation between resistance of potato cultivars and cyst size as a by-product. The data clearly show that cysts grown on potato genotypes that are (partially) resistant are up to 30% smaller in diameter than those growing on susceptible potato cultivars. This decrease corresponds to a reduction in volume of the cyst by 66% and is used as a proxy for egg numbers. So, generally, not only is the number of cysts reduced, but often the size and the quality of cysts is also impacted. The cyst size together with the cyst number can be used as an indicator for the resistance of a potato cultivar, but it is not a reliable marker of pathotype.

For future work, the focus should be to identify genomic virulence markers and therefore investigating the relationships between *G. pallida* populations will be important.

6.2.1.2 Determination of the relationships between different British G. pallida populations using gDNA analysis

In total, 36 British *G. pallida* populations and derived “single cyst” lines underwent Pool-Seq analysis, which resulted in the determination of allele frequencies from 1505 loci throughout the genome. These frequencies were used to generate phylogenetic trees with and without the “single cyst” lines (Figures 3-1 and 3-2). Luffness f1 is clearly a different group from the other populations, they are also more virulent against tested potato cultivars than other populations, apart from HA. Populations with the

pathotype Pa1 also form a cluster, though this is not as strongly supported by bootstrap values. This observation confirms the theory that Pa1 is one genotype in contrast to Pa2 and Pa3 (Trudgill, 1985). All other populations form a separate cluster that can be split in two subgroups, one containing population HA and the other containing all other assessed PCN populations.

These 1505 loci were also screened for potential SNP candidates that can be used to distinguish between phylogenetic groups and/or pathotypes. In total, 15 candidate SNPs that were indicative for one of the three phylogenetic groups were identified, and 3 of them, indicative for Pa1, were PCR amplified and Sanger sequenced. Following this further analysis, one SNP candidate with the chosen conditions and primers had the potential to be developed into a genomic pathotype marker. Future work would include assessing this locus to determine if it can reliably distinguish between pathotype Pa1 and the others, using as many different *G. pallida* populations as possible and, in particular, populations not used in this study. If that assessment is positive, a KASP assay could be designed that can discriminate pathotype Pa1 from all others. The other 12 candidate SNPs that are indicative for the other two phylogenetic groups could also be assessed to potentially identify more genomic markers.

Since 1505 loci with different allele frequencies throughout the genome is quite a low number, different methods for analysing the Pool-Seq data have been performed by the group of Benjamin Mimee at Saint-Jean-sur-Richelieu Research and Development Centre, Saint-Jean-sur-Richelieu, Québec, Canada to increase the number of informative loci. One recent analysis provided 3945 loci with different allele frequencies throughout all *G. pallida* populations, which allows filtering for further SNP candidates that could be used as genomic pathotype markers. In addition, a newly assembled genome for *G. pallida* (Thorpe et al., unpublished, paper in preparation), will provide information about the presence of possible effector genes in the proximity of these loci and if they differ in the phylogenetic groups, as well as if the candidate SNPs are close to each other.

Population HA and the “single cyst” lines derived from this are distinct from the other investigated populations/“single cyst” lines, not only in respect to the phylogenetic tree. Having up to three mitotypes in individual cysts of a “single cyst” line could indicate a new introduction, or that population HA is a hybrid of all three introductions

with all mitotypes in individual nematodes on different scmtDNA circles, which might be due to increased paternal leakage in the HA population. Depending on the number of scmtDNA circles, which can vary between individuals (and therefore also on a cyst level), the PCR primers resolve (mainly) one locus of the amplified *cytb* and s222 fragments. Therefore, the alternative PCR products could be below the detection limit. Population HA should be monitored and further characterised.

6.2.2 Exploitation of naturally *G. pallida* resistant potato wild species

The use of plant cultivars that are resistant to pathogens is a very effective and sustainable way to manage plant diseases and pests. For managing PCN, ideally as part of IPM, the application of resistant (and ideally tolerant) potato varieties is widely used. Many wild potato species and landraces, often native to Mexico and the Andean Highlands, possess resistances to pathogens, including PCN. The most commonly cultivated potato species in Europe, *Solanum tuberosum* ssp. *tuberosum,* originally lacked this genetic diversity and consequently did not show resistance to pathogens. This was demonstrated most poignantly during the potato famine in the middle of the 19th century, and as a consequence of this, potato breeding for resistance gained in importance.

One notable success from a resistance breeding programme is the continued control of *G. rostochiensis* in the UK through the deployment of the resistance gene *H1*. The *H1* resistance has been used in the UK for more than 50 years, yet it has not been overcome to date. This may be due to the very restricted genetic diversity of the original introduction of *G. rostochiensis* to Europe. Although the resistance *H1* is effective in reducing populations of *G. rostochiensis* (Ro1), it does not affect the multiplication of *G. pallida* populations. Therefore *G. pallida* populations could multiply with no restriction and are now the predominant species in the UK. *G. pallida* has a higher genetic variability than *G. rostochiensis* outside South America, and therefore is likely to be more challenging to control with a single natural resistance gene. It is therefore important to identify different sources of *G. pallida* resistance and to introgress them into breeding clones. These different resistance conferring genes can ideally be 'pyramided' to ensure broad and durable resistance to *G. pallida.*

In the second part of this study, one *G. pallida* resistance from the wild potato species, *S. spegazzinii* accession 7195, was mapped and introgressed into tetraploid potato cultivars (Chapters 4 and 5). This resistance to *G. pallida* showed to be effective against all three pathotypes occurring in Europe, and it looks like a new resistance locus, as no resistance for *G. pallida* has been described yet on chromosome VI. It is not known yet if this resistance is based on *R*-gene(s) or not. Therefore, the resistance discovered in the present work is a good candidate for breeding programs. For *G. pallida*, all so far described resistance loci are *R*-genes. Non-*R*-gene resistance genes against CN were described in *H. glycines* (Section 1.4.1.6).

6.2.2.1 Mapping a G. pallida resistance locus derived from S. spegazzinii accession 7195

As the biggest group of known genes/loci conferring resistance to pathogens are *R*-genes (Section 1.4.1), I used RenSeq to map the source of *G. pallida* resistance present in *S. spegazzinii*. The informative SNPs that were determined were clustered on chromosome XI in DM. The fact that some KASP assays with informative SNP candidates were only able to distinguish between the parental alleles to a variable extent indicated that the assays were not reliable and possibly PCR products from additional *R*-genes at different locations were being amplified. As a consequence, the KASP assays with the progeny clones from 13.A.02 were not interpretable and the approach was disregarded. The GenSeq approach, on the other hand, showed a clear correlation between being resistant and heterozygous in the informative SNPs that were used to design KASP markers on chromosome VI. With a combination of informative SNPs, obtained by GenSeq and GBS, the resistance locus could be narrowed down to a 118 kb region, containing 14 high-confidence gene models in the reference genome of DM (v6.01). So far, there is no indication that *R*-genes are present in this region. However, there is a possibility that in *S. spegazzinii*, but not in DM, a gene conversion event or translocation of *R*-genes has occurred, which is not uncommon and has been shown for example for *Mi-1* gene homologues in *Solanum* species (Sanchez-Puerta and Masuelli, 2011). If this were the case, the distance of the flanking markers would be larger than in DM, and it would explain why the RenSeq approach did not provide informative SNPs that were transferrable into robust KASP makers. The ultimate aim would be to identify and clone the gene(s) conferring

resistance. This could be achieved by either generating and sequencing a BAC library, by genome walking or by CRISPR-Cas9 targeted excision of genomic DNA followed by Nanopore-based sequencing (López-Girona et al., 2020). Once resistance genes have been identified, their mechanism of action can be determined. Further work would include the identification of the effector(s) that interact with the resistance protein.

This work clearly shows the importance of using a good reference genome when using RenSeq, as R-genes are known to duplicate and move within the genome. It would be best to use the parental parent 03.F.3a (35) as reference genome for the RenSeq analysis for population 13.A.02, as the used reference genome DM is not a close relative *to S. spegazzinii*.

6.2.2.2 Introgression of the resistance into tetraploid potato cultivars

Knowing the genetic location of resistance traits is not absolutely necessary to produce resistant plants by breeding. However, the identification of molecular markers linked to that resistance accelerates the breeding process. In addition, pyramiding different resistance genes to one pathogen is greatly accelerated with MAS and does not solely rely on using differential pathogen populations. Crosses between different maternal tetraploid potato genotypes and two diploid clones carrying the resistance were performed, and one, Alouette (a *G. pallida* susceptible potato cultivar) x 13.A.02(463), termed 18.UG1, produced two viable progeny clones. Three KASP markers (Figure 5-6) were applied and showed the same pattern as the maternal parent in both progeny clones studied. This can mean no introgression or the occurrence of a recombination event outside the region of the markers. A root trainer bio-assay revealed that the progeny clones 18.UG1(1) and 18.UG1(3) had the resistance introgressed, and this resulted in a reduction of female nematodes by 23- and 8-fold, respectively (Figure 5-8). There was a 37-fold reduction of female nematodes in the paternal parent 13.A.02(463) compared with the maternal parent DB337(37). This difference in reduction could be explained by gene dosage effects. In the diploid plants, the resistance gene is heterozygous with an occurrence of 50%, whereas in the tetraploid plants, the resistance gene is only present in a single dose (*i.e.*, 25%).

The progeny plants from the other crosses, which were provided by JHL, all had a *G. pallida* resistance in the maternal parent. According to the markers (Figure 5-6) all

progeny clones had the resistance introgressed. A root trainer bio-assay showed a significant reduction of female nematodes compared to the maternal parent in only one progeny clone of one cross with a maternal parent already having a source of resistance to *G. pallida*. It should be noted that the sample size is low. In this case a more sensitive method, like a pot bio-assay, should be applied in many replicates to obtain results with higher confidence. That is especially true where Innovator is the maternal parent, as the Innovator resistance to *G. pallida* is very strong, with an average number of female nematodes usually very low (<2) in a root trainer bio-assay.

In chapter 5, Figure 5-5, it was shown that clone 09.A03(6), which was used as paternal parent in some crosses, does not have the resistance gene mapped in Chapter 4; the clone shows reduced susceptibility which could be a different, minor resistance gene or susceptibility gene with an allele that confers reduced susceptibility. However, a cross between Vales Everest and 09.A03(6) shows fewer female nematodes than in the maternal parent Vales Everest, and in one case significantly fewer. This result indicates that the two resistance-conferring loci from the two parents act in different pathways and are therefore additive. Thus, it would be interesting to map this resistance-conferring locus and to find markers for this resistance. It is not surprising that another minor resistance locus was detected in *S. spegazzinii* accession 7195. Kreike et al. (1994) describe the major *G. pallida* resistance on chromosome 5, and minor resistance conferring loci on chromosome 4 and 7 in a different *S. spegazzinii* accession than used in this study. Caromel et al. (2003) describes QTLs for *G. pallida* resistance on chromosomes V, VI and XII.

S. spegazzinii was also described as a source of resistance to *G. rostochiensis* (Kreike et al. (1993), reviewed in Dalamu et al. (2012)). *S. spegazzinii* accession 7195 also confers resistance to *G. rostochiensis* Ro1, as shown in Figure 4-12. This resistance is different from the mapped resistance on chromosome VI, and it is not based on the presence of *H1.* Further studies of the *G. rostochiensis* resistance (RenSeq) are planned.

In summary, I have investigated the mitotypes and pathotypes of different British *G. pallida* populations and mapped *G. pallida* resistance in a wild potato species, *S. spegazzinii* accession 7195. Specific results of note include the following:

- I identified one SNP candidate that has the potential to be developed into a genomic pathotype marker to distinguish pathotype Pa1 from the others. This would be the first diagnostic molecular pathotype marker for *G. pallida* and would be of big value to simplify the determination of pathotypes of *G. pallida* populations. Both cyst size and mitochondrial introduction markers are not usable as reliable pathotype markers.
- Population HA was found to be a distinct phylogenetic lineage. Further analysis suggested that it may be a new introduction or a hybrid of the three known introductions to the UK. This is an exciting result as in either case it is important to monitor and further analyse this population (e.g., more virulence assays for South American pathotypes, investigating the genetic homogeneity of single nematodes within a cyst) in order to be able to manage this *G. pallida* population.
- One *G. pallida* resistance from the wild potato species *S. spegazzinii* accession 7195 was mapped using SNPs derived from GBS and GenSeq analyses, and the locus narrowed down to a 118 kb region on the reference DM. Further analysis is needed to determine whether an *R*-gene translocation/duplication event has occurred in *S. spegazzinii* or whether this dominant resistance is *R*-gene independent. If the resistance turns out to not be based on an R-gene, this would be the first example for *G. pallida*. The discrepancy of the results of RenSeg and GenSeq shows the importance of using a good reference genome and careful design of KASP assays.
- This resistance was successfully introgressed into a tetraploid potato cultivar and was found to confer resistance in a dosage dependant manner. This is very good news for potato breeders, as this is a broad-spectrum resistance to *G. pallida*.
- Some of the KASP markers that were used to map the resistance locus were found to have value as markers for resistance in other potato genetic backgrounds than *S. spegazzinii* and Mayan Gold. However, they are not ideal, as it would be useful to have at least two markers that are on both sides of the resistance locus in as many different genetic background as possible to be of

general use in MAS introgression of this new resistance into tetraploid potato breeding clones.

7 References

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