Genetic analysis of resistance of *Solanum tuberosum* L. to potato wart disease

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Abstract

The obligate biotrophic soil-borne fungus *Synchytrium endobioticum* is the causal agent of potato wart disease and is classified as a quarantine pest by the European and Mediterranean Plant Protection Organization. It produces sporangia with several hundred motile zoospores which infect meristematic tissue of below-ground parts of the plants, like tubers, stolons and stems of the potato, causing yield losses of up to 50-100 %. Typical symptoms are the formation of cauliflower-like irregular galls on the below-ground parts of the plant. The thick-walled resting sporangia are able to survive in the soil for several decades. More than 40 different pathotypes of *S. endobioticum* have been described. Pathotypes 1, 2, 6 and 18 are the most common and most aggressive forms of the fungus in Europe. Chemical control is difficult because accessibility of winter sporangia is limited and chemicals cannot penetrate the thick walls of the sporangium. Chemicals reported to be effective against *S. endobioticum* have also been described as phytotoxic and harmful to the environment. Strict phytosanitary measures and breeding of resistant potato cultivars are currently the methods of choice to control the disease. Another difficulty is the phenotypic resistance assessment of potato cultivars. To determine the resistance approximately 20 tubers have to be inoculated per pathotype and genotype, which are then evaluated microscopically and assigned to different resistance classes, ranging from extremely resistant to extremely susceptible. These amounts of tubers become available only after several years within the breeding process. Molecular markers that could detect resistant genotypes early in the breeding process, independently of the availability of tubers, would greatly facilitate breeding of potato wart resistant cultivars. Several resistance loci have been identified on different potato chromosomes with different resistance loci dependent on the genetic background of the used plant material. One major resistance locus has been identified in almost all genetic studies on wart resistance: the *Sen1* locus on chromosome 11. In this study, we generated a dihaploid potato population derived from a resistant tetraploid cultivar by a so-called prickle pollination with a wild potato species, *Solanum phureja*, which is known to possess high dihaploid induction ability, to analyze resistance against potato wart disease by reducing the genetic complexity implemented when working with tetraploid potato with its tetrasomic inheritance. Using genotyping data of a 12.8 k SNP array we could show that genetic analysis in dihaploids is much easier showing simpler segregation ratios in the progeny. Simultaneously the number of simplex markers is increased when compared to conventional crosses between two tetraploid genotypes. Putative introgressions of the pollinator genome in the dihaploid progeny were present in almost all genotypes and on almost all chromosomes. However, introgressions occurred as single events and did not disturb genetic analysis of the dihaploid genotypes. SNP marker data was used to generate 45 linkage maps, representing almost all of the 48 potato chromosomes. QTL mapping was performed for different phenotypic traits such as shoot length, number of nodes, number of tubers and tuber weight. QTL mapping revealed new quantitative trait loci but also confirmed already known QTLs described in the literature. Tubers of the dihaploid genotypes were tested for resistances to *S. endobioticum* pathotypes 6 and 18. Qualitative resistance mapping positioned the major resistance locus for both pathotypes on chromosome 11 in the *Sen1* region. The development of additional molecular markers further improved the mapping resolution, narrowing the resistance locus to less than 800 kilobase pairs. Eight molecular markers were segregating without recombination to resistance in our population. Two markers showed high diagnostic values in a small association panel, consisting of 50 German and Polish potato varieties. The markers were diagnostic in 89.5 % of the cultivars for resistance to pathotype 18 and in 86.6 % of the cultivars for resistance for pathotype 6. The markers represent the first diagnostic markers for the pathotypes 18 and 6. Sequencing of different pathotypes of *S. endobioticum* was performed to develop molecular markers to allow differentiation of the pathotypes. However, sequencing revealed only a very low polymorphism rate between the pathotypes. Markers developed in this study, allowed the distinction between pathotypes 1, 2 and 6 and pathotypes 8 and 18.

Keywords: dihaploid, *Synchytrium endobioticum*, molecular markers

Zusammenfassung

Der obligat biotrophe, bodenbürtige Pilz *Synchytrium endobioticum* ist der Verursacher des Kartoffelkrebses und wird von der „European and Mediterranean Plant Protection Organization“ als Quarantäneerreger eingestuft. Der Pilz bildet Sporangien mit mehreren hundert Zoosporen aus, die meristematisches Gewebe unterirdischer Pflanzenorgane, wie Knollen, Stolone und Sprosse, infizieren und Ernteverluste von bis zu 50-100 % verursachen. Typische Symptome sind tumorartige Auswüchse auf den unterirdischen Pflanzenorganen. Die dickwandigen Wintersporangien sind in der Lage für mehrere Jahrzehnte im Erdboden zu überleben. Mehr als 40 verschiedene Pathotypen von *S. endobioticum* wurden beschrieben. Die Pathotypen 1, 2, 6 und 18 sind die in Europa häufigsten und die aggressivsten Formen des Pilzes. Eine chemische Bekämpfung ist schwierig, da die Wintersporangien schlecht zu erreichen sind und die Chemikalien die Sporangienwand nicht durchdringen können. Chemikalien, die effektiv gegen *S. endobioticum* sind, sind phytotoxisch und umweltschädlich. Strenge phytosanitäre Maßnahmen und die Züchtung resistenter Kartoffelsorten sind zurzeit die Methoden der Wahl, um die Krankheit zu bekämpfen. Eine weitere Schwierigkeit stellt die phänotypische Resistenzprüfung von Kartoffelsorten dar. Zur Bestimmung der Resistenz müssen ungefähr 20 Knollen pro Pathotyp und Genotyp inokuliert werden, die anschließend mikroskopisch evaluiert und den verschieden Resistenzklassen, von extrem resistent bis extrem anfällig, zugeordnet werden. Diese Knollenmengen stehen erst nach mehreren Jahren im Züchtungsprozess zur Verfügung. Molekulare Marker, die resistente Genotypen frühzeitig und unabhängig von der Knollenverfügbarkeit identifizieren könnten, würden die Züchtung Kartoffelkrebs-resistenter Sorten erleichtern. Mehrere Resistenzloci wurden auf verschiedenen Kartoffelchromosomen identifiziert. Abhängig von dem genetischen Hintergrund des verwendeten Pflanzenmaterials unterschieden sich die Resistenzloci. Ein Hauptresistenzlokus wurde in nahezu allen Kartoffelkrebsstudien identifiziert: der *Sen1*-Lokus auf Chromosom 11. In der vorliegenden Arbeit wurde durch eine so genannte „Prickle Pollination“ mit *Solanum phureja*, die eine hohe Dihaploiden-Induzierbarkeit besitzt, eine dihaploide Kartoffelpopulation aus einer resistenten tetraploiden Sorte generiert, um die Kartoffelkrebsresistenz zu analysieren und die genetische Komplexität zu reduzieren, die mit Analysen in der tetraploiden Kartoffel und ihrer tetrasomalen Vererbung einhergeht. Mit den Genotypisierungsdaten eines 12.8 k SNP Arrays konnte gezeigt werden, dass genetische Analysen in dihaploiden Genotypen mit vereinfachten Spaltungsverhältnissen in einer dihaploiden Nachkommenschaft einfacher ist und parallel die Anzahl an Simplex-Markern im Vergleich zu konventionellen Kreuzungen zwischen zwei tetraploiden Genotypen erhöht ist. Vereinzelte Introgressionen des Bestäubergenoms in der dihaploiden Nachkommenschaft konnten zwar in allen Genotypen und auf allen Chromosomen festgestellt werden, störten die genetischen Analysen in den dihaploiden Genotypen aber nicht. Die SNP-Markerdaten wurden zur Erstellung von 45 genetischen Kopplungskarten genutzt, die nahezu alle 48 Kartoffelchromosomen repräsentieren. Eine QTL-Kartierung wurde für die phänotypischen Merkmale Sprosslänge, Anzahl der Nodien, Anzahl der Knollen und Knollengewicht durchgeführt, die sowohl neue als auch bereits bekannte QTLs detektierte. Die Knollen der dihaploiden Genotypen wurden für Resistenztests für die *S. endobioticum* Pathotypen 6 und 18 verwendet. Durch eine qualitative Resistenzkartierung konnte der Hauptresistenzlokus für beide Pathotypen in der *Sen1*-Region auf Chromosom 11 lokalisiert werden. Durch zusätzlich entwickelte molekulare Marker konnte die Kartierungsauflösung verbessert und das Resistenzintervall auf weniger als 800 Kilobasenpaare eingegrenzt werden. Acht molekulare Marker spalteten ohne Rekombination zur Resistenz in der Population. Zwei Marker zeigten einen hohen diagnostischen Wert in einem Assoziationssortiment, das aus 50 Kartoffelsorten bestand. Die Marker waren in 89,5 % der Sorten diagnostisch für die Resistenz gegen Pathotyp 18 und in 86,6 % der Sorten für die Resistenz gegen Pathotyp 6 und stellen die ersten diagnostischen Marker für diese Pathotypen dar. Eine Sequenzierung verschiedener Pathotypen wurde durchgeführt, um molekulare Marker zur Pathotypenunterscheidung zu entwickeln, jedoch konnte nur eine sehr geringe Polymorphismusrate zwischen den Pathotypen festgestellt werden. Die entwickelten Marker ermöglichen eine Unterscheidung der Pathotypen 1, 2 und 6 von den Pathotypen 8 und 18.

Schlagwörter: dihaploid, *Synchytrium endobioticum*, molekulare Marker

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Abbreviations

°C	degree Celsius
°N	latitude North
°S	latitude South
µm	micrometer
AFLP	amplified fragment length polymorphism
bp	base pair
CC	coiled-coil
cm	centimeter
CNL	coiled-coil NB-LRR
CRISPR/Cas	clustered regularly interspaced short palindromic repeats/ CRISPR associated protein
DiRK	„Diagnostik für eine Ressourcen-schonende Kartoffelstärke-Produktion“
DNA	deoxyribonucleic acid
DVL	Descriptive Variety List
EPPO	European and Mediterranean Plant Protection Organization
ETI	effector-triggered immunity
F1	first filial generation
FAO	Food and Agriculture Organization of the United Nations
g	grams
GBS	genotyping-by-sequencing
k	kilo, thousand
kbp	kilobase pairs
kg	kilograms
LD	linkage disequilibrium
LRR	leucine-rich repeat
m	meter
MAMP	microbial-associated molecular pattern
Mbp	Megabase pairs
mm	millimeter
NB	nucleotide-binding
NB-LRRs	nucleotide-binding leucine-rich repeats

NLR	NB-LRR-receptor
P1	pathotype 1
P18	pathotype 18
P2	pathotype 2
P6	pathotype 6
PAMP	pathogen-associated molecular pattern
PGSC	Potato Genome Sequencing Consortium
PLRV	*Potato leafroll virus*
PMTV	*Potato mop-top virus*
PRR	pattern recognition receptor
PTI	PAMP-triggered immunity
PVA	*Potato virus A*
PVM	*Potato virus M*
PVS	*Potato virus S*
PVX	*Potato virus X*
PVY	*Potato virus Y*
QTL	quantitative trait locus
R genes	resistance genes
RAD	restriction-site associated DNA
RFLP	restriction fragment length polymorphism
RGA	resistance gene analog
RNA	ribonucleic acid
RSe	Resistance loci for *Synchytrium endobioticum*
S. endobioticum	*Synchytrium endobioticum*
S. phureja	*Solanum phureja*
S. tuberosum	*Solanum tuberosum*
SNP	single nucleotide polymorphism
SSR	simple sequence repeat
TALEN	transcription activator-like effector nuclease
T-DNA	transfer DNA
TGC	Tomato Genome Consortium
TIR	toll interleukin-1 receptor
TNL	toll interleukin-1 receptor NB-LRR

1. General Introduction

1.1. Potato – a major staple crop

The potato originated in the Andean regions of South America in Peru and Bolivia (Bradshaw, 2009) and today 188 wild tuber-bearing species of potato are described (Spooner & Salas, 2006) with ploidy levels ranging from diploid (2*n* = 2*x* = 24) to hexaploid (2*n* = 6*x* = 72). Potato domestication from these wild species included selection for many phenotypic traits regarding tuber characteristics as well as foliage traits and a single domestication event was proposed that led to the cultivated potatoes known today (Spooner et al., 2005). In the 16th century the potato was introduced to Europe and subsequently to the rest of the world in the 17th century (Pandey & Kaushik, 2003). Today, the potato is grown in 149 countries over a wide range of latitudes from 65 °N to 50 °S and at altitudes from sea level to 4,000 m (Hijmans, 2001). It grows under a wide range of environmental conditions and shows a wide ecological adaptability (Camadro et al., 2004). Potato production is limited by high temperatures and therefore the production area is highly skewed towards the northern hemisphere, especially to the temperate zones of Europe (Hijmans, 2001).

1.1.1. *Solanum tuberosum* L. – taxonomy, genetics and general botany

The cultivated potato *Solanum tuberosum* L. belongs in the order Solanales and the suborder Solanineae to the Solanaceae family which comprises 3000-4000 species in approximately 90 genera (Gebhardt, 2016). The night shade family is highly diverse and contains many important crop plants like tomato, bell pepper and eggplant. Potato is an autotetraploid species with 48 chromosomes (2n = 4x = 48) and displays tetrasomic inheritance. The plant genome is medium-sized with a haploid genome size of approximately 840 Megabase pairs (Bradshaw, 2007a). It is an annual plant with short, erect, and branched stems. Leaves are compound and can be 30 to 60 cm long, with a terminal leaflet (Bradshaw, 2009). Potato flowers are hypogynous and actinomorphic and have fused, five-lobed corollas (Bradeen & Haynes, 2011). The flower color can vary from white to deep purple. Potato sets true seed in berries following natural pollination by insects, mainly bumble bees. Outcrossing is enforced in cultivated diploid species by a gametophytic self-incompatibility system. Most potato cultivars are clonally propagated through seed tubers, which allows multiplication of genetically uniform seedlings (Bradshaw et al., 2006).

1.1.2. Economic importance of the cultivated potato

Potato belongs to the most important food crops worldwide after maize, wheat and rice (Bradshaw, 2007a) with the highest nutritional productivity with 5,600 produced calories per cubic meter of water applied in cultivation, compared to 3,860 in maize, 2,300 in wheat and 2,000 calories in rice (FAO, 2008). In 2016, approximately 376 million tons were produced on an area of more than 19.2 million hectares all over the world (FAO, 2018). The most important countries for potato production are China, India, Russia, Ukraine and the United States of America as well as Germany (FAO, 2018). Potatoes are rich in vitamin C and contain high levels of potassium and dietary fibres (Mullins et al., 2006). They are consumed directly as well as in convenience food, such as potato chips and fries and are an important nutritional compound more dominantly in developed than in developing countries (Burlingame et al., 2009). Potato production is also important for industrial applications where potato starch is increasingly being used as a renewable raw material in paper and corrugating production, for chemicals and fermentation and in binders, adhesives and detergents. The use of potato starch in the biopharmaceutical industry and the cosmetic sector is increasing as well (Röper, 2002; Jobling, 2004).

1.1.3. Potato breeding

Breeding new potato cultivars with desired traits is time-consuming and challenging, as up to 40 traits regarding tuber quality and tolerances to biotic and abiotic stresses have to be considered and phenotyped in large populations over several years (Slater et al., 2014). Traditional potato breeding is carried out at the tetraploid level between cultivars, and is mainly executed by recurrent phenotypic selection of favorable traits. However, many unfavorable alleles can stay unnoticed in the tetraploid genome, which is why large populations of about 100,000 seedlings are needed to generate a new variety with desired traits (Lindhout et al., 2011). Crossing parents are typically selected on complementary phenotypic traits and generally are high-performing cultivars or breeding lines (Slater et al., 2014). Resulting populations are evaluated over a number of years and under different environmental conditions to identify superior genotypes based on their phenotype, which can take up to ten years (Jansky, 2009). A breeding scheme for conventional potato breeding is given in Table 1.

Table 1: Conventional potato breeding scheme with phenotypic selection of quality traits (modified from Becker, 2011). The number of genotypes and plants per genotype are listed for the respective years during the breeding process, as well as examples for relevant traits for selection. Genotype numbers are reduced progressively, while concurrently increasing the number of plants of potentially superior genotypes. The number of traits to be considered increases during the breeding process.

Year	Number of genotypes	Plants per genotype	Traits for selection
1	Crossings		
2	Seedling generation		
3	100,000	1	tuber shape, flesh color, eye depth
4	5,000	10	tuber shape, eye depth, starch content, maturity, raw flesh darkening, processing ability (chips)
5	500	25	processing ability (French fries), cooking properties, after-cooking darkening, taste
6	100	100	maturity, raw flesh darkening, alkaloid content, processing quality (chips and French fries), cooking properties, after-cooking darkening, taste, sugar content
7	10	2000	maturity, raw flesh darkening, alkaloid content, processing ability (chips and French fries), cooking properties, after-cooking darkening, taste, sugar content, storing abilities, resistances
8	0-5	Variety testing	

As the genome complexity in the autotetraploid species with its highly heterozygous constitution and tetrasomic inheritance makes analyses difficult, an alternative strategy for potato breeding is via the generation of dihaploids from a tetraploid cultivar. Breeding is then carried out at the diploid level, where dihaploids are crossed with diploid wild potato species, taking advantage of the disomic inheritance of useful resistance genes that can be introgressed from the wild potato species. However, most diploid species are self-incompatible and show severe inbreeding depression. To restore the tetraploid level, the resulting diploid hybrids need to be polyploidized, which is usually achieved through unilateral sexual polyploidizations with tetraploid potato by means of 4*x* x 2*x* crosses (Jansky, 2009).

1.1.3.1. Induction of dihaploid potatoes

To generate dihaploid potatoes from a tetraploid cultivar different techniques can be applied. An androgenic approach is via anther culture which has been applied in *S. tuberosum* as well as in other potato species (Irikura, 1975; Uhrig & Salamini, 1987; Rokka et al., 1996). However, the genotype has a strong influence on the success of the regeneration (Jacobsen & Sopory, 1978; Rokka et al., 1996).

A parthenogenic approach for the generation of dihaploids is through pollinations with specific clones of the diploid wild potato species *Solanum phureja*. In this so-called "prickle pollination" the development of dihaploid seed is induced (Hougas & Peloquin, 1957; Hutten et al., 1994). The most common inducer clones are IVP101, IVP35 and IVP48, with IVP101 showing the highest induction ability when compared with the other inducer clones (Hutten et al., 1994). The availability of a dominant seed-marker in the haploid inducer genotypes, the so-called "embryo spot", facilitates the identification of true dihaploid progeny, because undesirable hybrids within the progeny inherit an anthocyanin pigmentation which is visible at the base of the cotyledons of the embryo and also as nodal bands in plant seedlings (Peloquin & Hougas, 1959; Hermsen & Verdenius, 1973). The embryo-spot is visible through the seed-coat on both sides of the seeds, which produce plants with purple anthocyanin pigmentation in the nodes of seedlings (Figure 1).

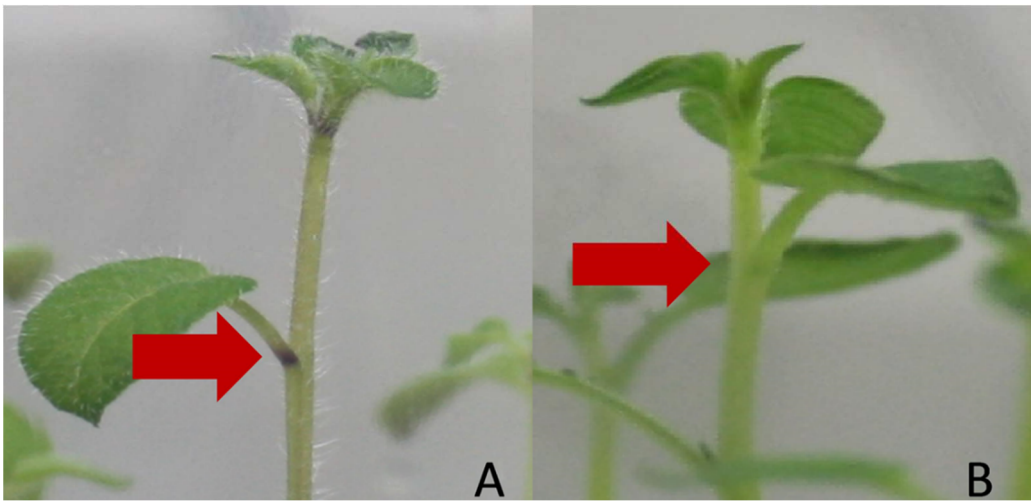

Figure 1: Plant seedlings derived from a cross with the dihaploid inducer *Solanum phureja* clone IVP 101. Hybrids of crosses with specific *S. phureja* clones show an anthocyanin pigmentation in the nodes of the seedlings (A), whereas true dihaploid genotypes do not show any anthocyanin pigmentation (B).

Different cytogenetic mechanisms underlying the dihaploid formation of crosses with *S. phureja* pollinators have been suggested. Hermsen and Verdenius (1973) suggested that *S. phureja* pollen triggers the development of unfertilized egg cells into embryos without making any genetic contribution to the dihaploid progeny, while Clulow et al. (1991) proposed that *S. phureja* chromosomes are eliminated after the fertilization of egg cells during cell division and that progeny from dihaploid induction may contain genetic information from *S. phureja*, although it does not show an embryo spot on the seed or an anthocyanin pigmentation in the nodes. Molecular and cytological introgression analyses of dihaploid populations confirmed the presence of *S. phureja* genome in

haploid genotypes (Clulow et al., 1993; Clulow & Rousselle-Bourgeois, 1997; Ercolano et al., 2004). The introgression rate may be influenced by the female parent (Straadt & Rasmussen, 2003).

1.1.4. Potato genomics

In 2011, the potato reference genome was published by the Potato Genome Sequencing Consortium (PGSC, 2011). A homozygous clone, DM1-3 516 R44, has been generated by duplicating a monoploid genotype which had been derived from the heterozygous clone of the *Phureja* Group of cultivated potato. DM1-3 516 R44, as well as a heterozygous diploid line RH89-039-16, have been sequenced for the potato reference genome in a whole-genome shotgun approach (PGSC, 2011). Contig assembly resulted in 727 Megabase pair (Mbp) sequences, of which 93.3 % were non-gapped. For the updated assembly of the potato reference genome (version 4.03) Sharma et al. (2013) integrated linkage data from a segregating diploid potato population derived from the originally sequenced DM clone, which resulted in 951 genome superscaffolds. Altogether, a total of 674 Mbp are contained in the 4.03 version of the assembly with a total of 39,031 predicted protein-coding genes (Gálvez et al., 2017). The first genome draft of the wild potato species *Solanum commersonii* was released (Aversano et al., 2015) and other members of the Solanaceae family have also been sequenced (TGC, 2012; Kim et al., 2014; Sierro et al., 2014; Bombarely et al., 2016).

1.1.4.1. Genetic mapping, molecular markers and QTL mapping in potato

Most of the genetic maps for potato were conducted in diploid genotypes. The first genetic linkage map for potato was constructed using restriction fragment length polymorphism (RFLP) markers identified in an interspecific cross of *S. phureja* and a hybrid of *S. tuberosum* and *Solanum chacoense* with tomato probes (Bonierbale et al., 1988), which was the first comparative mapping approach to reveal high co-linearity between the tomato and potato genome. A second RFLP map was obtained from an intraspecific backcross population derived from diploid *S. tuberosum* breeding lines using potato markers (Gebhardt et al., 1989; 1991) and high density linkage maps with more than 1,000 mapped RFLP markers of the tomato and potato genomes have been constructed (Tanksley et al., 1992). Another map integrating known RFLP markers, as well as morphological and isozyme markers was developed for diploid potato (Jacobs et al., 1995). Amplified fragment length polymorphism (AFLP) markers for potato have been developed and mapped to align genetic maps (van Eck et al., 1995; Rouppe van der Voort et al., 1997; 1998a), with an ultradense linkage map containing more than

10,000 AFLP markers developed by van Os et al. (2006). Simple sequence repeat (SSR) markers have been developed and used extensively in potato research and mapping approaches (Veilleux et al., 1995; Milbourne et al., 1998; Feingold et al., 2005; Ghislain et al., 2004; 2009). Sequencing of the potato genome enabled the discovery of a multitude of Single Nucleotide Polymorphisms (SNPs) within the potato genome. In their study, Uitdewilligen et al. (2013) estimated the SNP frequency to be 1 per 24 base pairs (bp) in exons and 1 per 15 bp in introns. Hackett et al. (2013) constructed tetraploid maps for potato with 3,839 mapped SNP markers.

Various quantitative trait loci (QTLs) have been identified in potato, with a major focus on different agronomic traits such as tuber yield and starch content (Schäfer-Pregl et al., 1998; Bradshaw et al., 2008; McCord et al., 2011; Schönhals et al., 2017). Multiple QTLs have been identified on various chromosomes for several tuber traits, such as for number of tubers, tuber weight, width and length (Rak et al., 2017). Quantitative resistance loci have also been identified for important potato diseases, such as late blight (Leonards-Schippers et al., 1994; Collins et al., 1999; Danan et al., 2011) and root cyst nematodes (Rouppe van der Voort et al., 1998b; Bryan et al., 2002).

1.1.4.2. SNP genotyping

Genotyping arrays are the most common tool for high-throughput SNP genotyping. Multiple platforms have been developed which offer many advantages when compared to gel-based genotyping platforms, like automation and standardization of results which in turn allow an easy and relatively fast analysis of a large number of individuals or samples. However, analysis of entire populations with several hundred genotypes can be costly and is limited to the genes or sequences that are included on the respective array, which can lead to an ascertainment bias if the chosen SNPs for the development of the array were originally discovered in a small number of genotypes and are not represented in different genetic backgrounds (Moragues et al., 2010; Thomson et al., 2012; De Donato et al., 2013). Several SNP arrays have been developed for potato. One of the most frequently used SNP arrays is featuring 8,303 SNP markers (Hamilton et al., 2011; Felcher et al., 2012) and has been used extensively in potato research (Hirsch et al., 2013; Lindqvist-Kreuze et al., 2014; Prashar et al., 2014; Obidiegwu et al., 2015; Rak et al., 2017). SNP markers on this array are evenly distributed across the twelve potato chromosomes to provide good genome coverage. More recently, a 20 k SNP array was developed by Vos et al. (2015). However, one major challenge in analyzing SNP marker data in tetraploid potato remains in the correct scoring of heterozygous allele dosages (Voorrips et al., 2011). Computational software such as fitTetra (Voorrips et al., 2011), TetraploidMap (Hackett & Luo, 2003) or GenomeStudio® (Illumina) could help estimating allele dosages in tetraploids. Other sequencing

techniques as alternatives to SNP genotyping arrays are the genotyping-by-sequencing (GBS) method (Elshire et al., 2011) and restriction-site-associated DNA (RAD) sequencing (Baird et al., 2008). Both techniques are based on the sequencing of a subset of the genome. Barcoding of samples allows multiplexed sequencing and therefore a relatively cheap identification of a large number of markers. Library preparation is achieved by digesting genomic DNA with restriction enzymes for GBS or digestion in combination with physical shearing for RAD sequencing. However, genotyping of tetraploids requires higher read depth compared to diploids to allow correct genotype calling and an accurate estimation of allele copy numbers. In highly heterozygous species such as potato with medium-sized genomes further complexity reduction by target enrichment might be necessary to achieve sufficient read depth (Uitdewilligen et al., 2013).

1.1.5. Resistance mechanisms and *R* genes in plants

Plants have developed effective strategies to recognize and respond to plant pathogens, which can be divided into two layers. On the cell surface, pathogen- or microbial-associated molecular patterns (PAMPs/MAMPs), such as bacterial flagellin or fungal chitin, are recognized by so-called pattern recognition receptors (PRRs), which induce the PAMP-triggered immunity (PTI). The second layer is induced by the release of pathogen virulence molecules, called effectors, into the plant cell. These effectors in the host cytoplasm may suppress PTI responses, leading to effector-triggered susceptibility. Effectors can be recognized intracellularly by a class of receptor proteins that consist of a nucleotide-binding (NB) and a leucine-rich repeat (LRR) domain which leads to an effector triggered immunity (ETI) response. Different mechanisms have been described for the recognition of pathogen effectors: direct recognition by physical association to the receptor or by an accessory protein that is part of the NB-LRR-receptor (NLR) or indirect recognition by the modification of an accessory protein, which may be its virulence target (guard model) or a structural mimic of the target (decoy model). The effector induces an alteration in the accessory protein which is then recognized by NLRs (Dodds & Rathjen, 2010). In general, ETI results in disease resistance and programmed cell death through a hypersensitive response at the infection site (Jones & Dangl, 2006). It has been proposed that responses in ETI occur more quickly and are more prolonged and robust than those in PTI (Tsuda & Katagari, 2010). An overview of the principles of plant immunity is given in Figure 2.

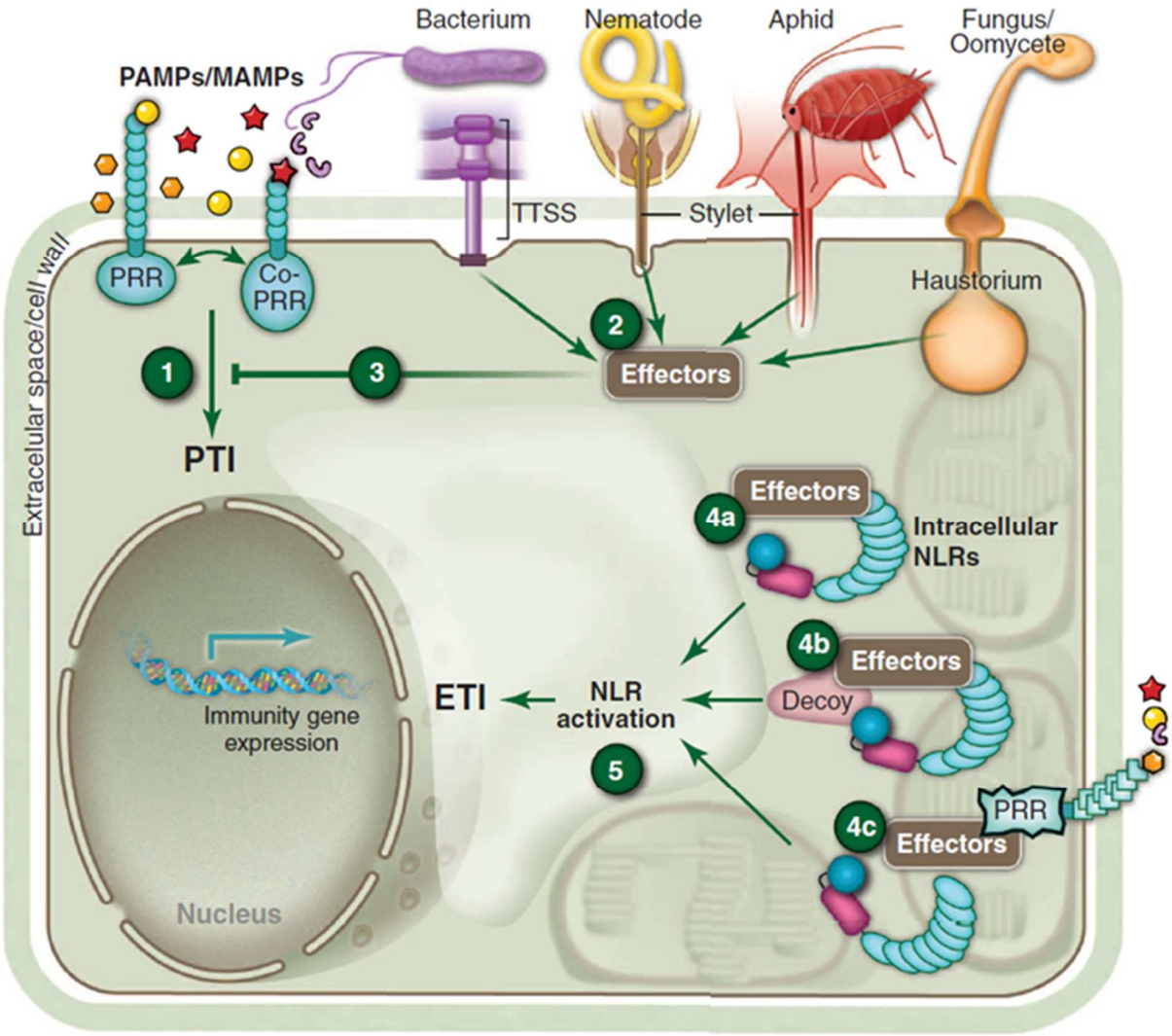

Figure 2: The principles of plant immunity (Dangl et al., 2013). Plant pathogens propagate in the extracellular space of a plant cell, releasing molecules such as lipopolysaccharides, flagellin and chitin. Those pathogen- or microbial-associated molecular patterns (PAMPs/MAMPs) are recognized by cell surface pattern recognition receptors (PRRs) and initiate the so-called PAMP-triggered immunity (PTI) of the plant (1). Effector proteins are released into the plant cell from the pathogens (2) and can suppress the PTI response (3). Effectors are recognized by plant nucleotide-binding (NB)-leucine-rich repeat (LRR) receptors (NLRs) by direct or indirect recognition mechanisms. Direct receptor ligand recognition (4a) leads to immune signaling by physically binding to the receptor. In a guard and decoy model, effectors cause an alteration in an accessory protein that either is its virulence target or structurally mimics the effector target (4b). The alteration of the protein is recognized by the NLRs. Interaction of an effector with an accessory protein may also lead to direct recognition by the NLRs (4c). These recognition mechanisms induce the effector triggered immunity (ETI) response (5).

The major class of resistance genes or resistance gene analogs (RGAs) in plants are NB-LRRs which can be further classified as toll interleukin-1 receptor (TIR) NB-LRRs (TNLs) or coiled-coil (CC) NB-LRRs (CNLs). The nucleotide binding site includes highly conserved motifs in a strict order, while LRRs have structural domains that are highly adaptable with diverse binding specificities (Marone et al., 2013). Numerous *R* genes have been identified in different plant species, for example in *Arabidopsis thaliana* (Meyers et al., 2003; Guo et al., 2011), *Oryza sativa* (Shang et al., 2009) and in *Brassica rapa* (Mun et al., 2009). In *S. tuberosum* 438 and 435 NB-LRR genes have been identified by Jupe et al. (2012) and Lozano et al. (2012), with 77 and 65 TNLs and 361 and 370 CNLs, respectively. Using a resistance gene enrichment sequencing approach Jupe et al. (2013) were able to increase the number of NB-LRRs from 438 to 755.

1.1.5.1. Resistances to pests and diseases in potato

As a clonally propagated plant, potato is highly vulnerable to pests and diseases. Many resistance genes have been introgressed from wild relatives of potato by crossing resistant wild species with susceptible potato varieties. Most resistance genes to viruses, fungi and nematodes in modern potato cultivars have been introgressed from only few closely related *Solanum* species, like *S. spegazzinii*, *S. acaule*, *S. vernei*, *S. stoloniferum* and *S. demissum* (Gebhardt & Valkonen, 2001) due to the introduction of undesirable traits of wild species together with the resistance genes (Simko et al., 2007). Resistance genes are often located in so-called resistance “hot-spots” in the potato genome where various genes for qualitative and quantitative resistances to different pathogens have been identified, and are often located in telomeric regions on the chromosomes (Jupe et al., 2012; 2013).

Resistance genes to the root cyst nematode *Globodera pallida* have been introgressed from the wild potato species *S. spegazzinii* and *S. vernei* (Gebhardt et al., 2011). Seven SNP markers on chromosome 5 were found to be significantly linked to nematode resistance. A haplotype model was developed to detect the SNP haplotype *c* that was linked with high resistance to *G. pallida*. This HC marker is highly diagnostic for pathotypes Pa2 and Pa3 (Sattarzadeh et al., 2006). Using RFLP markers Barone et al. (1990) mapped a major dominant resistance locus *Gro1* on chromosome 7 that confers resistance to several pathotypes of *G. rostochiensis*.

At least 40 viruses are known to infect potato, the most important being the *Potato leafroll virus* (PLRV) and *Potato virus Y* (PVY), followed by *Potato virus X* (PVX), *Potato virus A* (PVA), *Potato virus M* (PVM), *Potato virus S* (PVS) and the *Potato mop-top virus* (PMTV) with decreasing importance (Gebhardt & Valkonen, 2001). Two major resistance genes to PVX have been mapped, *Rx1* on chromosome 12 and

Rx2 on chromosome 5 in an RFLP analysis (Ritter et al., 1991). Resistance genes to PVY and PVA are located on chromosome 11, with genes Ry_{adg} and Ry_{sto} providing extreme resistance to PVY (Hämäläinen et al., 1997; 1998), and Na_{adg} conferring a hypersensitive resistance to PVA (Hämäläinen et al., 2000). For PVS the resistance gene *Ns* has been reported (Marczewski et al., 1998).

The most important fungal disease of potato is late blight, caused by the oomycete *Phytophthora infestans* (Judelson, 1997). Eleven *R* genes conferring resistance to late blight have been reported, of which *R1* has been mapped on chromosome 5 (Leonards-Schippers et al., 1992), *R2* on chromosome 4 (Li et al., 1998) and *R3*, *R6* and *R7* on chromosome 11 (El-Kharbotly et al., 1996). Other factors controlling quantitative resistance to *P. infestans* were found on almost every potato chromosome (Gebhardt & Valkonen, 2001). The only other fungus with identified and mapped resistance genes in potato is *Synchytrium endobioticum*.

1.2. *Synchytrium endobioticum* – the causal agent of potato wart disease

Potato wart disease is caused by *Synchytrium endobioticum* (Schilb.) Perc., a fungal pathogen of the order Chytridiales in the phylum Chytridiomycota. It is classified as a quarantine pest by the European and Mediterranean Plant Protection Organization (EPPO) and is one of the most severe and most important quarantine diseases of the cultivated potato (EPPO, 2004). *S. endobioticum* is an obligate biotroph and soil-borne pathogen and does not produce hyphae but sporangia with several hundred motile uniflagellated zoospores which infect meristematic tissue of potato when they are released into the soil upon decomposition of warts (EPPO, 2004). Potato is the primary host of *S. endobioticum*, where it infects tubers but also other below-ground parts of the plant causing yield losses of up to 50-100 % (Hampson, 1993; Melnik, 1998). Infections of potato roots are not known, but artificial infection of tomato roots has been described (Weiss, 1925). Likewise, other solanaceous species can be artificially infected without necessarily inducing the typical wart formation (Przetakiewicz, 2008). Potato wart disease is also known by black scab or black wart of potato (EPPO, 2004), as well as potato tumor or cancer (Obidiegwu et al., 2014). Until today, more than 40 different pathotypes have been described for *S. endobioticum*, with pathotype 1 being the most common pathotype (Przetakiewicz, 2015). Approximately 47 % of the currently registered varieties in Germany are resistant to pathotype 1 (Descriptive Variety List (DVL) of the Federal Plant Variety Office (Bundessortenamt), 2017). Pathotypes 1, 2, 6 and 18 are considered to be the most relevant and aggressive forms of the fungus (Stachewicz, 2002; Ballvora et al., 2011). However, only 3 % of the potato cultivars currently registered in Germany are resistant to pathotypes 1, 2, 6 and 18 (DVL, 2017).

1.2.1. Symptoms

Typical symptoms of potato wart disease are the formation of cauliflower-like, irregular warts on the below-ground parts of potato plants, like tubers, stolons and lower stems (Figure 3). Warts can vary in growth and shape from a few millimeters to several centimeters and may also cover the entire tuber (Franc, 2007). The wart tissue consists of hypertrophic, tumor-like dividing cells which contain thin-walled summer sporangia and thick-walled winter sporangia (Ballvora et al., 2011). Warts on the tubers initially have a white color, but turn brown to black upon decay (Franc, 2007). The size and number of the warts is dependent on the environmental conditions upon infection, the severity of the contamination of the soil with resting sporangia and the degree of susceptibility of the present potato cultivar (Stachewicz, 2002). Due to the soil-borne nature of the pathogen the presence of and the infection with *S. endobioticum* may stay undetected until harvest (Franc, 2007).

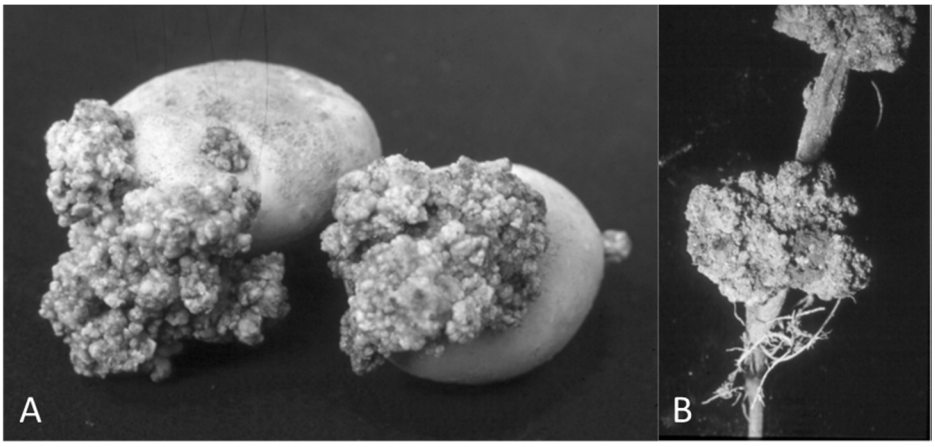

Figure 3: Typical wart symptoms on infected tubers (A) and the lower stem (B) of potatoes (Stachewicz, 2002). Warts can vary in their size and shape, depending on the environmental conditions, the virulence of the pathogen as well as the susceptibility of the potato cultivar.

1.2.2. Infection cycle

S. endobioticum does not produce hyphae upon infection, but sporangia with motile uniflagellated zoospores. Under favorable environmental conditions with temperatures between 8 and 18 °C and high soil humidity of 50-80 %, zoospores are released from decaying resting winter sporangia (Stachewicz, 2002). Up to 200-300 motile zoospores with a diameter of 2-4 µm migrate in the soil water to infect epidermis cells of meristematic tissues, e. g. potato eyes of the tubers. After infection the

host cells are enlarged and neighboring cells proliferate, resulting in characteristic wart symptoms of the disease (Franc, 2007). Haploid sori develop in the host cells, which produce new zoospores leading to secondary infections of meristematic tissue. In parallel to this asexual infection cycle, zoospores can also fuse to form diploid sporangia in a sexual infection cycle. Diploid sporangia develop into zygotes which penetrate the host cells, leading to multiple cell divisions of the host cells to form the thick-walled resting winter sporangia (Curtis, 1921). These are released into the soil upon decay of the wart tissue and may remain viable for up to 40 years (Laidlaw, 1985).

1.2.3. Disease control and dissemination

Due to the longevity of the resting sporangia and the high yield losses, *S. endobioticum* is classified as an A2 quarantine pest by the EPPO. Chemical control is not feasible, because of the limited accessibility of resting sporangia in the soil and the thick-walled nature of the resting sporangia (Hampson, 1977; Laidlaw, 1985). Furthermore, many chemicals have been reported to be phytotoxic or to lead to sterile soil (Hampson, 1977; Gunacti & Erkiliç, 2013). Therefore, strict phytosanitary measures are to be applied upon detection of infection to prevent dispersion of the pathogen. Contaminated plant material has to be destroyed and infected fields are not to be used for potato production for at least 20 years. Descheduling of the fields is only allowed when the absence of winter sporangia of *S. endobioticum* can be demonstrated by microscopic analysis of and extensive bioassays with soil samples of the infected field (EPPO, 2004). A PCR-based detection method for sporangia in soil samples was developed by van den Boogert et al. (2005). Potato varieties that are resistant against the present pathotype may be grown after ten years on partially descheduled fields. In so-called safety zones surrounding infected fields only potato varieties may be grown which are resistant to the pathotype found in the infected field (EPPO, 2004). Natural spreading of *S. endobioticum* is limited, due to the soil-borne nature of the pathogen and the need of moist soil for zoospore release and mobility. International trade of infected potato tubers with or without adhering soil throughout continents, as well as transport of soil from infected fields, soil-particles on farm machinery and irrigation water are the major sources for distribution of the disease (Obidiegwu et al., 2014). Due to the strict control measures and regulatory strategies applied in many countries, distribution of the disease is sporadic and limited to few infection sites. Occurrence of potato wart disease has been reported predominantly in European countries but also in North and South America, Asia, Africa and Oceania (Figure 4). Records on occurrence of the disease are sometimes inconsistent or have not been officially confirmed (Obidiegwu et al., 2014).

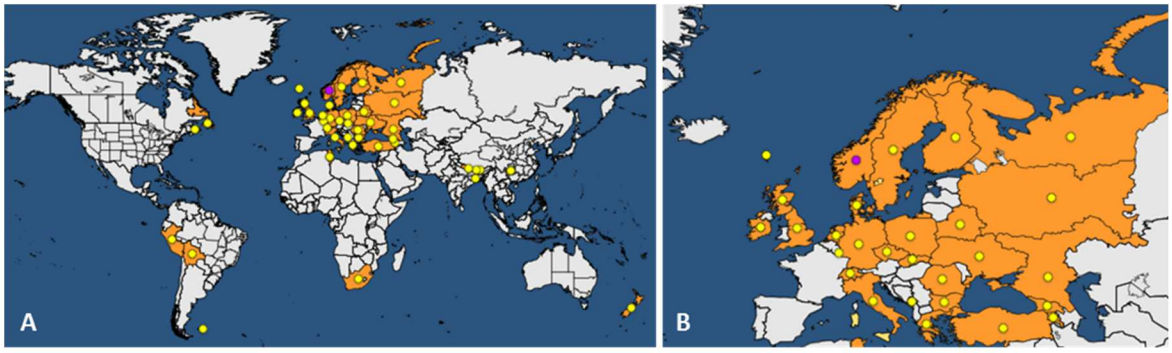

Figure 4: Current status of the global distribution of potato wart disease (A) and the occurrence in European countries (B) as reported by EPPO in November 2017. Countries where the pathogen is present are colored in orange with a yellow dot. In Norway the pathogen is reported to be under eradication (violet dot). The figure has been modified from the EPPO homepage (https://gd.eppo.int/taxon/SYNCEN/distribution).

1.2.4. Phenotypic resistance assessment

The phenotypic resistance screenings for potato wart are usually carried out under laboratory conditions, due to the unsteadiness of environmental conditions present on contaminated testing fields that could influence the resistance assessment. In most countries, resistance tests are performed according to the methods described by Spieckermann (Spieckermann & Kothoff, 1924) or Glynne and Lemmerzahl (Glynne, 1925; Lemmerzahl, 1930).

For the Spieckermann test, wart compost containing winter sporangia is prepared for which fresh warts are cleaned from adhering soil and cut into small pieces of approximately 1 cm. These pieces are then mixed with clean sand in a ratio of 1 kg of wart tissue to 3 kg of sand and incubated at a temperature of 18-25 °C. The mixture is moderately moistened on a daily basis for two months and then weekly for another two months. After the incubation time the compost is air-dried for two months and can then be used for resistance testing and pathotype identification. Sporangium density and vitality should be determined using a highly sensitive cultivar before the original testing. The compost mixtures can be used for up to two years. Tuber eye fields of 2 x 2 cm are cut out of the tubers to be tested and are moistened before 1 to 1.5 g of the compost mixture is put on top of the eye fields. The tuber pieces are incubated in the dark at 16-18 °C and moistened regularly for eight weeks. After this incubation period the tuber pieces are evaluated under a stereo microscope for their resistance reaction (EPPO, 2004).

In most countries of the European Union, however, the Glynne-Lemmerzahl method is used to determine resistance to *S. endobioticum* (Flath et al., 2014). This method uses fresh wart tissue containing summer sporangia and is based on the infection of young sprouts with zoospores. Eye fields with emerging sprouts of 1-2 mm in length of whole tubers or tuber pieces are ringed with warm vaseline using a syringe. Those rings are filled with water and a piece of fresh wart tissue. The water is essential to ensure zoospore mobility. Furthermore, only the uncut surface of the wart pieces should be put into contact with the water. After an incubation time of 48 hours at temperatures of 8-12 °C the wart tissue is removed and the tubers are covered with moist sterile soil and incubated for three to four weeks at 16-18 °C. Afterwards, tubers are evaluated under a stereo microscope to determine their resistance reaction (EPPO, 2004). The German and Polish Glynne-Lemmerzahl methods are most commonly used, which differ in the plant material used, treatment of the plant material before and after inoculation with wart tissue, incubation temperatures and incubation periods (Flath et al., 2014).

Disease symptoms are scored according to the modified scheme developed by Langerfeld and Stachewicz (1994) where sprouts are scored from 1 (extremely resistant) to 5 (extremely susceptible) according to their reaction phenotype (Figure 5; Table 2). Resistance phenotyping according to Hille (1965) with some deviations regarding the resistance classification is more common for the Spieckermann test.

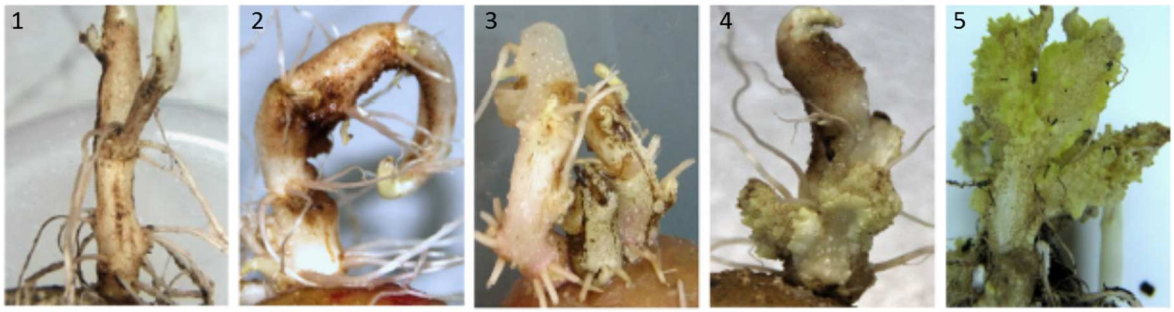

Figure 5: Classification of reaction types for resistance against *S. endobioticum* according to their phenotypes obtained with the German version of the Glynne-Lemmerzahl method (Flath et al., 2014). Sprouts are evaluated microscopically after the incubation period and scored from 1 (extremely resistant) to 5 (extremely susceptible) according to the description in Table 2.

Table 2: Modified classification of reaction types regarding the resistance phenotyping against *S. endobioticum* according to Langerfeld and Stachewicz (1994).

Reaction type	Group	Classification	Description
1	R1	Extremely resistant	Early defense necrosis; no visible sorus formation
2	R1	Resistant	Late defense necrosis; sorus formation partially visible; sori immature or necrotic before maturity
3	R2	Weakly resistant	Very late defense necrosis; single ripe sori or sorus fields developed, but completely surrounded by necrosis; up to five non-necrotic sori, clear necrosis in other zones of the same tuber piece; high attack of the control cultivar is essential
4	S1	Slightly susceptible	Scattered infections; sori or sorus fields non-necrotic, few in number, containing winter sporangia; late necrosis can be present on other infection sites on the sprout; the sprout can be slightly malformed (thickened)
5	S2	Extremely susceptible	Dense infection fields; numerous ripe non-necrosed sori and sorus fields; fields with dense non-necrotic infection sites; predominant tumor formation

1.2.5. Identification of pathotypes of *S. endobioticum*

The most important *S. endobioticum* pathotypes 1, 2, 6, 8 and 18 can be differentiated using a potato cultivar set varying in their resistances to the respective pathotypes as suggested by the EPPO (Table 3) using the aforementioned Spieckermann test (Spieckermann & Kothoff, 1924), the Glynne-Lemmerzahl test (Glynne, 1925; Lemmerzahl, 1930) or field tests. However, different countries may use different cultivars for pathotype identification and results may differ between executing laboratories and protocols used (Flath et al., 2014).

Table 3: A set of differential potato cultivars suggested for pathotype identification according to the EPPO in 2004. Cultivars are listed as resistant (R) or susceptible (S) to *S. endobioticum* pathotypes 1, 2, 6, 8 and 18.

Cultivar	Pathotype 1	Pathotype 2	Pathotype 6	Pathotype 8	Pathotype 18
`Deodara´	S	S	S	S	S
`Tomensa´	S	S	S	S	S
`Eersteling´	S	S	S	S	S
`Producent´	R	S	S	S	S
`Combi´	R	S	S	S	S
`Saphir´	R	S	R	R	R
`Delcora´	R	R	R	S	S
`Miriam´	R	R	R	R	S
`Karolin´	R	R	R	R	R
`Ulme´	R	R	R	R	R
`Belite´	R	R	R	R	-

As a PCR-based alternative, molecular markers have been developed for the detection of *S. endobioticum* (Niepold & Stachewicz, 2004; van den Boogert et al., 2005; van Gent-Pelzer et al., 2010) and microarray-based hybridization for the detection of *S. endobioticum* has been described (Abdullahi et al., 2005). However, these methods do not allow the distinction between different pathotypes of *S. endobioticum*. Bonants et al. (2015) presented a real-time TaqMan PCR assay to discriminate *S. endobioticum* pathotype 1 from the other pathotypes 2, 6, 8 and 18.

1.2.6. The genetics of resistance to *S. endobioticum*

The genetics of wart resistance has been analyzed early and was one of the first traits subjected to Mendelian genetic analysis (Salaman & Lesley, 1923). Black (1935) hypothesized that two or more genes must be involved in the expression of resistance by observing segregation ratios of resistant and susceptible plants in a F1 progeny of crosses between susceptible parents. Salaman and Lesley (1923) reported that suppressor genes are involved in the resistance mechanism. Hehl et al. (1999) were the first to map genes for wart resistance in a diploid mapping population. They located the dominant gene *Sen1* on chromosome 11 conferring resistance to *S. endobioticum* pathotype 1. *Sen1* is closely linked to two potato homologues of the *N* gene which provides resistance to the tobacco mosaic virus in tobacco. A second dominant gene for resistance to pathotype 1, *Sen1-4*, could be mapped on chromosome 4 by Brugmans et al. (2006) who also used a diploid population. The first resistance loci for pathotypes 2, 6 and 18 were identified by Ballvora et al. (2011) in two tetraploid half-sib families,

where resistance segregated as a quantitative trait. QTLs for resistance to pathotypes 2, 6 and 18 could be identified on chromosome 1, to pathotype 18 on chromosome 9 and to pathotype 1 on chromosome 11, co-localized with *Sen1*. The authors also showed that resistances to pathotypes 2, 6 and 18 are highly correlated, but independent from resistance to pathotype 1. Groth et al. (2013) analyzed the progeny of a cross of the tetraploid cultivars `Saturna´ and `Panda´ and mapped QTLs for resistance to pathotypes 1, 2, 6 and 18 on chromosomes 6, 8 and 11. Another QTL for resistance against pathotype 1 was identified on chromosome 11, which again co-localized with the *Sen1* locus. Additionally, resistance QTLs for pathotypes 2, 6 and 18 were identified on chromosomes 7 and 10, for pathotypes 6 and 18 on chromosome 2 and for pathotype 2 on chromosome 1. Obidiegwu et al. (2015) evaluated the progeny of a cross between a wart resistant and a susceptible tetraploid breeding clone by extensive genotyping with SSR and SNP markers and identified multiple resistance loci on chromosomes 1, 3, 4, 10, 11 and 12 for pathotypes 1, 2, 6, and 18. The *Sen1* locus on chromosome 11 was identified as a major factor for resistance to all four pathotypes of *S. endobioticum* with the other QTLs having minor effects regarding resistance. An overview of all known wart resistance loci is shown in a physical map of the 12 potato chromosomes in Figure 6 as published by Obidiegwu et al. (2015).

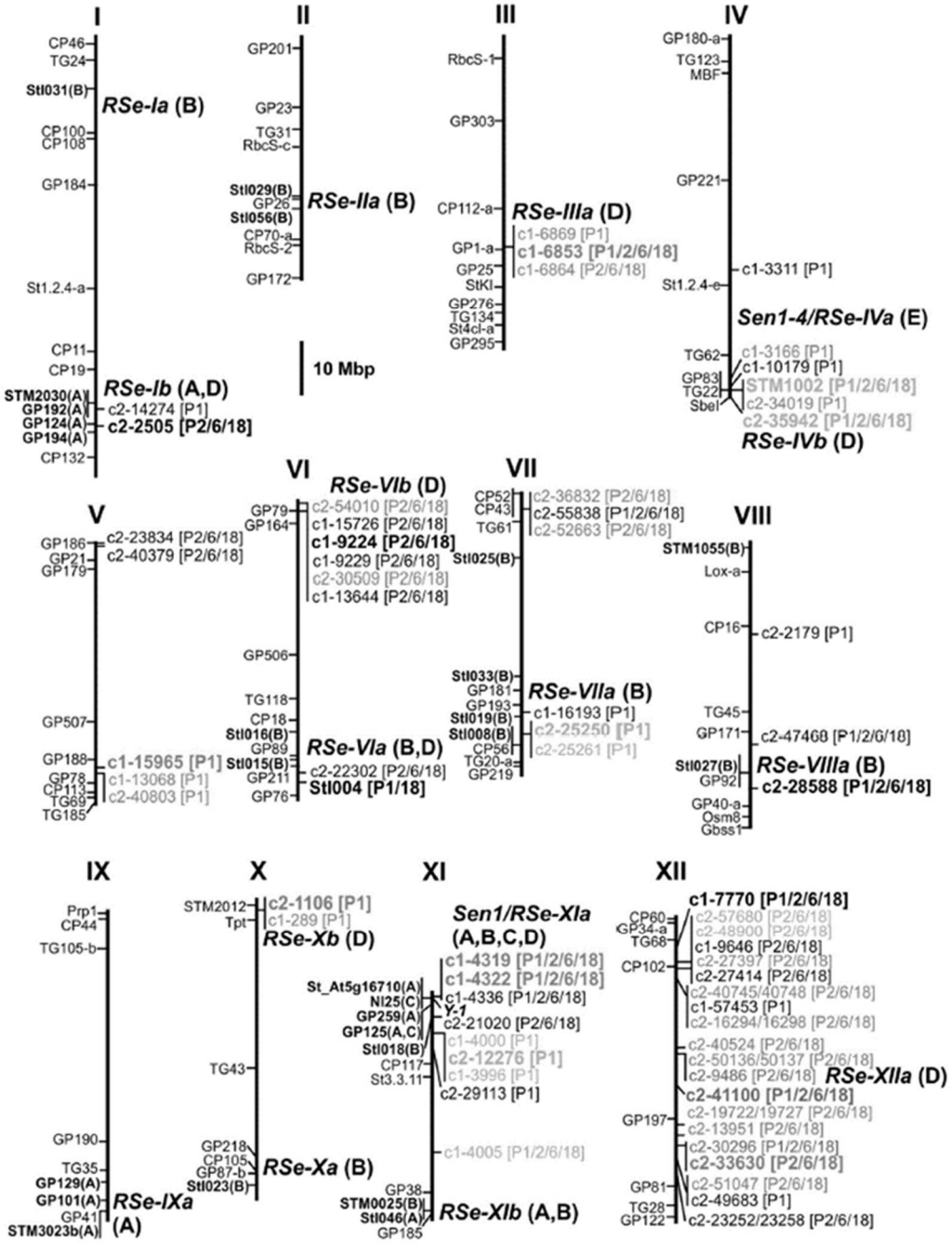

Figure 6: Physical maps of the twelve potato chromosomes with known wart resistance loci (Obidiegwu et al., 2015). Wart resistance loci (RSe) are shown on the right side of the respective chromosomes with the corresponding references in parenthesis: A for Ballvora et al. (2011), B for Groth et al. (2013), C for Hehl et al. (1999), D for Obidiegwu et al. (2015) and E for Brugmans et al. (2006). Additionally, markers linked to wart resistance loci are depicted in bold letters on the left side. Markers linked to resistance which were identified by Obidiegwu et al. (2015) are shown on the right side with their respective pathotype specificity in brackets (P1 for pathotype 1, P2 for pathotype 2, P6 for pathotype 6 and P18 for pathotype 18). Additional markers are shown on the left for general orientation.

1.3. The collaboration project DiRK and the overall goal of the project

The collaboration project DiRK („Diagnostik für eine Ressourcen-schonende Kartoffelstärke-Produktion") had the overall goal to establish precision breeding for starch potatoes, based on diagnostic molecular markers for resistances against the different pathotypes of *S. endobioticum*. Diagnostic markers for potato wart resistances should be combined with other resistance properties like resistances against the quarantine nematode pathogen *Globodera pallida*, as well as immunity against potato virus Y, for which diagnostic markers are already available, to develop high-yielding starch potatoes to ensure a sustainable and profitable resource production for the starch-processing industry.

As the phenotypic resistance evaluation for potato wart disease is very laborious and often ambiguous, DNA-based molecular markers that are closely linked to resistance loci would considerably facilitate the detection of resistance to potato wart disease, circumventing the phenotypic resistance assessment based on the inoculation of a few dozen tubers. Marker-assisted selection for potato wart disease will facilitate precision breeding for starch potatoes.

As the genetic composition of resistance against potato wart disease is complicated being a quantitative trait that is controlled by multiple loci the analysis of tetraploid plant material can be complex. To reduce this genome complexity a dihaploid potato population derived from a cross of a tetraploid cultivar and the dihaploid inducer *S. phureja* was used for genetic analyses.

1.3.1. Thesis objectives

Based on the aforementioned overall goal of the collaboration project, the following thesis objectives were determined which were analyzed in the three manuscripts presented in this thesis:

i. to determine whether genetic analysis of a dihaploid potato population is a convenient tool to analyze the genetics of potato wart resistance and additional phenotypic traits of a tetraploid potato cultivar (manuscripts 1 and 2).
ii. to determine the extent of introgression of the dihaploid inducer genome of the wild potato species *S. phureja* and whether it disturbs genetic analyses of dihaploid potato genotypes (manuscript 1).
iii. to identify molecular markers for resistance against *S. endobioticum* pathotypes 6 and 18 that are linked to resistance loci for potato wart disease (manuscript 2).

iv. to analyze whether resistances for the *S. endobioticum* pathotypes 6 and 18 are conferred by different resistance loci (manuscript 2).

v. to develop molecular markers with diagnostic value that can be applied in populations derived from cultivars with different genetic backgrounds (manuscript 2).

vi. to identify whether pathotypes of *S. endobioticum* differ genetically and to develop molecular markers that can be used for pathotype identification (manuscript 3).

2. Manuscripts and Publications

2.1. Maximization of Markers Linked in Coupling for Tetraploid Potatoes via Monoparental Dihaploids

Annette Bartkiewicz[1], Friederike Chilla[1], Diro Terefe-Ayana[1,2], Jens Lübeck[3], Josef Strahwald[3], Eckhard Tacke[4], Hans-Reinhard Hofferbert[4], Marcus Linde[1], Thomas Debener[1]

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Type of authorship:	First author
Type of article:	Research article
Contribution to the publication:	Planned and performed the experiments, analyzed the data, prepared all tables and figures and wrote the manuscript
Status of publication:	Submitted to Frontiers in Plant Science
Supplements:	Supplementary material mentioned in the manuscript is provided as electronic appendix

Maximization of Markers Linked in Coupling for Tetraploid Potatoes via Monoparental Dihaploids

Annette Bartkiewicz[1], Friederike Chilla[1], Diro Terefe-Ayana[1,2], Jens Lübeck[3], Josef Strahwald[3], Eckhard Tacke[4], Hans-Reinhard Hofferbert[4], Marcus Linde[1], Thomas Debener[1]*

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Abstract

Dihaploid potato populations derived from a single tetraploid donor constitute an efficient strategy to analyze markers segregating from a single donor genotype. Analysis of marker segregation in populations derived from crosses between autotetraploids is complicated by a maximum of eight segregating alleles, multiple dosages of the markers and problems related to linkage analysis of marker segregation in repulsion. Here, we present data on two monoparental dihaploid populations generated by prickle pollination of two tetraploid varieties with *Solanum phureja* and genotyped with the 12.8 k SolCAP single nucleotide polymorphism (SNP) array. We show that in a population of monoparental dihaploids, the number of biallelic SNP markers segregating in linkage to loci from the tetraploid donor genotype is much larger than in putative crosses of this genotype to a diverse selection of 125 tetraploid varieties. Although this strategy is more laborious than conventional breeding, the generation of dihaploid progeny for efficient marker analysis is straightforward if morphological markers and flow cytometry are utilized to select true dihaploid progeny. The level of introgressed fragments from *S. phureja*, the dihaploid inducer, is very low, supporting its suitability for genetic

analysis. Mapping with single-dose markers allowed the analysis of quantitative trait loci (QTLs) for four phenotypic traits.

Keywords: dihaploids, potato, SNPs, linkage, monoparental, QTLs, mapping

Introduction

The cultivated potato (*Solanum tuberosum* L.) is a highly heterozygous autotetraploid outcrossing species with 48 chromosomes (2*n* = 4*x* = 48) that shows tetrasomic inheritance and has a haploid genome size of approximately 840 Megabase pairs (Bradshaw, 2007). Progeny from crosses between tetraploid genotypes display complex segregation patterns that severely complicate genetic analyses compared with progeny from diploid parents (Mann et al., 2011).

As a maximum of eight alleles can segregate in progenies from crosses between tetraploid genotypes, marker analysis is also complicated because full resolution of the marker genotypes can only be achieved by the precise determination of allele dosages. Although the latter problem has been solved with recent technological advances in generating single nucleotide polymorphism (SNP) markers (Voorrips et al., 2011; Hackett et al., 2013), detection of linkage between traits and markers that are linked in repulsion remains elusive. Therefore, most of the mapping approaches in tetraploids are based on markers that are linked in coupling.

Most of the genetic studies in potato have been conducted in diploid genotypes (Bonierbale et al., 1988; Gebhardt et al., 1989; 1991; Tanksley et al., 1992), which are often obtained as dihaploids from tetraploid cultivars by androgenesis or parthenogenesis. An androgenic approach to obtain dihaploids from a tetraploid cultivar is via anther culture (Uhrig & Salamini, 1987; Rokka et al., 1996), although it has been reported that many tetraploid cultivars do not respond well to this method (Irikura, 1975) and that there is a strong influence of the respective genotype on the success rate (Jacobsen & Sopory, 1978). Song et al. (2005) successfully used 57 primary dihaploid lines derived from an anther culture for the development of genetic markers for extreme resistance to potato virus Y (Ry_{sto}) in a bulked segregant analysis.

A parthenogenic approach to generate dihaploids is so-called “prickle pollination” crosses with specific *Solanum phureja* pollinators that induce the development of dihaploid seed (Hougas & Peloquin, 1957; Hutten et al., 1994). The availability of a dominant seed marker (“embryo spot”) in the haploid inducer genotypes facilitates the removal of undesirable hybrids within the progeny because tetraploid and triploid hybrids of the cross inherit a purple anthocyanin pigmentation at the base of the cotyledons, which is also visible as nodal bands in plant seedlings (Peloquin & Hougas, 1959; Hermsen & Verdenius, 1973). Different cytogenetic mechanisms have been proposed for the dihaploid formation of crosses

with *S. phureja* pollinators. Dihaploids from such crosses have been regarded as parthenogenetically developed, and it was speculated that *S. phureja* pollen triggers the development of unfertilized egg cells into embryos without making any genetic contribution to the embryo itself (Hermsen & Verdenius, 1973). Clulow et al. (1991) suggested that *S. phureja* chromosomes are eliminated from embryonic cells during cell divisions after the fertilization of egg cells, resulting in dihaploid progeny. This finding was also reported in later introgression analyses (Clulow & Rousselle-Bourgeois, 1997; Straadt & Rasmussen, 2003; Ercolano et al., 2004). Dihaploid populations derived by parthenogenesis have been used to identify markers that are linked to nematode resistance (Pineda et al., 1993); however, the number of genotypes used in the RFLP mapping approach was very small with 37 dihaploid individuals.

The first genetic analyses in potatoes were reported as early as 1910 by Salaman, who analyzed the inheritance of male sterility, haulm characteristics, tuber shape and color, as well as eye depth without considering the tetraploidy and tetrasomic inheritance of the cultivated potato. Therefore, genetic analyses were often limited to the inheritance of dominant traits such as the tuber skin color (Black, 1933). The construction of linkage maps was first reported by Bonierbale et al. (1988), who used restriction fragment length polymorphism (RFLP) markers and tomato probes, followed by an RFLP map obtained from diploid *S. tuberosum* lines (Gebhardt et al., 1989; 1991) and high density maps of the tomato and potato genomes (Tanksley et al., 1992). Amplified fragment length polymorphism (AFLP) and simple sequence repeat (SSR) markers have been used extensively in potato research and mapping approaches (van Os et al., 2006; Veilleux et al., 1995; Milbourne et al., 1998; Ghislain et al., 2004, 2009; Feingold et al., 2005). Over the last years, most of the aforementioned markers have been replaced by SNP markers, for which extensive resources are available in potato (Hamilton et al., 2011; Felcher et al., 2012; Uitdewilligen et al., 2013; Vos et al., 2015).

However, traditional potato breeding is carried out at the tetraploid level between tetraploid cultivars, and it is mainly executed by the phenotypic selection of favorable traits (Carputo & Frusciante, 2011). Genetic analysis of traits and trait combinations from particularly interesting tetraploid genotypes is prone to the abovementioned problems. A solution to such problems would be the generation of a larger population of dihaploids extracted from single elite tetraploids (monoparental dihaploids). In these populations, 25 % of all markers from a particular genomic region should segregate in linkage to one of the four chromatids.

In this study, we present the generation of two large monoparental dihaploid populations consisting of 215 and 87 individuals, respectively, which were derived from two different tetraploid cultivars. We analyzed the populations and additional unrelated tetraploid varieties with a large set of SNPs and determined the degree of genome introgression from the *S. phureja* pollinators. The markers were

used for mapping of quantitative trait loci (QTLs) in the larger population as well as for analyzing the number of useful single-dose markers in simulated crosses with different tetraploid cultivars.

Material and methods

Plant material

Two tetraploid breeding lines of industrial starch potatoes, P208 and P809, were used for the construction of two dihaploid populations by so called “prickle pollination” with the dihaploid inducer clones IVP101 and IVP35 of the diploid wild potato species *Solanum phureja*. Dihaploid genotypes derived from the cross with P208 were used for SNP genotyping and genetic mapping. A subset of the dihaploid progeny derived from P809, as well as 125 tetraploid German and Polish cultivars, were also used for SNP genotyping.

Generation of dihaploid potato populations

Pollinations with the dihaploid inducers were performed in the greenhouse on emasculated flowers of P208 and P809. Seeds of the cross of P208 with *S. phureja* IVP35 were preselected for the occurrence of an embryo spot. Seeds derived from the crosses were surface sterilized by incubation for 30 seconds in 70 % ethanol and two minutes in 0.5 % sodium hypochlorite + Tween20, followed by three washing steps of five minutes each in sterile distilled water. Seeds were germinated *in vitro* on Murashige Skoog medium (Murashige and Skoog, 1962) containing 3 % sucrose and solidified with 8.4 g plant agar (Duchefa Biochemie B.V., Haarlem, The Netherlands) per liter. The emerging seedlings were cultivated at 23 °C in a 16 h light / 8 h dark cycle with light intensities of approximately 61 µmol m^{-2} s^{-1}.

Ploidy determination

Putative dihaploid seedlings were visually selected by a lack of anthocyanin pigmentation in the nodes of the *in vitro* seedlings. The ploidy of the selected seedlings was subsequently determined by flow cytometry with a CyFlow Ploidy Analyzer (Partec, Münster, Germany). Leaf tissue (~1 cm^2) from *in vitro* plantlets was chopped with razor blades in nuclei extraction buffer. Plant nuclei were stained with 4',6-diamidino-2-phenylindole using the CyStain UV Precise P kit (Partec, Münster, Germany). Analyses were performed according to the manufacturer’s protocol, counting at least 1,000 nuclei per sample. The parental genotypes with known ploidy were used as standards for diploid and tetraploid genotypes.

DNA extraction

For DNA extraction, approximately 30 mg of dried leaf tissue was homogenized with a TissueLyser II (Qiagen, Hilden, Germany). DNA was extracted using the DNeasy Plant Mini Kit (Qiagen, Hilden, Germany) according to the manufacturer's protocol. The DNA concentration was determined using a NanoDrop 2000 spectrophotometer (Thermo Fisher Scientific Inc., Waltham, Massachusetts, USA).

Simple Sequence Repeat markers

SSR markers were PCR-amplified from 40 ng genomic DNA using primers for markers STI0032, StI031, STI051 (Feingold et al., 2005), STM0031 and STM1052 (Milbourne et al., 1998) for the P208 population and StI047, StI0030 (Feingold et al., 2005) and STM1106 (Milbourne et al., 1998) for the P809 population, to check for introgression of the pollinator genome of *S. phureja*. Forward primers were M13-tailed (5'-GTAAAACGACGGCCAGT-3') at the 5'-end, and a second M13-forward primer labelled with IRD700 (Eurofins MWG, Ebersberg, Germany) was used (Schuelke, 2000). The PCR mixture consisted of a total volume of 20 µl containing 0.125 µM of the IRD700-labelled M13-forward primer, 0.025 µM of the marker-specific forward primer, 0.25 µM of the marker-specific reverse primer, 1 unit of DCS *Taq* polymerase (DNA Cloning Service e. K., Hamburg, Germany), 1 x Williams buffer (100 mM Tris-HCl (pH 8.0), 500 mM KCl, 20 mM $MgCl_2$, 0.01 % gelatin) and 0.15 mM of each dNTP. PCR conditions were as follows: initial denaturation for 5 minutes at 94 °C, followed by 25 cycles of 45 seconds at 94 °C, 1 minute at 63 °C and 1 minute at 72 °C, eight cycles of 30 seconds at 94 °C, 45 seconds at 52 °C and 1 minute at 72 °C, and a final extension of 10 minutes at 72 °C. After PCR, 100-250 µl of formamide loading dye (98 % formamide, 10 mM EDTA, 0.05 % pararosaniline) was added, and samples were denatured for three minutes at 95 °C. For each sample, 0.3 µl of diluted PCR product was separated on 6 % polyacrylamide gels (Sequagel XR, National Diagnostics, Nottingham, UK) on a LI-COR DNA Analyzer 4300 (LI-COR, Lincoln, Nebraska, USA) according to the manufacturer's protocol.

Amplified Fragment Length Polymorphism markers

AFLP analysis was performed for the P208 population as previously described by Vos et al. (1995) with minor modifications. For each genotype, 250 ng of DNA was digested with 10 units *Hind*III and 3 units *Mse*I restriction enzymes (New England Biolabs Inc., Ipswich, Massachusetts, USA). Preamplification was performed with adapter-specific primers. Preamplified samples of five or six genotypes were pooled into 42 bulks for the final amplification, for which an IRD700 end-labelled *Hind*III primer (Eurofins MWG, Ebersberg, Germany) with three selective bases (5'-AGACTGCGTACCAGCTT-AAC-3') and 16 different *Mse*I primers (5'-GACGATGAGTCCTGAGTAA-ANN-3') with three selective bases (AAA,

AAC, AAG, AAT, ACA, ACC, ACG, ACT, AGA, AGC, AGG, AGT, ATA, ATC, ATG, ATT) at the three prime end were used. Fragments were size separated as described above. AFLP analysis was performed subsequently in the individual genotypes of the respective bulks when *S. phureja*-specific marker bands were detected in the bulks.

SNP genotyping using the 12.8 k SolCAP potato SNP array

Using the 12.8 k SolCAP potato genotyping array, 219 genotypes of the P208 population and 39 genotypes of the P809 population, as well as the parental genotypes and the two pollinator clones of *S. phureja*, were genotyped for 12,808 SNPs (http://solcap.msu.edu/potato_infinium.shtml). P208 and the two *S. phureja* clones were genotyped with two repeats. In addition, 125 tetraploid German and Polish potato cultivars, which are listed in supplementary Table S4, were also genotyped. Custom genotyping was performed by Neogene Genomics (Neogene Genomics, Lincoln, Nebraska, USA).

Kompetitive Allele Specific PCR assay

SNP array results were validated using Kompetitive allele specific PCR (KASP) markers for the eight SNP markers solcap_snp_c2_10957, solcap_snp_c2_17747, solcap_snp_c2_25560, solcap_snp_c2_32982, solcap_snp_c2_35942, solcap_snp_c2_41768, solcap_snp_c2_42407 and solcap_snp_c2_52712. KASP primers were designed by LGC Genomics (LGC, Hoddesdon, UK) in a KASP by Design assay based on the context sequence information provided by the Solanaceae Coordinated Agricultural Project (http://solcap.msu.edu/data/potato_69011_map_context_DM_v3_superscaffolds.txt). PCR was performed with 50 ng of genomic DNA in a 10 µl reaction volume on an ABI StepOnePlus instrument (Thermo Fisher Scientific, Waltham, Massachusetts, USA) according to the protocol provided by LGC genomics.

Genetic mapping

Genetic mapping was performed for the P208 population using only single-dose SNP markers indicated by a 1:1 segregation. Skewed markers or markers with missing values for more than 7% of the genotypes were not considered. Linkage analysis was performed in JoinMap®4 (Van Ooijen, 2006) using the mapping function of Haldane (1919) and the regression mapping algorithm (Stam, 1993). For the construction of the linkage maps LOD scores between 6 and 15 were chosen. For each potato chromosome, two to four linkage maps were constructed.

Morphological characterization and QTL mapping

For 168 genotypes of the P208 population, three clones per genotype were potted in 5-liter pots with standard potting soil “Einheitserde” (type P) and were phenotyped in the greenhouse under semi-controlled conditions for the four morphological traits: number of tubers, tuber weight, shoot length, and number of nodes. Mean values were calculated for shoot length and number of nodes, which was measured and counted for three clones per genotype. Tubers were harvested from three clones, and the mean value was calculated for the tuber number per plant. All tubers were weighed, and the mean value for weight per tuber was calculated by dividing the overall tuber weight by the overall tuber number for each genotype. Initial testing for a normal distribution of the phenotypes was performed with a Shapiro-Wilk normality test using R software version 3.1.3 (R development core team, 2011). Phenotypic data were Box-Cox transformed (Box & Cox, 1964) using the Free Statistics Software version 1.2.1 (Wessa, 2016) for QTL analysis with MapQTL®6 (Van Ooijen, 2009) with a permutation test with 1,000 permutations and subsequent interval mapping and Multiple-QTL mapping with default settings.

Results

Generation of the dihaploid populations and selection of true dihaploids by morphology and flow cytometry

A subset of seeds from pollinations with the two inducer lines, 1178 seeds for P208 and 498 seeds for P809, were used for in vitro germination. Emerging seedlings were preselected morphologically for anthocyanin pigmentation at the nodes of the plantlets. As this pigmentation is inherited from the haploid inducer, it is only visible in triploid and tetraploid progeny. The diploid ploidy level of the morphologically selected seedlings was subsequently confirmed by flow cytometry where genotypes were clearly classified in di-, tri- and tetraploids in relation to the diploid and tetraploid genotypes that were used as standards.

For the P208 x *S. phureja* IVP101 cross, we obtained 112 dihaploid genotypes (11.5 %), whereas 106 dihaploid genotypes (51.5 %) were generated from the P208 x *S. phureja* IVP35 cross, where seeds were pre-selected for an embryo spot before germination (Table 1). Altogether, 218 dihaploid genotypes for P208 were generated. The P809 x *S. phureja* IVP101 cross resulted in 89 dihaploid genotypes (17.9 %). Non-germinated seeds were not taken into consideration for the percentages.

Table 1: Establishment of two monoparental dihaploid populations derived from two different tetraploid cultivars.

Cross	Number of seeds used for *in vitro* germination	Number of dihaploid genotypes	Percentage of dihaploid genotypes
P208 x *S. phureja* IVP101	972	112	11.5 %
P208 x *S. phureja* IVP35	206	106	51.5 %
P809 x *S. phureja* IVP101	498	89	17.9 %

Test of introgression of *Solanum phureja* DNA into the dihaploid potato populations

Introgression analysis was performed using different SSR markers that showed fragments specific for the male dihaploid inducer. The 218 dihaploid genotypes derived from P208 were screened with five SSR markers: STI0032, StI031, STI051, STM0031 and STM1052. Genotype K4-7 showed at least one *S. phureja*-specific marker band for the markers (Figure 1). The 89 dihaploid genotypes of P809 were screened with three SSR markers: StI0030, STI047 and STM1106. With SSR marker StI0030, genotype P35-3 showed a putative introgression of *S. phureja* DNA. SSR marker StI047 additionally identified a second genotype, P10-2, with a putative introgression (Figure 2), whereas with the SSR marker STM1106, none of the genotypes showed a *S. phureja*-specific marker band. AFLP analysis with bulks of five to six genotypes of the P208 population was performed and revealed one additional genotype, B35F-11, which showed three *S. phureja* specific marker bands for the primer combination *Hind*III-AAC/*Mse*I-ACT (data not shown).

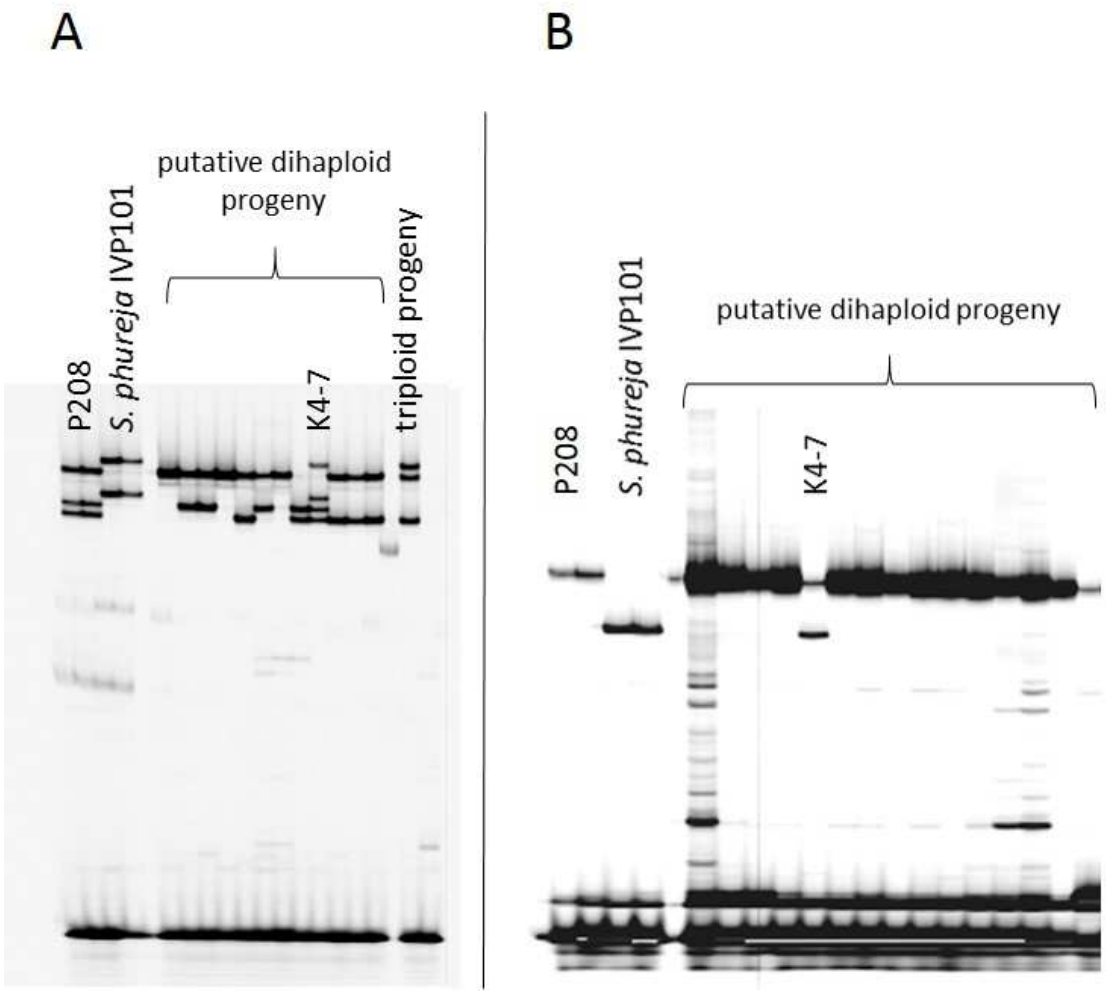

Figure 1: Polyacrylamide gel electrophoresis of SSR markers StI031 (A) and STI051 (B) analyzed in a subset of putative dihaploid genotypes of the P208 x *S. phureja* IVP101 cross. Genotype K4-7 showed a *S. phureja*-specific allele for both SSR markers in addition to the maternal alleles.

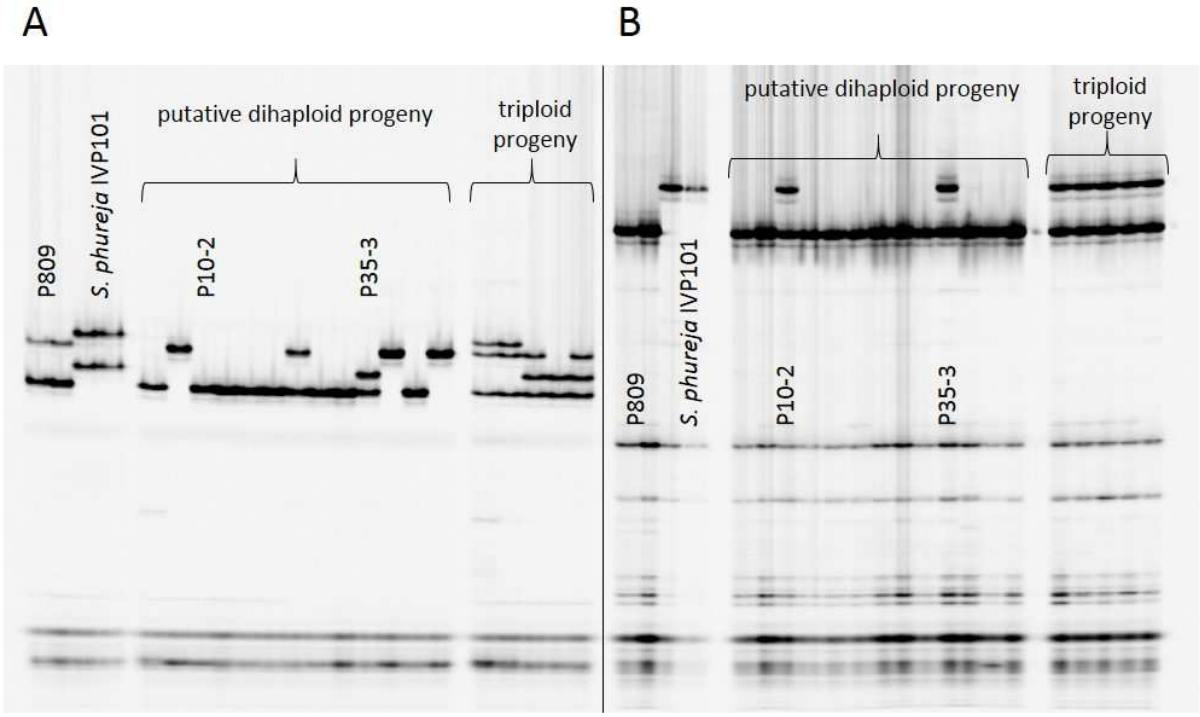

Figure 2: Polyacrylamide gel electrophoresis of SSR markers StI047 (A) and StI0030 (B) analyzed in a subset of putative dihaploid genotypes of the P809 x *S. phureja* IVP101 cross. Genotype P35-3 showed a *S. phureja*-specific marker band for SSR markers StI047 and StI0030. With SSR marker StI0030, genotype P10-2 was additionally identified to show a *S. phureja* introgression.

All 218 genotypes of the P208 population and a subset of 39 genotypes of the P809 population, as well as the parental genotypes and the two pollinator clones *S. phureja* IVP101 and IVP35, were genotyped for 12,808 SNPs using the SolCAP potato genotyping array. The SNP marker data were used to check for additional *S. phureja* introgressions in the dihaploid genotypes. Genotypes K4-7 and B35F-11 were excluded from further analysis because they showed putative introgressions in the SSR and AFLP analyses, as well as one additional genotype that did not allow proper SNP genotyping. For the P208 x *S. phureja* IVP101 cross, 647 SNP markers were analyzed that were homozygous for one allele in P208 and heterozygous or homozygous for the other allele in the pollinator. For the P208 x *S. phureja* IVP35 cross, 633 SNP markers were suitable for introgression analysis, and for the P809 x *S. phureja* IVP101 cross, 795 SNP markers showed according allele configurations. Introgression analysis revealed twelve SNPs for each of the P208 x *S. phureja* IVP101 and P208 x *S. phureja* IVP35 crosses, and 27 SNPs for the P809 x *S. phureja* IVP101 cross, indicating a putative introgression of the pollinator DNA of *S. phureja* into the dihaploid progeny (Table S1).

The percentages of dihaploid genotypes that showed a putative *S. phureja* introgression for the individual SNP markers ranged from 0.9 % to 57.66 % for the P208 x *S. phureja* IVP101 cross, from 0.94 % to 66.04 % for the P208 x *S. phureja* IVP35 cross and from 2.56 % to 69.23 % for the P809 x *S. phureja* IVP101 cross (Table S1). *S. phureja*-specific markers could be detected on all 12 chromosomes, excluding chromosomes 1 and 10 in the dihaploid progeny derived from P809. SNP markers solcap_snp_c2_54921 and solcap_snp_c2_52621 could not be located on any potato chromosome in the potato genome browser.

In the two populations derived from P208, introgression rarely occurred with more than one marker per chromosome. Introgressions on the same chromosome showed only a few overlapping genotypes. In the P809 population, introgression markers were more commonly located on the same chromosomes. Most notably, two marker pairs on chromosomes 4 (solcap_snp_c2_26773 and solcap_snp_c1_3311) and 8 (solcap_snp_c2_29491 and solcap_snp_c1_6140) showed large numbers of overlapping genotypes, with 18 of 19 and 22 of 24 genotypes, respectively, showing introgression of the pollinator genome (Table S2).

Nearly all dihaploid genotypes displayed single SNP markers that were specific for *S. phureja*. Only two genotypes, one for each of the crosses with P208, did not show any *S. phureja*-specific allele configuration in the SNP genotyping. The percentages of *S. phureja* markers that putatively introgressed into the individual dihaploid genotypes were very low, ranging from 0.16 % and 1.24 % for the P208 x *S. phureja* IVP101 cross and from 0.16 % to 1.26 % for the individual dihaploids from the P208 x *S. phureja* IVP35 cross, with an average of 0.53 % and 0.56 %, respectively (Table S3). For the P809 x *S. phureja* IVP101 cross, all genotyped individuals of the dihaploid progeny showed an introgression of the *S. phureja* genome ranging from 0.63 % to 1.89 %, with an average of 1.34 % (Table S3).

To validate these results, a KASP assay was performed in the P208 population for eight introgression markers. For three markers, separation of the different SNP genotypes was not possible in the cluster plots. KASP assays were consistent with the SNP array genotyping for three markers in the dihaploid genotypes. The two remaining markers (solcap_snp_c2_32982 and solcap_snp_c2_42407) showed different results in the KASP assay and on the SNP array for one or two genotypes, respectively (Table 2), indicating calling errors.

Table 2: Comparison of SNP genotyping results between the SolCAP SNP array and the KASP assay in the P208 population. Percentages of not available (NA) marker data for both methods are also listed.

SNP marker	Consistent results	Differing results	NA SNP array	NA KASP assay
solcap_snp_c2_17747	96.35 %	0 %	1.37 %	2.28 %
solcap_snp_c2_25560	97.26 %	0 %	0.91 %	1.83 %
solcap_snp_c2_32982	98.17 %	0.46 %	0.46 %	0.46 %
solcap_snp_c2_35942	91.78 %	0 %	7.76 %	0.46 %
solcap_snp_c2_42407	92.24 %	0.91 %	6.39 %	0.46 %

Genetic mapping in a dihaploid potato population

SNP marker data obtained from genotyping with the 12.8 k SolCAP SNP array was used for genetic mapping in the dihaploid P208 population. The SNPs were quality filtered, and only markers with less than 15 missing values within the dihaploid potato population were considered for mapping. Of these 9,953 markers, 9,286 markers (93.3 %) showed identical SNP calling results in two repetitions for P208. Altogether, 647 SNP markers showed missing values in at least one repetition in P208, and different genotyping results were identified for 20 SNP markers (0.2 %) in the two repetitions. These markers were excluded from further analyses. Of the 9,286 SNP markers that showed identical results regarding allele configurations in the two repetitions of P208, 4,682 SNP markers (50.4 %) segregated in the dihaploid P208 population (Table 3). For construction of the genetic maps, 2,548 single-dose SNP markers displaying a 1:1 segregation were used.

Table 3: Summary of the SNP marker data derived from the SNP array in the dihaploid potato population derived from P208.

Filtering criteria	no. of SNPs	segregation ratios	no. of SNPs
all SNPs	12,808		
after quality filtering	10,376		
less than 15 missing values	9,953		
identical in both samples of P208	9,286		
segregating in the DH population	4,682		
allele configuration AA:AB	1,358	1:1 segregation	1,129
		5:1 segregation	32
		distorted	197
allele configuration BB:AB	1,701	1:1 segregation	1,419
		5:1 segregation	32
		distorted	250
allele configuration AA:AB:BB	1,573		
allele configuration AA:BB	51		

Altogether, 45 linkage groups were constructed, with LOD scores between 6 and 15, with a total of 2,387 mapped SNP markers, from which 1,290 markers were excluded during the mapping process to provide a better overview because they showed an identical segregation pattern to previously mapped markers. One hundred and sixty two markers could not be mapped on any linkage group or were excluded manually. For each of the potato chromosomes, four linkage groups were constructed, excluding chromosomes 2 and 6, where only three and two linkage groups could be constructed, respectively (Table 4). The total length of the genetic maps was 2,675.6 cM, with an average of 55.24 SNP markers per linkage group. The genetic maps for the four linkage groups of potato chromosome 9 are shown in Figure 3. Genetic maps for all twelve potato chromosomes are shown in supplementary Figures S1-S12.

Table 4: Number of linkage groups per chromosome that were constructed for the P208 population with the average number of SNP markers per linkage group.

chromosome	no. of LGs	average no. of loci per LG
1	4	61.75
2	3	64.67
3	4	41.25
4	4	55.25
5	4	55.25
6	2	100.5
7	4	55.25
8	4	37
9	4	67
10	4	46.25
11	4	30.25
12	4	48.5

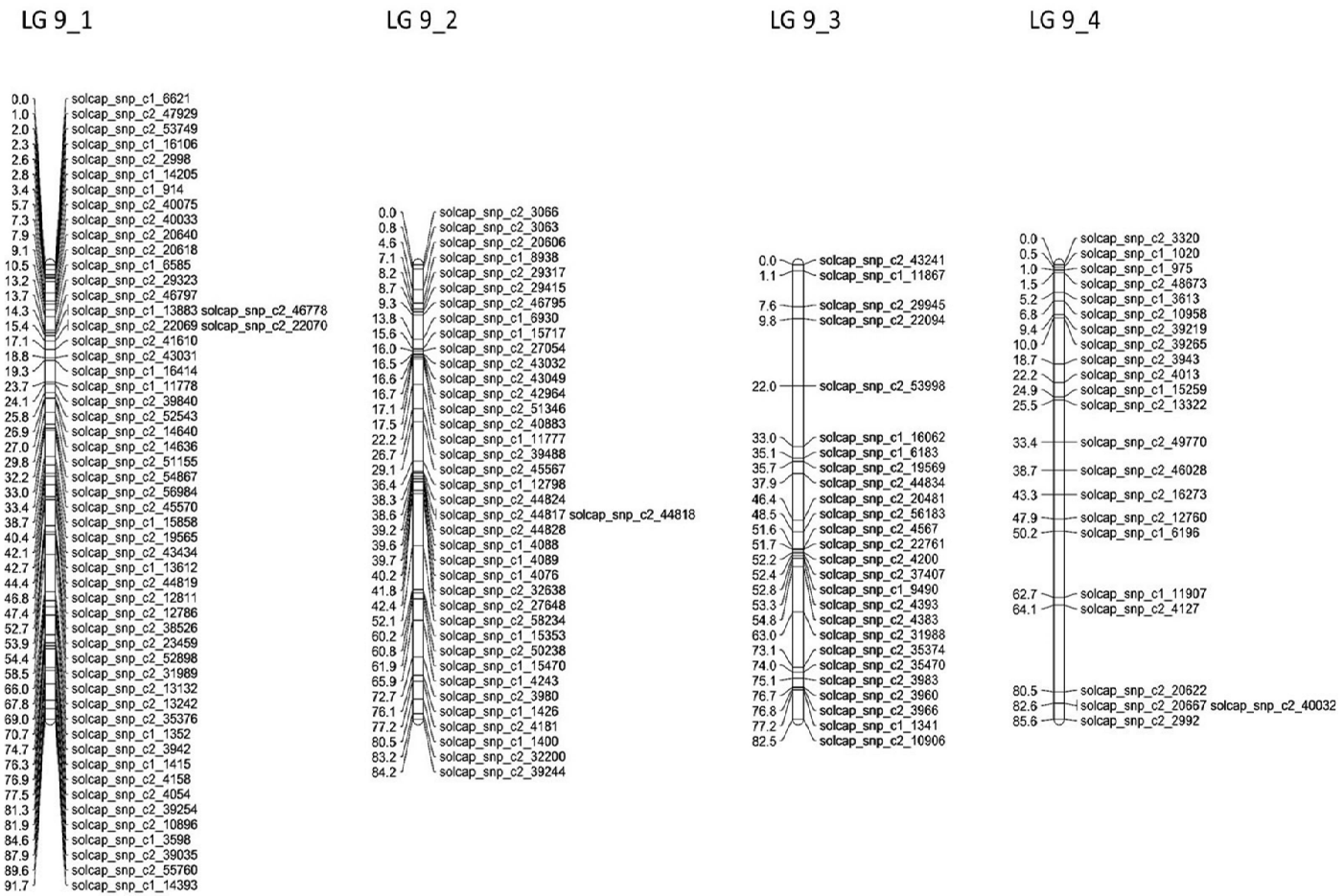

Figure 3: Linkage groups constructed in JoinMap®4 with single-dose SNP markers segregating in the P208 population. The four linkage groups represent potato chromosome 9. In total, 268 SNP markers could be mapped for this chromosome, with an average of 67 SNP markers per linkage group. SNP markers showing the same segregation pattern in the population as previously mapped markers were excluded during the mapping process for reasons of clarity.

To evaluate whether a monoparental population allows the mapping of more markers in linkage from the donor genotype compared to a conventional cross with a second tetraploid parent, we analyzed theoretical numbers of putatively segregating single-dose markers in conventional crosses between P208 and a second tetraploid parent. Using the SNP marker data available from the SNP array for 125 tetraploid cultivars of different breeding origins (http://www.bdp-online.de/de/GFPi/Abteilungen_Projekte/Abteilung_Kartoffeln/EU_Projekt__Cornet-SynTest/Participating_Breeders/), all 2,548 single-dose markers identified for P208 were considered, and the tetraploid cultivars were screened for markers with one or more doses of the same marker allele. In a cross with such a parent, those markers would no longer segregate as single-dose markers but would display more complex segregation patterns. The number of useful single-dose markers in a tetraploid cross segregating in linkage to loci on one of the four chromatids of P208 would be reduced to 543 to 928 SNP markers, representing 21.31 % to 36.42 % of the useful SNPs in our dihaploid population (Table 5). Detailed SNP marker information for each tetraploid cultivar is provided in supplementary Table S4.

Table 5: Number of single-dose markers derived from P208 in putative progenies from crosses to various tetraploid varieties. The numbers of putative single-dose markers in a biparental cross for 125 tetraploid cultivars are listed, as well as the percentage of useful single-dose markers in a biparental cross when compared to the 2,548 single-dose markers in the P208 population.

Number of single-dose markers in a cross between P208 and a second tetraploid cultivar	Number of tetraploid cultivars	Percentage of useful SNP markers compared to single-dose markers in P208
<600	2	<23.55 %
601-700	23	23.59 % - 27.47 %
701-800	66	27.51 % - 31.40 %
801-900	32	31.46 % - 35.32 %
>900	2	>35.36 %

Morphological characterization and QTL mapping

For 168 genotypes of the P208 population, three clones per genotype were phenotyped for shoot length, number of nodes, number of tubers and tuber weight. For the remaining genotypes of the P208

population, acclimatization from *in vitro* culture was not successful, or the overall plant vigor was very low, and therefore they were not included in the analysis.

The average shoot length ranged from 2.0 cm to 167.33 cm, and the mean number of nodes was between 3.0 and 36.3. The average tuber number ranged from 0 to 101.67 tubers per plant, with a mean weight per tuber between 0.23 g and 200.29 g (Figure S13-S16).

Only the data for the average number of nodes showed a normal distribution, with a p-value of 0.1726 based on the Shapiro-Wilk normality test (Table S5). The phenotypic data for the remaining traits was transformed using a Box-Cox transformation with subsequent testing for a normal distribution. Box-Cox-transformed data for the average number of tubers and average tuber weight showed a normal distribution with a p-value of 0.3171 and 0.3685, respectively. A normal distribution of the average shoot length was assumed for the QTL analyses. Box-Cox-transformed data for all phenotypic traits showed significant positive correlations (Table S6).

For the QTL analyses, a genome-wide LOD score of 3.4 was chosen after permutation tests with 1,000 permutations for all phenotypic traits in MapQTL®6, except for the average number of nodes, for which a genome-wide LOD score of 3.2 was chosen. Significant QTLs were found for all phenotypic traits by interval mapping (Table 6). Two QTLs for shoot length were detected on potato chromosomes 2 and 4, two QTLs for the number of nodes were found on chromosomes 4 and 5 and one significant QTL each was detected for the tuber number and tuber weight on potato chromosome 4. No additional QTLs were detected using the Multiple-QTL mapping approach, but the QTL for shoot length located on chromosome 2 could not be detected using this approach. Intervals could be minimized in the Multiple-QTL mapping approach in comparison to the interval mapping, except for the QTL on chromosome 4 for the number of nodes. The QTL interval for tuber numbers located on chromosome 4, for example, could be minimized from approximately 30 cM in the interval mapping to less than 3 cM in the Multiple-QTL mapping approach (Figure 4). QTL charts for all phenotypic traits are shown in supplementary Figures S17-S22.

Table 6: QTLs for tuber weight (TW), number of tubers (TN), shoot length (SL) and number of nodes (N) using an Interval mapping (IM) and a Multiple-QTL mapping approach (MQM). The positions of the respective QTLs are listed, as well as the SNP markers with the highest LOD score and the explained variance.

trait	mapping approach	chr.	LG	position [cM]	LOD score	marker with highest LOD score	physical position	position [cM]	LOD score	expl. variance [%]
TW	IM	4	4_1	56.758-91.810	3.65-12.92	solcap_snp_c2_29872	2,714,003	86.160	12.92	28.5
TW	MQM	4	4_1	82.861-91.810	9.73-12.92					
TN	IM	4	4_1	62.067-91.810	3.79-6.07	solcap_snp_c2_11569	6,210,370	78.504	6.07	14.5
TN	MQM	4	4_1	76.792-79.504	5.31-6.07					
SL	IM	2	2_1	41.238-42.917	3.72-3.77	solcap_snp_c1_13236	31,663,265	42.917	3.73	9.7
				44.612-57.206	3.61-4.48	solcap_snp_c2_38952 /solcap_snp_c2_5213	28,230,202/ 28,112,384	51.912/ 51.925	4.48	11.6
				58.994-61.852	3.41-3.52	solcap_snp_c2_38007	21,868,087	61.852	3.52	9.2
SL	MQM	2	2_1	-	-	-	-	-	-	-
SL	IM	4	4_1	75.870-91.810	3.47-6.74	solcap_snp_c2_29872	2,714,003	86.160	6.74	16.9
SL	MQM	4	4_1	82.861-85.861	3.91-5.78	solcap_snp_c2_45927	3,562,152	82.861	3.91	9.0
N	IM	4	4_1	84.861-91.810	3.35-4.05	solcap_snp_c2_29872	2,714,003	86.160	4.00	10.4
N	MQM	4	4_1	83.861-91.810	3.20-4.25				4.25	10.0
N	IM	5	5_4	97.000-110.467	3.32-3.70	solcap_snp_c2_50317	NA	108.824	3.57	9.3
N	MQM	5	5_4	96.000-108.824	3.65-4.06				3.81	8.9

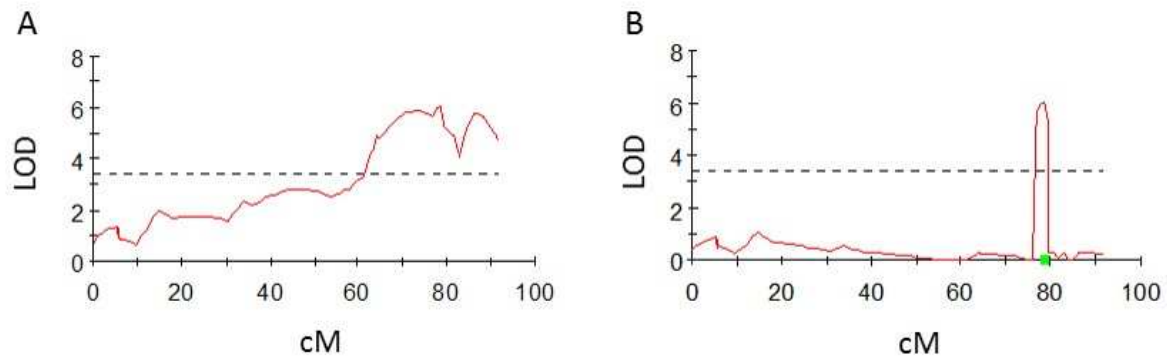

Figure 4: LOD profile on a linkage group for chromosome 4 for the average number of tubers using interval mapping (A) and Multiple-QTL mapping (B). LOD values above the significance threshold of 3.4 (dashed line) were detected with both approaches. The QTL intervals could be narrowed down from approximately 30 cM to less than 3 cM using the Multiple-QTL mapping approach.

Discussion

Although, haploid techniques have been used for decades to improve cultivar breeding in potato (Hougas & Peloquin, 1958; Hougas et al., 1958; Chase, 1963), conventional breeding programs are almost exclusively conducted at the tetraploid level. In contrast, genetic studies have mostly been carried out at the diploid level with dihaploid genotypes crossed to diploid potato species. Genetic analyses in which monoparental dihaploid lines have been used directly are very rare, and the population sizes were very small with only 37 (Pineda et al., 1993) and 57 dihaploid individuals (Song et al., 2005). In this study, we show that the generation of monoparental dihaploid populations is a useful tool to dissect interesting traits of an autotetraploid genotype for genetic analyses. To our knowledge, this is the first study to show the direct use of a large population of monoparental dihaploids for the construction of genetic linkage maps and QTL mapping.

The procedure we chose to select dihaploid progeny using a combination of morphological markers and flow cytometry, although more laborious than conventional tetraploid crosses, is an efficient strategy for the generation of such populations because most of the unwanted triploid and tetraploid progeny can be easily identified phenotypically by the presence of anthocyanin pigmentation in the nodes. As flow cytometry allows the rapid high-throughput screening of large numbers of samples, the selection of dihaploid genotypes is a straightforward approach. The frequencies of dihaploids obtained from *S. phureja* IVP101 crosses were relatively low at 11.5 % and 17.9 %, but considerably higher after pre-selection for an embryo spot in the cross of *S. phureja* IVP35 with 51.5 % dihaploid genotypes (Table 1). Hutten et al. (1994) showed that when comparing the dihaploid induction ability of different dihaploid inducers of *S. phureja*, clone IVP101 performed better than clones IVP35 and IVP48,

indicating that pre-selection for an embryo spot on the seeds before *in vitro* germination is an efficient way to increase the number of dihaploid genotypes. However, pre-selection for an embryo spot on the seeds was found to be very laborious and time-consuming due to the dark seed color of seeds derived from P208. Pre-selection for anthocyanin pigmentation in the nodal bands of the seedlings, however, was very efficient for selecting true dihaploids, and diploid ploidy was confirmed by flow cytometry (data not shown).

It has been reported that dihaploid induction via anther culture may be preferable to *S. phureja* pollination with higher frequencies of dihaploids and faster shoot formation (Schwarzfischer et al., 2002), although both haploid techniques are laborious and time-consuming. One major disadvantage that has been reported in dihaploids derived from *S. phureja* pollinations is the introgression of the pollinator genome in the dihaploid progeny (Clulow et al., 1991). Our results showed that one and two dihaploid genotypes of the P208 population and the P809 population, respectively, contained significant introgressions, indicating that elimination of *S. phureja* chromosomes occurs after fertilization and may be genotype-specific for the tetraploid parental genotype.

Genotyping with the 12.8 k SNP array revealed additional putative *S. phureja* introgressions in the dihaploid progeny. The number of SNP markers that could be used for introgression analysis was relatively low as only 633 to 795 SNP markers could be considered that were homozygous for one allele in the tetraploid parental genotypes and heterozygous or homozygous for the second allele in the pollinator genotype. SNP markers specific for the *S. phureja* inducers occurred in almost all genotypes and on nearly all of the potato chromosomes (Table S1). These results are in contrast to those of Straadt & Rasmussen (2003), who observed no introgression of pollinator DNA in 30 dihaploid genotypes derived from crosses with *S. phureja* IVP101 using AFLP markers for their introgression analysis but also indicated that the introgression rate may be influenced by the tetraploid *S. tuberosum* seed parent. Rarely, more than a single introgression marker could be detected on the same chromosomes in the P208 population, but more often in the P809 population. Markers indicating introgression located on the same chromosome were further analyzed to distinguish true introgression events from putative artifacts due to genotyping errors. Therefore, adjacent markers on either side of the introgression markers were evaluated according to Bourke et al. (2015), who analyzed the occurrence of double reduction rates in tetraploids and used a strict criterion to distinguish true double reduction events from genotyping errors. They assumed a double reduction only when three consecutive markers showed the expected allele configurations. When applying this criterion in our populations, none of the markers would fulfill the requirements for a true introgression. However, the marker density was relatively low, and sometimes the distances between two markers were quite large or the markers showing an introgression did not have adjacent markers with differential allele configurations in the parental genotypes on both sides. An exception to this phenomenon are the

markers solcap_snp_c2_29491 and solcap_snp_c1_6140 on chromosome 8, which are located proximately to one another and show highly consistent occurrences in the same genotypes. These markers could indicate a true introgression of the pollinator genome, although they do not fulfil the criterion used by Bourke et al. (2015). Therefore, our results indicate that the overall percentages of the *S. phureja* genome in the dihaploid populations are very low and do not disturb genetic analyses at the diploid level. This result is supported by verification of selected SNP array marker data for the P208 population using a KASP assay. KASP markers have been used in various plant species for SNP genotyping and validation (Cortés et al., 2011; Rosso et al., 2011; Byers et al., 2012) and for the determination of allele dosages in polyploids (Cuenca et al., 2013). Of the five SNP markers that could be analyzed in the population, two markers, solcap_snp_c2_32982 and solcap_snp_c2_42407, showed differing results in the KASP assay for one or two genotypes, respectively (Table 2).

Genetic mapping was performed in the P208 population using single-dose marker data obtained from the 12.8 k SNP array. Our approach did not include multidose markers, an approach used by many researchers to improve the map density (da Silva et al., 1995; Kriegner et al., 2003; Aitken et al., 2007; Hackett et al., 2013), because herein we focused only on markers segregating in linkage from the tetraploid donor genotype. Furthermore, single-dose markers allow higher mapping precision, and with a population size of 218 progeny from which only 168 could be phenotyped, map resolution was considered to be sufficient using only single-dose markers. The fraction of markers that displayed distorted segregation was 16.7 % of all segregating simplex markers and is within the range of other observations in diploid potatoes where 6 to 57 % of markers showed distorted segregation (Gebhardt et al., 1991; Felcher et al., 2012; Manrique-Carpintero et al., 2016;). With a total of 45 linkage groups that were constructed with 2,387 mapped markers (93.7 % of the simplex markers), only two of the twelve potato chromosomes were represented by less than four linkage groups, which could either be due to large homozygous stretches on these chromosomes or because insufficient numbers of markers were mapped. However, the number of mapped markers in this study is considerably higher compared to linkage maps that were constructed using mostly AFLP and SSR markers for tetraploid (Bradshaw et al., 2004; 2008; Meyer et al., 1998; Bryan et al., 2004) or diploid potato populations (Gebhardt et al., 1989; 1991; Bryan et al., 2002), with only a few hundred mapped markers. Notable exceptions are the high-density map constructed by van Os et al. (2006) with more than 10,000 AFLP markers and the tetraploid maps constructed by Hackett et al. (2013) with 3,839 mapped SNP markers.

We attempted to evaluate whether a monoparental population allows the mapping of more markers in linkage from the donor genotype compared to a conventional cross with a second tetraploid parent in which the heterozygosity for the same marker alleles present in the donor genotype leads to more complex banding patterns. Therefore, we analyzed the theoretical number of segregating single-dose markers of P208 crossed with different tetraploid genotypes for which SNP information from the SNP

array was available. In summary, we found that a significant number of markers currently segregating as single-dose markers in our dihaploid population had a match with at least one dose in the putative parents (Table 5; Table S4), which would reduce the number of useful single-dose markers of P208 to only 21.31 % (543) up to 36.42 % (962) of SNP markers from the 2,548 simplex SNP markers segregating in the monoparental dihaploid population. Thus, a much larger fraction of single-dose markers from the maternal parent can be analyzed in our monoparental population because no markers from a second parent will complicate marker segregation patterns. Additionally, all additional single-dose markers from a second tetraploid parent will automatically segregate in repulsion and therefore are only of limited use for mapping maternal traits in conventional crosses. Although gamete formation on the maternal side is not different from a conventional tetraploid cross, segregation patterns are much simpler because only combinations of two alleles are present. While this is not different from the frequently used populations from two dihaploids, only maternal alleles segregate. Alternative approaches to analyzing the genetics of the tetraploid donor are less effective and more time and resource-consuming. Intercrosses of single dihaploids would require an additional generation and would suffer from self-incompatibility that renders a number of cross combinations unsuccessful. Furthermore, to capture the whole genetic variation of a tetraploid, a large number of successful cross combinations would be needed.

To demonstrate the additional utility of our monoparental dihaploid population, QTLs were analyzed for four phenotypic traits: number of tubers per plant, tuber weight, shoot length and number of nodes using an interval and a Multiple-QTL mapping approach. Many QTL studies have been conducted in potato for various agronomic traits and yield characteristics (Bonierbale et al., 1993; Freyre & Douches, 1994, Schäfer-Pregl et al., 1998; Bradshaw et al., 2008; McCord et al., 2011), as well as quantitative resistances to late blight (Leonards-Schippers et al., 1994; Collins et al., 1999; Danan et al., 2011, Massa et al., 2015) and to cyst nematodes (Rouppe van der Voort et al., 1998; Bryan et al., 2002). More recently, Schönhals et al. (2017) mapped QTLs for tuber yield, starch content and starch yield in three populations of tetraploid varieties and breeding clones and identified genomic regions on all twelve chromosomes with QTLs for the analyzed traits. Rak et al. (2017) identified multiple QTLs for several tuber traits in a biparental potato population and found three QTLs for the number of tubers on chromosomes 4, 5 and 10 and two QTLs for tuber weight, width and length on chromosomes 5 and 6. In this study, we identified one QTL each for tuber number and tuber weight on chromosome 4, explaining 14.5 % and 28.5 % of the variance of these phenotypic traits (Table 6). QTLs for both traits are located closely to one another, where the markers with the highest LOD score span a genomic region of less than 3.5 Mbp, indicating that the underlying genes for tuber number and tuber weight are tightly linked. This locus on the short arm of chromosome 4 represents an additional QTL for tuber number to that of Rak et al. (2017), which is located approximately 60 Mbp farther on the long arm of

the same chromosome. Additional QTLs were identified for shoot length on chromosomes 2 and 4 and for number of nodes on chromosomes 4 and 5. In the interval mapping, the marker with the highest LOD score was the same for both traits, explaining 16.9 % of the variance in shoot length and 10.4 % of the variance in the number of nodes (Table 6). Additionally, shoot length and number of nodes were highly correlated with a correlation coefficient of 0.91 (Table S5), indicating that these traits are controlled by the same underlying gene on chromosome 4 but are additionally controlled by different genes located on chromosomes 2 and 5 for shoot length and number of nodes, respectively. A QTL for plant height on chromosome 5 has also been described by Bradshaw et al. (2004) and Hackett et al. (2014) and is located in the same chromosomal region as the QTL we found for the number of nodes. These results show that QTL mapping in a monoparental dihaploid population is suitable to confirm known as well as to detect additional QTLs in a single tetraploid cultivar. Nevertheless, QTL mapping is highly dependent on the genetic background of the studied populations and must be analyzed in a tetraploid genetic background for applications in commercial breeding.

Conclusions

This is the first study, to our knowledge, to present the direct use of a large monoparental dihaploid potato population consisting of more than 200 genotypes for genetic analyses. Introgression of the *S. phureja* pollinator genome was very low within the progeny. Construction of genetic linkage maps using single-dose SNP markers as well as QTL mapping of four phenotypic traits was successful, with a considerably higher number of SNP markers segregating in linkage than in conventional crosses between two tetraploid genotypes. Although the construction of dihaploid populations is more laborious than in conventional breeding, our approach represents a promising strategy with monoparental dihaploids as a useful tool for genetic analysis of a single tetraploid potato cultivar circumventing problems associated with tetraploid genetics.

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Author Contributions Statement

AB performed the experiments, analyzed the data, prepared all tables and figures and wrote the manuscript. FC generated the populations and determined the ploidy of genotypes by flow cytometry.

DT planned and supervised the experiments. JL, JS, ET and HH provided the plant material for the crossings and planned the experiments. ML planned and supervised the experiments and corrected the manuscript. TD planned and supervised the experiments and wrote part of and corrected the manuscript.

Conflict of Interest Statement

The authors state that there are no conflicts of interest related to this publication.

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2.2. Improved genetic resolution for linkage mapping of resistance to potato wart in monoparental dihaploids with potential diagnostic value in tetraploid potato varieties

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Improved genetic resolution for linkage mapping of resistance to potato wart in monoparental dihaploids with potential diagnostic value in tetraploid potato varieties

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Abstract

We analyzed a segregating monoparental dihaploid potato population comprising 215 genotypes derived from a tetraploid variety that is highly resistant to *Synchytrium endobioticum* pathotypes 18 and 6. The clear bimodal segregation for both pathotypes indicated that a major dominant resistance factor in a simplex allele configuration was present in the tetraploid donor genotype. Compared to that in previous analyses of the same tetraploid donor in conventional crosses with susceptible tetraploid genotypes, a segregation pattern with a reduced genetic complexity of resistance in dihaploids was observed here. Using the 12.8 k SolCAP SNP array, we mapped a resistance locus to the *Sen1* region on potato chromosome 11. The improved mapping resolution provided by the monoparental dihaploids allowed for the localization of the genes responsible for the resistance to both pathotypes in an interval spanning less than 800 kbp on the reference genome. Furthermore, we identified eight molecular markers segregating without recombination to P18 and P6 resistance. Also, two developed markers display improved diagnostic properties in an independent panel of tetraploid varieties. Overall, our data provide the highest resolution mapping of wart resistance genes at the *Sen1* locus thus far.

Keywords: *Synchytrium endobioticum*, SNP array, molecular marker, pathotype, recombination

Key message

We achieved improved mapping resolution of the major wart resistance locus *Sen1* in a dihaploid population using SNP data and developed additional markers with diagnostic value in tetraploid varieties.

Introduction

The obligate biotrophic soil-borne fungus *Synchytrium endobioticum* (Schilb.) Perc. belongs to the Chytridiomycetes class and is the causal agent of potato wart disease. This pathogen is classified as an A2 quarantine pest by the European and Mediterranean Plant Protection Organization (EPPO 2004) and is globally distributed (Obidiegwu et al. 2014). This pathogen can infect potato tubers, stolons and stems and cause yield losses of up to 50-100% (Hampson 1993; Melnik 1998). The typical symptoms of potato wart include the formation of cauliflower-like irregular galls that vary in size and shape (Franc 2007). The wart tissue consists of hypertrophic, tumor-like dividing cells with thin-walled summer sori and thick-walled winter sori that can survive in soil for up to 30 to 40 years (Laidlaw 1985). Controlling this disease is very difficult due to the limited ability of fungicides to access the winter sporangia. Furthermore, chemicals that are effective against potato wart disease are also phytotoxic (Hampson 1977; Gunacti and Erkliç 2013). Strict phytosanitary measures and the breeding and cultivation of resistant cultivars are the only feasible strategies for controlling potato wart disease. More than 40 different pathotypes of this pathogen have been reported (Baayen et al. 2006; Çakır et al. 2009; Przetakiewicz 2015), and pathotypes 1, 2, 6, 8 and 18 are considered the most important forms of the fungus (Stachewicz 2002). Pathotype 18 is also considered to be among the most aggressive pathotypes, and only a few resistance genes have been characterized thus far.

Two dominant resistance genes, *Sen1* and *Sen1-4*, have been identified in diploid potato mapping populations. *Sen1* is located on potato chromosome 11 and confers resistance to *S. endobioticum* pathotype 1 (Hehl et al. 1999). In addition, according to Hehl et al. (1999), the *Sen1* locus is closely linked to resistance gene-like sequences that are homologous to the *N* gene, which is responsible for TMV resistance in tobacco. Brugmans et al. (2006) identified a *Sen1-4* locus, which is responsible for resistance to pathotype 1, on chromosome 4.

In two half-sib families, Ballvora et al. (2011) identified three Simple Sequence Repeat (SSR) markers that were linked to genes, which are responsible for resistance to *S. endobioticum* and mapped on chromosomes 1, 9 and 11: *Sen/2/6/18-I* on chromosome 1 confers resistance to pathotypes 2, 6 and 18; *Sen18-IX* on chromosome 9 confers resistance to pathotype 18; and *Sen1-XI* on chromosome 11 confers resistance to *S. endobioticum* pathotype 1. The resistance to pathotypes 2, 6 and 18 is highly

correlated but independent of the resistance to pathotype 1. The *Sen* alleles that increased or decreased resistance to potato wart were inherited from both the resistant and the susceptible parents (Ballvora et al. 2011). Furthermore, Groth et al. (2013) detected a major Quantitative Trait Locus (QTL) responsible for resistance to pathotype 1 near the *Sen1* locus on chromosome 11. QTLs responsible for resistance to pathotypes 1, 2, 6 and 18 have been detected on potato chromosomes 6, 8 and 11, and QTLs responsible for resistance to pathotypes 2, 6 and 18 have been detected on chromosomes 7 and 10. A QTL responsible for resistance to pathotypes 6 and 18 has been detected on chromosome 2, and a QTL responsible for resistance to pathotype 2 has been detected on chromosome 1. Additionally, Obidiegwu et al. (2015) used the 8.3 k SolCAP SNP array to genotype a tetraploid potato population and identified new and previously known loci responsible for resistance to various *S. endobioticum* pathotypes on chromosomes 1, 3, 4, 10, 11 and 12; *Sen1* was a major resistance locus on chromosome 11, and several minor resistance loci were observed on various other chromosomes.

Several linkage maps have been constructed in potato (Bonierbale et al. 1988; Gebhardt et al. 1989; Jacobs et al. 1995; Van Os et al. 2006). Most potato linkage maps are based on diploid potato populations to facilitate genetic segregation and inheritance models in the tetraploid species (Bonierbale et al. 1988; Jacobs et al. 1995; Van Os et al. 2006; Felcher et al. 2012). Primary dihaploid lines derived from anther culture have been successfully used to develop markers for resistance against potato virus Y (Song et al. 2005), and a parthenogenic approach has been used to map nematode resistance (Pineda et al. 1993). However, the population sizes in both studies were very small with only 57 and 37 dihaploid individuals.

In this study, we analyzed a large dihaploid population consisting of 215 genotypes, and subsets of these genotypes were screened for resistance to *S. endobioticum* pathotypes 18 and 6, which are among the most significant pathotypes responsible for potato wart. The dihaploid individuals were genotyped using the 12.8 k SolCAP SNP array, and the marker data were used to identify loci responsible for resistance to potato wart. Additional molecular markers were developed to fine map the major resistance locus on chromosome 11, and the resistance locus was narrowed to approximately 780 kbp in the region of the known major *Sen1* resistance locus. This fine mapping is a significant improvement in the resolution of genes responsible for resistance to pathotypes 18 and 6 at the *Sen1* locus. The tightly linked markers were tested in tetraploid varieties and showed potential diagnostic value in different genetic backgrounds.

Material and methods

Plant material

The tetraploid breeding line P208, which is resistant to *S. endobioticum* pathotypes 1, 2, 6 and 18, was used to construct a dihaploid population using the so-called "prickle pollination" with dihaploid inducer clones, i.e., IVP101 and IVP35, of the diploid wild potato species *Solanum phureja*. Altogether, 215 dihaploid genotypes were used for the resistance phenotyping, genotyping, and genetic mapping. In addition, 50 tetraploid potato cultivars were used to determine the diagnostic value of selected molecular markers developed in this study.

Generation of dihaploid potato populations

The pollinations with the dihaploid inducers were performed in a greenhouse on emasculated P208 flowers (Bartkiewicz et al. submitted). The seeds of the cross of P208 and *S. phureja* IVP35 were preselected based on the occurrence of an embryo spot. The seeds derived from the crosses were surface sterilized by a 30-second incubation in 70% ethanol, two-minute incubation in 0.5% sodium hypochlorite + Tween 20, and three five-minute washing steps with sterile distilled water. The seeds were germinated *in vitro* on Murashige Skoog medium (Murashige and Skoog 1962) solidified using 8.4 g plant agar (Duchefa Biochemie B.V., Haarlem, The Netherlands) per liter and containing 3% sucrose. The emerging seedlings were cultivated at 23°C under a 16 h light / 8 h dark cycle with light intensities of approximately 60 µmol m^{-2} s^{-1}.

Ploidy determination

The putative dihaploid seedlings were visually selected based on the lack of anthocyanin pigmentation in the nodes of the *in vitro* seedlings. Subsequently, the ploidy of the selected seedlings was determined by performing flow cytometry using a CyFlow Ploidy Analyzer (Partec, Münster, Germany). The leaf tissue (~1 cm^2) from the *in vitro* plantlets was chopped using razor blades in nuclei extraction buffer. The plant nuclei were stained with 4',6-diamidino-2-phenylindole using a CyStain UV Precise P Kit (Partec, Münster, Germany). The analyses were performed according to the manufacturer's protocol, and at least 1,000 nuclei were counted per sample. Parental genotypes with known ploidy were used as standards for the diploid and tetraploid genotypes.

Resistance phenotyping

Resistance to *S. endobioticum* pathotypes 18 and 6 (hereafter abbreviated as P18 and P6) was determined as previously described by Ballvora et al. (2011), but the tubers were not covered with a moist soil/peat mixture after the inoculation. For each tuber-bearing genotype, between five and 40 tubers were inoculated. The disease symptoms were scored from 1 (highly resistant) to 5 (highly susceptible). The mean scores were calculated according to $M = [a + 2b + 3c + 4d + 5e]/n$, where *a*, *b*, *c*, *d* and *e* are the number of tubers scored 1 to 5, and *n* is the total number of scored tubers. For the qualitative resistance mapping, the genotypes were considered resistant at a mean resistance score lower than 2.49 and susceptible at a mean resistance score higher than 3.51. Additionally, only genotypes with at least five successfully inoculated and scored tubers were considered. Few genotypes with medium resistance scores were excluded for the qualitative resistance mapping.

DNA extraction

For the DNA extraction, approximately 30 mg of dried leaf tissue were homogenized using a TissueLyser II (Qiagen, Hilden, Germany). The DNA was extracted using a DNeasy Plant Mini Kit (Qiagen, Hilden, Germany) according to the manufacturer's protocol. The DNA concentration was determined using a NanoDrop 2000 spectrophotometer (Thermo Fisher Scientific Inc., Waltham, Massachusetts, USA).

SNP genotyping using the 12.8 k SolCAP potato SNP array

Using the 12.8 k SolCAP potato genotyping array, the 215 dihaploid genotypes and the parental genotypes were genotyped for 12,808 SNPs. The parental genotypes were genotyped with two repeats. Custom genotyping was performed by Neogene Genomics (Neogene Genomics, Lincoln, Nebraska, USA).

Kompetitive Allele Specific PCR assay

The SNP array results were validated using the following Kompetitive Allele Specific PCR (KASP) markers of the SNP markers that were most significantly linked to potato wart resistance: solcap_snp_c2_33740, solcap_snp_c2_33712, solcap_snp_c1_4319, solcap_snp_c1_4322, solcap_snp_c2_6082, solcap_snp_c2_6287, solcap_snp_c1_2275, solcap_snp_c2_6309 and solcap_snp_c2_6285. The KASP primers were designed by LGC Genomics (LGC, Hoddesdon, UK) using

a KASP by Design assay based on the context sequence information provided by the Solanaceae Coordinated Agricultural Project (http://solcap.msu.edu/data/potato_69011_map_context_DM_v3_superscaffolds.txt). PCR was performed using 50 ng of genomic DNA in a 10 µl reaction volume on an ABI StepOnePlus instrument (Thermo Fisher Scientific, Waltham, Massachusetts, USA) according to the protocol provided by LGC genomics.

Construction of pools for the bulked segregant analysis (BSA)

Three bulks were constructed based on the results of the disease resistance screening. Bulk1 comprised three highly resistant genotypes (mean resistance scores ≤ 1.5), bulk2 comprised five resistant genotypes (mean resistance scores between 1.6 and 1.7) and bulk3 comprised seven susceptible genotypes (mean resistance scores ≥ 4.2). The bulks were used for the initial marker analysis. Then, the markers showing banding patterns specific to the highly resistant and resistant bulks were tested in the individual genotypes of the bulks and the entire dihaploid population.

Simple Sequence Repeat markers

Simple Sequence Repeat (SSR) markers were developed for the *Sen1* region on potato chromosome 11 using the SSRLocatorI software (Da Maia et al. 2008). The markers were PCR amplified from 40 ng of genomic DNA using the primers listed in Supplementary Table S1. The forward primers were M13-tailed (5'-GTAAAACGACGGCCAGT-3') at the 5'-end and a second M13-forward primer labelled with IRD700 (Eurofins MWG, Ebersberg, Germany) was used (Schuelke 2000). The PCR mixes with a total volume of 20 µl contained 0.125 µM of the IRD700 labelled M13-forward primer, 0.025 µM of the marker-specific forward primer, 0.25 µM of the marker-specific reverse primer, 1 unit of DCS *Taq* polymerase (DNA Cloning Service e.K., Hamburg, Germany), 1 x Williams buffer (100 mM Tris-HCl (pH 8.0), 500 mM KCl, 20 mM $MgCl_2$, and 0.01% gelatine) and 0.15 mM of each dNTP. The PCR conditions were the same as those described by Omondi et al. (2017). After performing the PCR, 100-250 µl of formamide loading dye (98% formamide, 10 mM EDTA, and 0.05% pararosaniline) were added, and the samples were denatured for three minutes at 95°C. For each sample, 0.3 µl of diluted PCR product were size separated on 6% polyacrylamide gels (Sequagel XR, National Diagnostics, Nottingham, UK) on a LI-COR DNA Analyzer 4300 (LI-COR, Lincoln, Nebraska, USA) according to the manufacturer's protocol.

Single Strand Conformation Polymorphism markers

Single Strand Conformation Polymorphism (SSCP) markers were developed for the *Sen1* region on potato chromosome 11 and PCR amplified from 40 ng of genomic DNA using the primers listed in Supplementary Table S1. The PCR mixes were the same as those described above for the SSR markers. The PCR conditions were as follows: initial denaturation for 5 minutes at 94°C; 30 cycles of 45 seconds at 94°C, 1 minute at 63°C and 1 minute at 72°C; ten cycles of 30 seconds at 94°C, 45 seconds at 52°C and 1 minute at 72°C; and a final extension of 10 minutes at 72°C. After performing the PCR, an equal amount of SSCP dye (95% formamide, 0.01 M NaOH, 0.05% xylene cyanol, and 0.05% bromophenol blue) was added, and the samples were denatured for three minutes at 95°C. For each sample, 1 µl of the diluted PCR product was size separated on 0.5 x MDE gels (0.5 x MDE ® gel solution (Lonza Group Ltd., Basel, Switzerland), 0.6 x long run TBE buffer (80.4 mM Tris, 7.5 mM boric acid, and 1.5 mM EDTA), 5% glycerine, 0.05% APS, and 10 µl TEMED. The IRD-labelled single strands were detected using the Odyssey® Infrared Imaging System (LI-COR, Lincoln, Nebraska, USA).

Y1delATT-marker

The Y1delATT-marker developed by Obidiegwu et al. (2015) was PCR-amplified from 20 ng of genomic DNA using the forward primer 5'-CTGGTAGGGGAAAAAGAACGTG-3' and reverse primer 5'-GAAATCTGAGTGAGCCATAGTC-3'. The PCR mixes with a total volume of 25 µl contained 0.5 µM of each primer, 6% dimethyl sulfoxide (Roth GmbH, Karlsruhe, Germany), 0.4 M Betaine (Sigma-Aldrich, St. Louis, Missouri, USA), 1 unit of Bioline *Taq* polymerase (Bioline, Luckenwalde, Germany), 1 x Williams buffer (100 mM Tris-HCl (pH 8.0), 500 mM KCl, 20 mM $MgCl_2$, and 0.01% gelatine) and 0.16 mM of each dNTP. The PCR conditions were the same as those described by Obidiegwu et al. (2015). The PCR products were detected by agarose gel electrophoresis.

RNA isolation

For the extraction of RNA from the leaf tissue (30-50 mg) of three resistant dihaploid genotypes, i.e., B35B-1, B35A-7 and B35F-6, and three susceptible dihaploid genotypes, i.e., B35C-8, B35F-10 and K12-3, the samples were frozen in liquid nitrogen and homogenized using a TissueLyser II (Qiagen, Hilden, Germany). The RNA extraction was performed using an RNeasy Plant Mini Kit (Qiagen, Hilden, Germany) according to the manufacturer's protocol. Contaminating DNA was removed from the extracted RNA using a DNA-free™ Kit (Ambion, Thermo Fisher Scientific, Waltham, Massachusetts, USA) according to the manufacturer's protocol.

RNA-Seq data analysis

Three resistant dihaploid genotypes and three susceptible dihaploid genotypes were selected for the transcriptome sequencing on the Illumina platform using the 2 x 125 bp paired end mode. Transcriptome sequences specific to the resistant genotypes were identified using the following three different approaches: (1) mapping the assembled reads against known Solanaceae resistance gene analogs (RGAs), (2) using RGAs specifically identified in the *Sen1* region on potato chromosome 11 and (3) identifying contigs specific to the resistant dihaploid genotypes. Then, the identified contigs were used for the marker development.

PCR markers derived from the RNA-Seq analysis

The PCR markers derived from the RNA-Seq analysis were amplified from 20 ng of genomic DNA using specific primers for three resistant-specific contigs, i.e., Kc8103 (forward primer 5'-GGGAAGTGCATGATTCAGAGC-3', reverse primer 5'-GGCAGTTCCGTTATCCTAGTG-3'), Kc49 (forward primer 5'-TTGCTTTGTTTTCCCTCCGG-3', reverse primer 5'-CATCAACTGGCTTCATTGGA-3') and Kc19 (forward primer 5'-GTTCACTGTTTCATTTATGGACTGA-3', reverse primer 5'-TTCAATTTTCCCCGGATCTT-3'), in a total volume of 25 µl. The reactions were performed according to the manufacturer's protocol using MyTaq^{TM} DNA polymerase (Bioline, Luckenwalde, Germany). The PCR products were detected by agarose gel electrophoresis.

Marker-trait associations

The marker-trait associations were determined using R software version 3.1.3 (R development core team 2011) by performing a non-parametric Kruskal-Wallis rank test. The mean values of the resistance phenotyping and the genotyping results of the SNP array were utilized. The PCR marker data were transformed into a 1/0 matrix representing the presence (1) and absence (0) of the respective marker band. After the FDR adjustment, a p-value of 0.05 was chosen as the significance threshold indicating that a marker is significantly linked to resistance to *S. endobioticum* pathotypes 18 and 6.

Genetic linkage mapping

The genetic mapping of the P208 population was performed using only single dose SNP markers indicated by a 1:1 segregation. Skewed markers or markers with missing values for more than 15 genotypes were not considered. To construct the linkage maps, LOD scores between 6 and 15 were

chosen. For each potato chromosome, two to four linkage maps were constructed. A linkage analysis was performed in JoinMap®4 (Van Ooijen 2006) using the mapping function of Haldane (1919) and the regression mapping algorithm (Stam 1993). To fine map the resistance locus, SSR-, SSCP- and PCR marker data were included. The same mapping parameters were used as described for the genetic linkage mapping.

Results

Phenotypic analysis of wart resistance

Of the 215 dihaploid genotypes, 181 genotypes produced tubers for inoculation with *S. endobioticum* P18 and P6. Tubers of 170 genotypes were successfully inoculated with P18, with five to 38 tubers and mean resistance scores ranging from 1.3 to 4.6 (Figure 1a). Of the 181 tuber-bearing genotypes, 160 genotypes produced enough tubers to also be inoculated with P6. For 150 genotypes, five to 30 tubers were successfully inoculated with P6 with mean resistance scores ranging from 1.0 to 4.7 (Figure 1b). The resistance to both pathotypes displayed a clear bimodal distribution and was highly correlated (Figure 2) with a correlation coefficient of r = 0.8 (p-value < 2.2e-16).

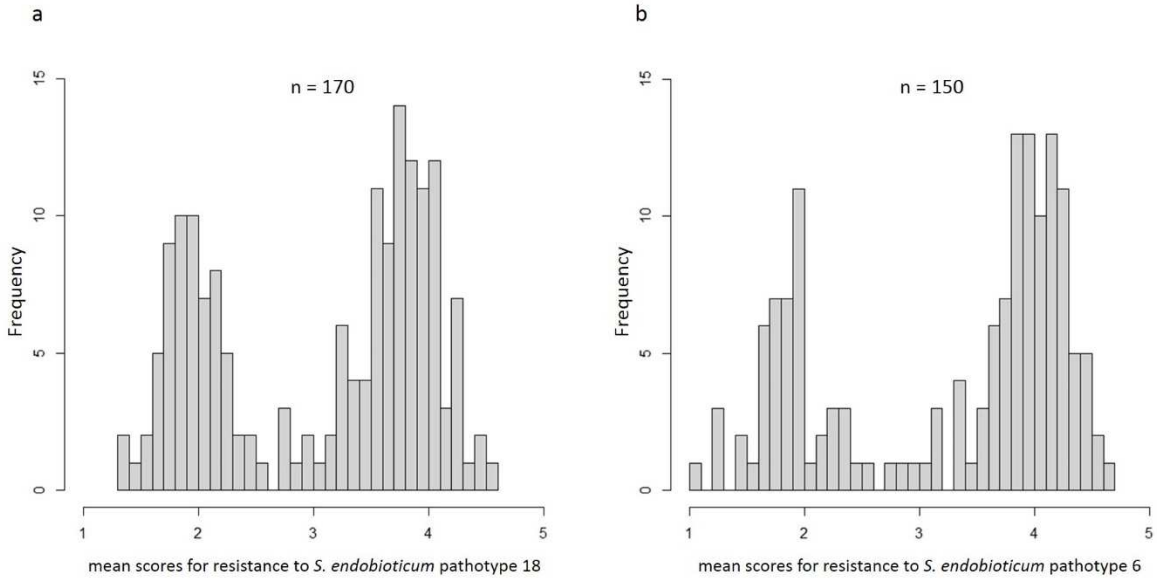

Figure 1: Distribution of the mean scores of the resistance to *S. endobioticum* P18 (a) and P6 (b). Altogether, 170 and 150 genotypes were successfully inoculated with P18 and P6, respectively.

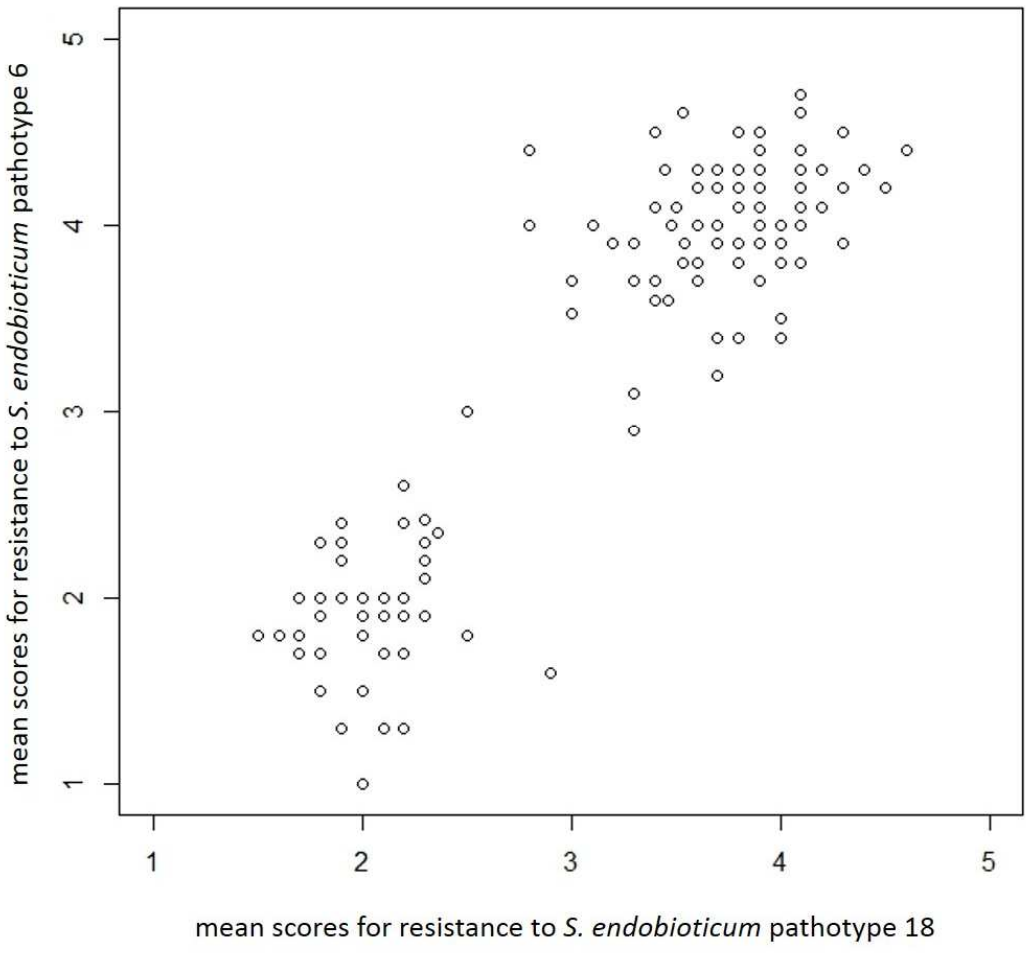

Figure 2: Correlation of resistance to *S. endobioticum* P18 and P6. Mean resistance scores of 145 genotypes are plotted, for which phenotypic resistance data were available for both pathotypes. Resistance to both pathotypes is highly correlated with a correlation coefficient of r = 0.8 (p-value < 2.2e-16).

Mapping of wart resistance loci

The mapping of the resistance to the two wart pathotypes was conducted using the following two strategies: first, a simple QTL analysis was performed based on the quantitative wart scores and Kruskal-Wallis tests, and second, a conventional qualitative mapping approach was used in which the resistance scores were recoded as resistant (mean scores less than 2.49) or susceptible (mean scores higher than 3.51).

Quantitative analysis of marker-trait associations

The phenotypic resistance scores were used to calculate the marker-trait associations. The SNP genotyping using the 12.8 k SolCAP SNP array resulted in 4,679 segregating SNP markers within the population. These markers were used to perform Kruskal-Wallis tests to determine the association of resistance to P18 and P6. Ninety-nine significant SNP markers were identified for resistance to P18, including 82 markers located on potato chromosome 11. Of these markers, the 20 markers with the lowest p-values, i.e., the markers that are most significantly linked to resistance, were all located within a 5.6 Mbp region on the distal end of chromosome 11. Sixteen markers were located on chromosome 10 (Table 1). For one marker, no physical position could be identified in the potato genome browser. Eighty-seven significant markers were identified for P6, all of which were located on chromosome 11 (Table 1). The 14 markers with the lowest p-values were all located at the distal end on chromosome 11, which is consistent with the results observed for P18. Altogether, 76 markers were identical to those identified to be significantly linked to the resistance to P18. Information regarding the individual SNP markers that were significantly linked to P18 and P6 is provided in Supplementary Tables S2 and S3.

Table 1: Number of SNP markers significantly linked to the resistance to *S. endobioticum* P18 and P6. The number of markers is listed for both pathotypes in the respective physical regions on chromosomes 10 and 11 according to the Spud DB Genome Browser PGSC v4.03. The number of identical markers for both pathotypes is also listed.

Chromosome	Physical region [Mbp]	Number of P18 markers	Number of P6 markers	Identical markers for both pathotypes
11	0 – 5.6	40	34	34
	6.2 – 9.6	12	13	12
	10.0 – 14.3	21	20	20
	20.8 – 28.0	5	6	5
	30.5 – 41.8	4	13	4
10	55.7 – 58.1	16	-	-

For nine of the markers that were most significantly linked to resistance to both P18 and P6, a KASP assay was performed to validate the SNP marker data. Both methods showed high consistency regarding the genotyping, and only one marker, i.e., solcap_snp_c2_6082, differed for two dihaploid genotypes (Table 2).

Table 2: Comparison of genotyping results between the 12.8 k SolCAP SNP array and KASP assay for the nine markers that were most significantly linked to resistance to *S. endobioticum* P18 and P6 in 215 dihaploid genotypes. Percentages of not available (NA) marker data for the KASP assay are also listed.

SNP marker	Consistent results	Differing results	NA KASP assay
solcap_snp_c2_33740	100 %	0 %	0 %
solcap_snp_c2_33712	99.08 %	0 %	0.91 %
solcap_snp_c1_4319	98.63 %	0 %	1.37 %
solcap_snp_c1_4322	99.08 %	0 %	0.91 %
solcap_snp_c2_6082	96.35 %	0.91 %	2.74 %
solcap_snp_c2_6287	97.72 %	0 %	2.28 %
solcap_snp_c1_2275	99.08 %	0 %	0.91 %
solcap_snp_c2_6309	96.35 %	0 %	3.65 %
solcap_snp_c2_6285	99.08 %	0 %	0.91 %

Qualitative fine mapping of wart resistance

A genetic linkage map was generated using 2,548 single dose SNP markers displaying a 1:1 segregation. Altogether, 45 linkage groups were obtained (Bartkiewicz et al. submitted). The nine markers that were most significantly associated with the resistance to *S. endobioticum* P18 and P6 could be mapped on a single linkage group.

For the qualitative resistance screening, the genotypes were classified as either resistant (mean resistance score ≤ 2.49) or susceptible (mean resistance score ≥ 3.51). After excluding individuals with medium resistance scores ranging from 2.5 to 3.5, 144 and 137 genotypes were clearly classified as resistant or susceptible to P18 and P6, respectively. Of these genotypes, 61 genotypes were resistant and 83 genotypes were susceptible to P18, corresponding to a 1:1 segregation ratio for P18 ($\chi^2 = 3.361$; $p > 0.05$). The segregation of resistance scores for P6 was skewed towards a 1:2 ratio ($\chi^2 = 0.179$; $p > 0.05$) with 48 resistant and 89 susceptible genotypes.

The qualitative resistance mapping allowed for the mapping of the resistance locus for P18 on chromosome 11 between the two SNP marker groups solcap_snp_c2_33740/solcap_snp_c2_33712 and solcap_snp_c1_4322/solcap_snp_c1_4319, spanning a physical distance of 1.15 Mbp. The recombinant genotypes for these two marker groups are listed in Table 3. The qualitative mapping of the resistance to P6 placed the resistance locus in the same interval.

To further narrow the marker interval around the resistance locus, 51 SSR- and 53 SSCP-markers were developed for the 1.15 Mbp genomic region (Table S1). The comparison of the RNA-Seq data between the resistant and susceptible dihaploid genotypes allowed for the development of three PCR markers that showed specific marker bands in the resistant genotypes. Additionally, the Y1delATT marker from

previous publications (Obidiegwu et al. 2015) was used for the fine mapping. The markers were tested in a “bulked segregant” analysis using three DNA pools consisting of genomic DNA of highly resistant, resistant and susceptible genotypes. The entire dihaploid population was screened for marker bands specific to the highly resistant and resistant pools. Altogether, seven SSR-markers, four SSCP-markers, three PCR markers and the Y1delATT marker were used to fine map the resistant locus (Figure 3). The approximate physical positions of these markers based on the reference genome and the number of recombinant genotypes are listed in Table 3. Six SSR markers, one SSCP marker and one PCR marker showed no recombination to P18 and P6 resistance. SNP markers solcap_snp_c1_4322/solcap_snp_c1_4319 and SSR marker RK36 flank the resistance locus on each side with one recombinant genotype.

By mapping the additionally developed molecular markers onto the reference genome, the genomic region around the resistance locus was narrowed from approximately 1.15 Mbp to approximately 777 kbp.

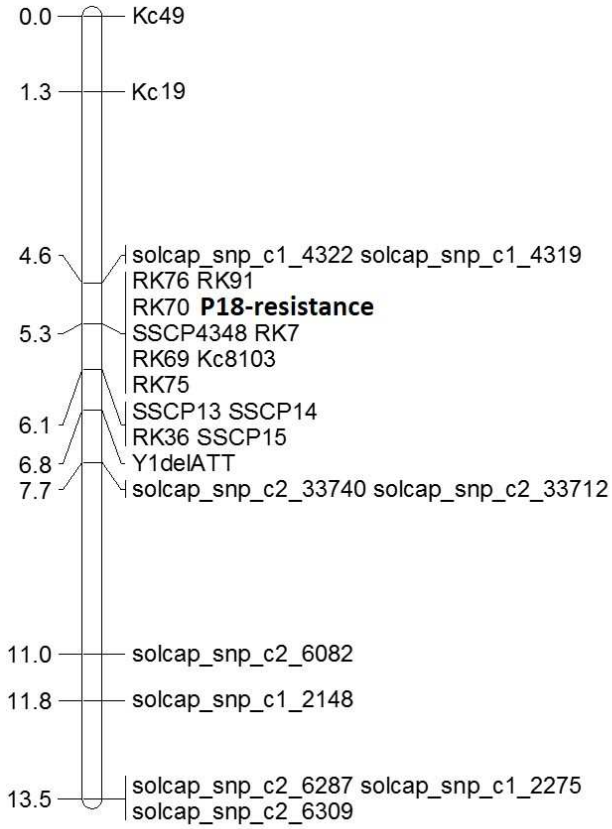

Figure 3: Fine mapping of the locus responsible for the resistance to *S. endobioticum* P18 on chromosome 11. Eight markers showed no recombinant genotypes to the resistance locus (P18-resistance) at 5.3 cM, while four markers were recombinant for one genotype at 6.1 cM. For the Y1delATT marker, two genotypes were recombinant.

Table 3: Molecular markers used to fine map the major locus responsible for the resistance to *S. endobioticum* P18 on chromosome 11. Markers flanking the resistance locus are listed along with their physical position and the number of recombinant genotypes for each marker.

Marker	Marker type	Physical position [bp]	Recombinant genotypes
solcap_snp_c1_4322/ solcap_snp_c1_4319	SNP	939,581	K8-1
SSCP4348	SSCP	1,163,786	None
Kc8103	PCR	1,407,791	None
RK7	SSR	1,610,809	None
RK75	SSR	1,630,787	None
RK76	SSR	1,637,061	None
RK70	SSR	1,665,423	None
RK69	SSR	1,667,558	None
RK91	SSR	1,683,357	None
RK36	SSR	1,716,722	K14-3
SSCP13	SSCP	1,768,997	K14-3
SSCP14	SSCP	1,771,582	K14-3
SSCP15	SSCP	1,776,834	K14-3
Y1delATT	PCR	1,844,035	K14-3; B35H-4
solcap_snp_c2_33740/ solcap_snp_c2_33712	SNP	2,089,292	K14-3; B35H-4; B35G-8

The phenotypic effects of these markers were calculated in the entire dihaploid population, including the genotypes with medium resistance scores for P18 that were previously excluded (Table 4). The mean scores of the group with the markers ranged from 2.01 to 2.08, and the mean scores of the group without the markers ranged from 3.77 to 3.80. The phenotypic distribution of the resistance to P18 for markers showing no recombinant genotypes and markers showing one recombinant genotype, K14-3, is shown in Figure 4. The phenotypic resistance scores ranged from 1.3 to 2.9 in the group with a present marker and 2.8 to 4.6 in the group without the marker for the individual genotypes. The recombinant genotype K14-3 (Figure 3, Table 3) had a mean resistance score of 1.9.

Table 4: Marker-trait association of the most significant markers linked to resistance to *S. endobioticum* P18. For each marker, the number of recombinant genotypes, the p-value of the Kruskal-Wallis test, and the mean resistance scores of the groups with and without markers are listed.

Marker	Number of recombinant genotypes	p-value Kruskal-Wallis	Mean marker present	Mean marker absent
solcap_snp_c1_4319 solcap_snp_c1_4322	1	<2.2e-16	2.063676	3.799902
SSCP4348 Kc8103 RK7 RK75 RK76 RK70 RK69 RK91	0	<2.2e-16	2.014091	3.797981
RK36 SSCP13 SSCP14 SSCP15	1	<2.2e-16	2.015846	3.779905
Y1delATT	2	<2.2e-16	2.045909	3.777788
solcap_snp_c2_33740 solcap_snp_c3_33712	3	<2.2e-16	2.076567	3.77466

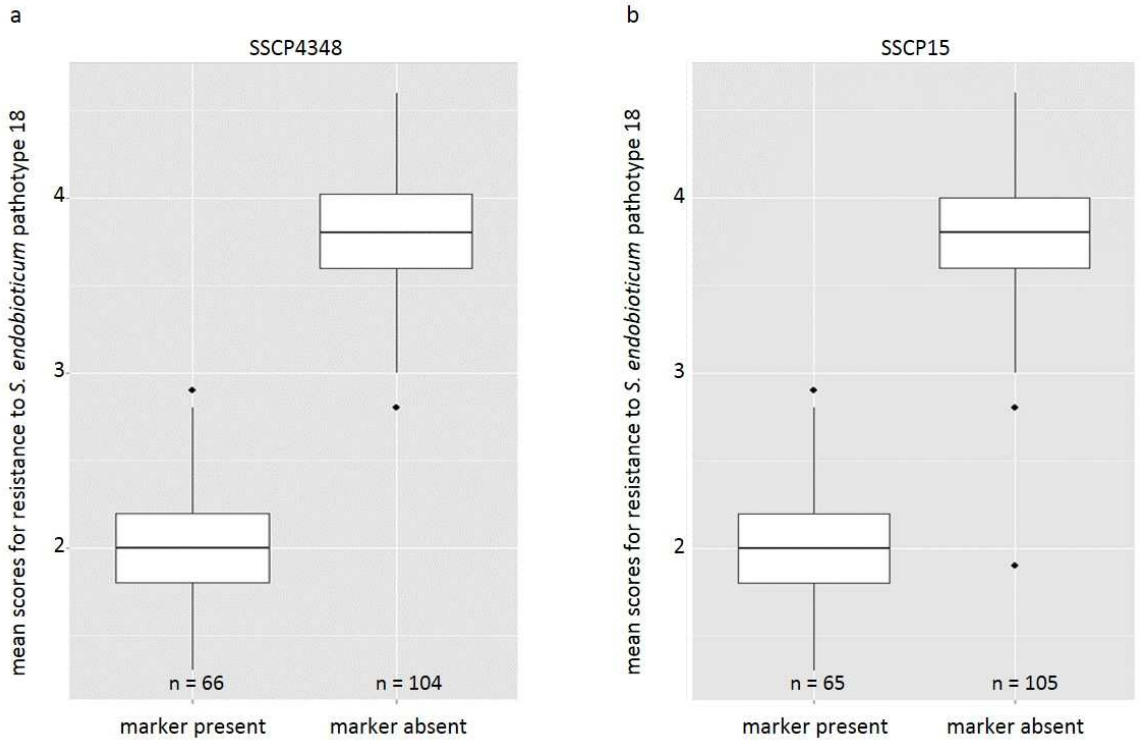

Figure 4: Distribution of mean scores of resistance to *S. endobioticum* P18 for markers SSCP4348 (a) and SSCP15 (b) in the dihaploid population. Distribution of the phenotypes is shown in dependence of the presence and absence of the respective markers in 170 genotypes. The boxes represent the 25th and 75th quartiles, and the medians are indicated by the bold line.

Diagnostic value of the developed markers

To test the diagnostic value of the newly developed molecular markers, selected markers were also tested in a subset of 38 tetraploid potato varieties with available phenotypic resistance scores for P18 (Table 5). The best results were obtained with markers Kc8103 and RK36, each of which identified four non-matching varieties. `Jutrzenka´ and `Saphir´ were both classified as resistant to P18 but showed susceptible genotypes for both markers. In contrast, the varieties `Merano´ and `Milek´ were susceptible to P18 but showed a resistant genotype with both markers (Figure 5). Fourty-five varieties with known resistances for P6 were tested with the two markers and six non-matching genotypes were identified. The other tested markers were either not polymorphic in the tetraploid varieties or did not show a clear segregation pattern for resistant and susceptible varieties.

Table 5: Tetraploid potato varieties with known resistances to *S. endobioticum* P18 and their obtained genotypes for markers Kc8103 and RK36. Classification of 38 varieties is provided; R1 indicates highly resistant and resistant varieties, R2 indicates weakly resistant varieties, S1 indicates slightly susceptible varieties and S2 indicates highly susceptible varieties. Eight varieties were classified as resistant (R1 or R2), and 30 varieties were classified as susceptible (S1 or S2). The genotyping results for markers Kc8103 and RK36 are also listed. For both markers, varieties `Jutrzenka´ and `Saphir´ as well as `Merano´ and `Milek´ showed non-matching genotypes.

Variety	Resistance classification	Genotypes for markers Kc8103 and RK36
`Alegria´	S2	susceptible
`Altus´	S1/S2	susceptible
`Avano´	S1	susceptible
`Birte´	S2	susceptible
`Burana´	S2	susceptible
`Campina´	S2	susceptible
`Combi´	S1/S2	susceptible
`Concordia´	S2	susceptible
`Cumbica´	S2	susceptible
`Deodara´	S1/S2	susceptible
`Desirée´	S1/S2	susceptible
`Finka´	S2	susceptible
`Gawin´	R1/R2	resistant
`Heidi´	S2	susceptible
`Igor´	R1	resistant
`Ikar´	R1/R2	resistant
`Jasia´	S1	susceptible
`Jutrzenka´	R1/R2	susceptible
`Laura´	S2	susceptible
`Lilly´	S2	susceptible
`Megusta´	R2	resistant
`Merano´	S1/S2	resistant
`Milek´	S1/S2	resistant
`Miriam´	S1/S2	susceptible
`Natascha´	S2	susceptible
`Opal´	S2	susceptible
`Panda´	S1	susceptible
`Romanze´	S2	susceptible
`Saphir´	R1	susceptible
`Seresta´	S1	susceptible
`Soraya´	S2	susceptible
`Sleza´	R2	resistant
`Talent´	S1/S2	susceptible
`Toccata´	S2	susceptible
`Tomensa´	S1/S2	susceptible
`Troja´	S2	susceptible
`Ulme´	R1/R2	resistant
`Venezia´	S2	susceptible

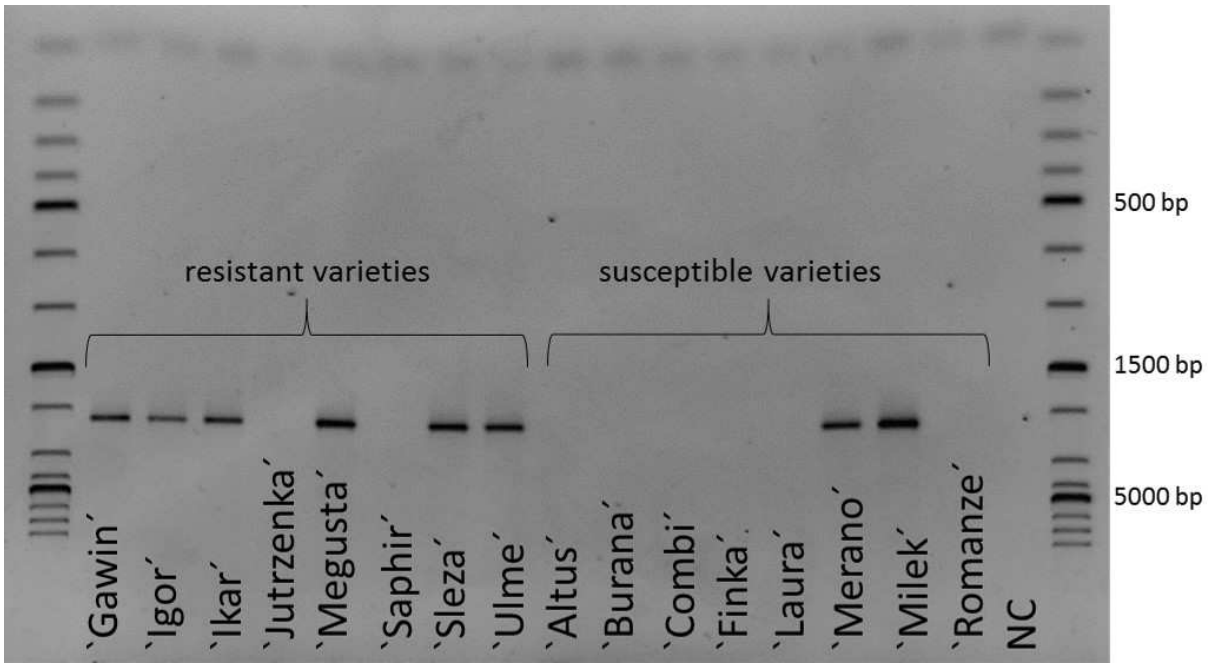

Figure 5: Agarose gel electrophoresis of PCR marker Kc8103 in 16 tetraploid potato varieties with known resistance to *S. endobioticum* P18. Resistant varieties show a marker band at 2197 bp, which is not present in the susceptible varieties. Varieties `Jutrzenka´ and `Saphir´ as well as `Merano´ and `Milek´ show non-matching genotypes for this marker.

Discussion

In this study, we present molecular markers that tag a major resistance locus on potato chromosome 11, and no recombinant genotypes were observed in our dihaploid potato population. To our knowledge, this study is the first to narrow the position of the resistance locus for P18 and P6 to less than 800 kbp, establishing the basis for further genetic analyses of wart resistance and the identification of the underlying resistance gene or genes.

The distributions of the phenotypic resistance scores were bimodal for both pathotypes (Figure 1) and showed a 1:1 segregation ratio for P18 after excluding a few genotypes with medium resistance scores between 2.49 and 3.51, indicating that one major resistance gene is responsible for the resistance. The phenotypic distribution of resistance segregating as a monogenic character has been described in earlier studies for pathotype 1 (Hehl et al. 1999; Brugmans et al. 2006; Obidiegwu et al. 2015). The bimodal phenotypic distribution of resistance to pathotypes 2, 6 and 18 has also been observed by Obidiegwu et al. (2015); however, the distribution did not fit a 1:1 segregation ratio; in the SNP genotyping, 79 genotypes with intermediate resistance scores were excluded, and the resistance mapping was performed using 54 selected genotypes. Using the resistant parent used in the study conducted by Obidiegwu et al. (2015), we observed a clearer segregation of resistance, indicating the presence of a major dominant factor responsible for the resistance segregation in our population. The

difference from the tetraploid population used by Obidiegwu et al. (2015) could be explained by additional factors contributed by the second tetraploid parent in their study that could have modified the interactions. Furthermore, the ploidy level could account for the variation resulting in genotypes with intermediate resistance scores because loci displaying dose-dependent effects lead to a broader distribution in tetraploid progeny than in dihaploids. In our study, we excluded 26 medium scored genotypes to avoid false positives in the classification of the genotypes as resistant. Nevertheless, the developed markers were tested in all genotypes and showed the expected results, even in the medium scored genotypes. The segregation of the resistance scores for P6 was slightly skewed towards susceptibility and did not correspond to a 1:1 segregation. This skewed distribution for P6 resistance could be easily explained by the fact that certain genotypes resistant to P18 did not produce enough tubers to be tested with P6. Because the resistance scores for both pathotypes were highly correlated (Figure 2), these genotypes could also be resistant to P6 with a high probability, resulting in a 1:1 segregation for P6. The high correlations between the resistances to pathotypes 2, 6 and 18 have been previously described (Ballvora et al. 2011; Groth et al. 2013).

Using the SNP genotyping data, the qualitative resistance mapping of the clearly classified genotypes identified the resistance loci for both pathotypes on potato chromosome 11 (Figure 3) in the *Sen1* region, which is known as a major locus responsible for resistance to potato wart pathotype 1 (Hehl et al. 1999; Gebhardt et al. 2006; Ballvora et al. 2011, Groth et al. 2013) and pathotypes 2, 6 and 18 (Obidiegwu et al. 2015). Of the 82 markers that were significantly linked to the resistance to P18 located on chromosome 11, 92.7% (76 markers) were also significantly linked to the resistance to P6 (Table S2, S3). In addition, 16 markers identified on chromosome 10 were significantly linked to the resistance to P18. For P6, eleven additional markers identified on chromosome 11 were not identified to be significantly linked to the resistance to P18. The resistance locus for P18 on chromosome 10 is consistent with the resistance locus identified by Groth et al. (2013), indicating that the resistance to P18 is controlled by a second minor resistance locus on chromosome 10 in addition to the major resistance locus on chromosome 11, which is not the case for P6. A KASP assay of the nine most significantly linked SNP markers confirmed the genotyping results of the SNP array (Table 2). Only one marker showed different genotyping results for two genotypes. However, one genotype was excluded from the resistance analyses because only three and two tubers were successfully inoculated with P18 and P6, respectively. The other genotype was excluded from the resistance analysis of P18 because it showed a medium resistance score of 3.3.

The development of additional SSR, SSCP, and PCR markers allowed for the fine mapping of the major resistance locus on chromosome 11 by narrowing the locus from approximately 1.15 Mbp to approximately 777 kbp. The physical distances of markers showing one and zero recombinant genotypes are 33,365 bp between markers RK91 (no recombinant genotype) and RK36 (K14-3 as a

recombinant genotype) and considerably higher between solcap_snp_c1_4322/solcap_snp_c1_4319 (K8-1 as a recombinant genotype) and SSCP4348 (no recombinant genotype) with 224,205 bp, indicating that there are possibly additional markers in this region to further narrow down the resistance locus which stayed undetected in this study. Altogether, the developed markers in this study allowed a clear distinction between resistant and susceptible genotypes in our dihaploid population (Table 4; Figure 4).

Although wart resistance is highly dependent on the genetic background of the respective varieties (Khiutti et al. 2012), we determined the diagnostic value of our developed makers by screening 38 tetraploid potato varieties with known resistance to P18 and 45 varieties with known resistance to P6. The markers Kc8103 and RK36 showed the most promising results. Of the 38 tested varieties for P18, four non-matching genotypes were observed, and the markers were diagnostic in 89.5% of the cases (Table 5; Figure 5). Of the 45 tested varieties for P6 the markers were diagnostic in 86.6% of the cases with six non-matching genotypes. To the best of our knowledge, these two markers that were developed in our study are the first markers to show potential diagnostic value for resistance to P18 and P6. Thus far, only one marker, i.e., Nl25, has been reported to show high linkage to the *Sen1* locus and, therefore, resistance to pathotype 1 (Gebhardt et al. 2006). Within the CORNET project SynTest (Establishment of a harmonised methodology for testing the resistance of potato cultivars to potato wart disease in the EU) the usability of three DNA markers (Nl25, GP125 and Stl046) was tested to evaluate the resistance to pathotype 1. With seven non-matching varieties out of 89 tested, the marker Nl25 was diagnostic in 92% of the cases (K. Flath, personal communication). Unfortunately, reliable phenotypic resistance data for P18 and P6 are not available for more tetraploid varieties to further substantiate the diagnostic value of markers Kc8103 and RK36 in different genetic backgrounds. Nevertheless, our results indicate that the same resistance locus plays a role in the resistance reaction in different genetic backgrounds displayed by different potato varieties with additional resistance loci likely present on various other chromosomes.

Conclusions

In this study, we analyzed resistance to potato wart P18 and P6 using a monoparental dihaploid population derived from a highly resistant tetraploid cultivar. The resistance to both pathotypes can be resolved into a major factor at the *Sen1* locus on chromosome 11 of potato. The resistance to P18 is additionally influenced by minor QTLs on chromosome 10. By converting the resistance scores into qualitative scores, we fine mapped the resistance to P18 and P6 to a genomic interval of less than 800 kbp using several linked markers without recombination. This study provides the highest resolution in mapping the resistance to P18 and P6 thus far and opens opportunities for screening candidate genes

in the future. Furthermore, several developed markers showed potential diagnostic value for resistance to *S. endobioticum* P18 and P6 in 38 and 45 tetraploid varieties with different genetic backgrounds. To the best of our knowledge, these markers are the first to possess diagnostic value for this pathotype. The use of DNA marker techniques will provide a cost-effective evaluation of P18 and P6 resistance. This will considerably speed up the breeding progress. Potato cultivars with improved resistance will open new markets in Eastern Europe and Russia and will enable a more efficient control of potato wart disease.

Author Contribution Statement

AB planned and performed the experiments, analyzed the data, prepared all tables and figures and wrote the manuscript. FC generated the dihaploid potato population and determined the ploidy of genotypes by flow cytometry. DT performed the RNA-Seq experiment and developed the PCR markers derived from the RNA-Seq analysis. JL, JS, ET and HH provided the plant material for the crossings and tuberized the dihaploid genotypes. KF performed the wart resistance phenotyping. ML planned and supervised the experiments and corrected the manuscript. TD planned and supervised the experiments and wrote part of and corrected the manuscript.

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Conflict of interest

The authors declare that they have no conflict of interest.

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2.3. Genomic and Transcriptomic Resources for Marker Development in *Synchytrium endobioticum*, an Elusive but Severe Potato Pathogen

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Genetics and Resistance

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ABSTRACT

Synchytrium endobioticum is an obligate biotrophic fungus that causes wart diseases in potato. Like other species of the class Chytridiomycetes, it does not form mycelia and its zoospores are small, approximately 3 µm in diameter, which complicates the detection of early stages of infection. Furthermore, potato wart disease is difficult to control because belowground organs are infected and resting spores of the fungus are extremely durable. Thus, *S. endobioticum* is classified as a quarantine organism. More than 40 *S. endobioticum* pathotypes have been reported, of which pathotypes 1(D1), 2(G1), 6(O1), 8(F1), and 18(T1) are the most important in Germany. No molecular methods for the differentiation of pathotypes are available to date. In this work, we sequenced both genomic DNA and cDNA of the German pathotype 18(T1) from infected potato tissue and generated 5,422 expressed sequence tags (EST) and 423 genomic contigs. Comparative sequencing of 33 genes, single-stranded confirmation polymorphism (SSCP) analysis with polymerase chain reaction fragments of 27 additional genes, as well as the analysis of 41 simple sequence repeat (SSR) loci revealed extremely low levels of variation among five German pathotypes. From these markers, one sequence-characterized amplified region marker and five SSR markers revealed polymorphisms among the German pathotypes and an extended set of 11 additional European isolates. Pathotypes 8(F1) and 18(T1) displayed discrete polymorphisms which allow their differentiation from other pathotypes. Overall, using the information of the six markers, the 16 isolates could be differentiated into three distinct genotype groups. In addition to the presented markers, the new collection of EST from genus *Synchytrium* might serve in the future for molecular taxonomic studies as well as for analyses of the host–pathogen interactions in this difficult pathosystem.

Synchytrium endobioticum (Schilb.) Percival is the causative agent of potato wart disease. It is a soilborne obligate biotrophic organism that belongs to the fungal phylum Chytridiomycota. *S. endobioticum* is an economically important quarantine organism, and infection of the host can lead to unmarketable tubers and complete yield losses (Hampson 1993; Melnik 1998). Once fields are contaminated with *S. endobioticum*, potato production is forbidden until the soil is proven to be free of sporangia (Obidiegwu et al. 2014). However, the sporangia can survive in the soil for 30 years or longer (Arora and Khurana 2004; Laidlaw 1985). After 43 years, in favorable conditions, disease recurrence may occur even from single spores of *S. endobioticum* (Przetakiewicz 2015b). Because the thick-walled winter sporangia are extremely durable, effective chemical control measures are not available (Obidiegwu et al. 2014). Strict quarantine measures and the use of resistant cultivars have been used to control the fungus but local occurrence of the fungus has been reported in nearly all European countries (Çakır 2005; De Boer 2001; Flath et al. 2014; Przetakiewicz 2015a).

S. endobioticum exhibits a life cycle with a haploid asexual reproduction phase which can change under stress conditions into sexual reproduction with a diploid phase. In spring uniflagellate zoospores of approximately 3 µm in diameter are released from infected tissue and may penetrate meristematic tissue of young tubers or potato stolons, leaving their flagellum outside. After infection, the potato host cell greatly enlarges and surrounding cells divide irregularly, forming the tumor-like tissue of the wart. The zoospore inside develops into a haploid, thick-walled sorus containing several prosori or zoosporangia. These so-called summer sporangia can release several hundred haploid zoospores which can infect new tissues. Under unfavorable conditions, the zoospores can act as isogametes and fuse to a diploid biflagellate zygote. The zygotes can penetrate host tissues in the same way as the zoospores. The infected cells are buried deep in the tissue, forming thick-walled resting or winter sporangia which are released to the soil. The diploid nucleus divides repeatedly, with a first reduction division forming uninucleate haploid zoospores which are released after rupturing of the cell wall (Curtis 1921; Lange and Olson 1981).

More than 40 *S. endobioticum* pathotypes have been reported (Baayen et al. 2006; Çakır et al. 2009; Przetakiewicz 2015a), and 5 of these—namely, pathotypes 1(D1), 2(G1), 6(O1), 8(F1), and 18(T1)—are the most important and widespread pathotypes in Europe (Anonymous 2004; Flath et al. 2014; Obidiegwu et al. 2014). At present, a conventional, time-consuming and laborious inoculation assay using differential cultivars is the only method available for distinguishing different *S. endobioticum* pathotypes (Anonymous 2004; Baayen et al. 2006; Obidiegwu et al. 2014). Classification using differential cultivars may also incur bias in the assignment of pathotypes because different countries use different potato differential cultivars, and the inoculation assay itself may result in inconsistencies

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*The *e-Xtra* logo stands for "electronic extra" and indicates that five supplementary figures and three supplementary tables are published online.

between different laboratories using the same cultivars (Flath et al. 2014). Therefore, there is a need to complement the use of differential cultivars with molecular diagnostic techniques.

As an alternative to the time-consuming detection methods, highly sensitive polymerase chain reaction (PCR)-based methods have been proposed. The described protocols include simple PCR and real-time PCR-based detection (Bonants et al. 2015; Niepold and Stachewicz 2004; van den Boogert et al. 2005; van Gent-Pelzer et al. 2010), microarray-based detection (Abdullahi et al. 2005), and colorimetric detection using a peptide nucleic acid probe and a cyanine dye (Duy et al. 2015). In addition, TaqMan PCR assays based on ribosomal DNA sequences have also been developed (Smith et al. 2014; van Gent-Pelzer et al. 2010). More recently, a real-time TaqMan PCR assay was described to differentiate pathotype 1(D1) from the other four pathotypes: 2(G1), 6(O1), 8(F1), and 18(T1). However, these methods are either inconsistent or need expensive instruments and consumables. Therefore, simple, affordable, and reliable molecular diagnostic techniques for *S. endobioticum* are still missing.

Genomic and transcriptomic sequence information from plant pathogens is important for the understanding of several aspects of plant–pathogen interaction. It may be used to discriminate pathotypes and is crucial to reveal genetic diversity within pathogen populations which, in turn, facilitates pathogen control strategies (McDonald and Linde 2002a,b). It may also lead to the identification of pathotype-specific effector genes (Jones and Dangl 2006) on which disease management measures can be based. However, at present, there are only eight *S. endobioticum* gene sequences available in public databases, including GenBank, European Nucleotide Archive (ENA), and DNA Data Bank of Japan (DDBJ), and these sequences originate from the 18S and 28S ribosomal RNA gene sequences. Although such nuclear ribosomal sequences have been reported as fungal DNA barcode markers (Schoch et al. 2012), the sequencing of intergenic nontranscribed spacers of ribosomal genes from four *S. endobioticum* pathotypes showed no sequence polymorphisms (Smith et al. 2014), indicating that ribosomal gene sequencing is poorly suited for dissecting *S. endobioticum* pathotypes.

Therefore, the objectives of the study presented here are to provide high-quality genomic and transcriptome sequence data of *S. endobioticum*, develop molecular markers for diagnosis, and assess polymorphisms and differentiate pathotype groups.

MATERIALS AND METHODS

Fungal pathotypes. Eleven isolates representing different known pathotypes and five isolates of unknown *S. endobioticum* pathotypes from five countries were used in this study (Table 1). These five isolates (numbers SE4, SE5, SE6, SE7, and SE13) were from a newly characterized recent finding and are in the process of being assigned to a pathotype (J. Przetakiewicz, personal communication). Fourteen of the isolates were obtained as fresh wart tissue from the Julius Kühn Institute (Germany) and Plant Breeding and Acclimatization Institute (IHAR) (Poland). Two of the pathotypes (numbers SE2 and SE11) were obtained as wart compost from Germany and Northern Ireland (Table 1). The pathotype nomenclature is according to the standard code (Baayen et al. 2006; Langerfeld et al. 1994). The German *S. endobioticum* pathotype 18(T1) was selected for sequencing based on its aggressiveness and its wide distribution (Stachewicz 2002). Susceptible 'Tomensa' potato was used for propagation and maintenance of the fungus.

DNA and RNA extraction. Sixteen days postinoculation, the proliferating tumor-like tissue of 14 isolates (SE1, SE3 to SE10, and SE12 to SE16) (Table 1) was harvested in 100-mg aliquots and frozen in liquid nitrogen. Dried winter spores (30 mg) of two isolates (SE2 and SE11) (Table 1) were also aliquoted from the wart compost. The frozen tissue or dry winter spores were then homogenized with a bead mill (Retsch GmbH, Haan, Germany). DNA was extracted from the fine powder of the 16 isolates using the DNeasy Plant Mini Kit (Qiagen, Hilden, Germany) according to the manufacturer's instructions. Similarly, 100 mg of ground tissue was used from the German pathotype 18(T1; number SE 16) (Table 1) for RNA extraction using the RNeasy Plant Mini Kit (Qiagen) according to the manufacturer's instructions. Removal of the remaining DNA from the extracted RNA was performed using the DNase-free kit from Ambion (Ambion, Cambridgeshire, UK), as recommended by the manufacturer. The DNA and RNA concentrations were determined using a NanoDrop 2000 spectrophotometer (Thermo Fisher Scientific Inc., Waltham, MA), and the integrity was checked on a 1% agarose gel.

Whole-genome or transcriptome shotgun sequencing. High molecular weight DNA and RNA samples (20 µg) from the German pathotype 18(T1) were delivered on dry ice to Eurofins Genomics (Eurofins Genomics GmbH, Ebersberg, Germany), where genomic DNA and cDNA library preparations were performed and sequenced on an Illumina HiSeq 2500 in the 2-by-150-bp paired-end mode. Two channels of the Illumina HiSeq 2500 (one for the genomic library and the other for the cDNA library) were used for the sequencing. The whole-genome and transcriptome shotgun sequencing were performed using a single source of tissue from Tomensa potato infected with the German *S. endobioticum* pathotype 18(T1, SE 16) (Table 1).

Sequence analyses and selection of *S. endobioticum* sequences. The Illumina TruSeq adapters were trimmed from the raw reads using CLC Genomics Workbench 7.5 (Qiagen). The sequences with a Phred quality score of less than 30 were removed. The trimmed reads were mapped to the potato, tomato, and *Batrachochytrium dendrobatidis* genome sequences separately using the default parameters of CLC Genomics Workbench 7.5. In addition, the cDNA reads were mapped to potato and tomato transcripts. The potato genome sequence (*Solanum tuberosum* Group Phureja DM) and the tomato genome sequence (*S. lycopersicum* 'Heinz 1706') were downloaded from public websites (The Potato Genome Sequencing Consortium 2011; The Tomato Genome Consortium 2012). The draft genome of *B. dendrobatidis* was obtained from the Broad Institute public website (https://www.broadinstitute.org/).

The sequence reads that were not mapped to the potato and tomato genomes and transcriptomes were collected as non-Solanaceae and, therefore, putative *Synchytrium endobioticum* reads. The reads mapped to *B. dendrobatidis* were also collected and included in the non-Solanaceae reads.

TABLE 1. List of investigated *Synchytrium endobioticum* pathotypes and isolates

Number	Pathotypes/isolates	Country of collection	Source material
SE1	1(D1)	Germany	Fresh wart tissue
SE2	1(D1)	Northern Ireland	Wart compost
SE3	39(P1)	Poland	Fresh wart tissue
SE4	D12[a]	Denmark	Fresh wart tissue
SE5	D14[a,b]	Denmark	Fresh wart tissue
SE6	P2/15[a]	Poland	Fresh wart tissue
SE7	D22[a]	Denmark	Fresh wart tissue
SE8	2(Ch1)	Poland	Fresh wart tissue
SE9	3(M1)	Poland	Fresh wart tissue
SE10	2(G1)	Germany	Fresh wart tissue
SE11	2(G1)	Germany	Wart compost
SE12	6(O1)	Germany	Fresh wart tissue
SE13	D25[a,c]	Denmark	Fresh wart tissue
SE14	8(F1)	Germany	Fresh wart tissue
SE15	18(T1)	Greece	Fresh wart tissue
SE16	18(T1)[d]	Germany	Fresh wart tissue

[a] Newly described isolates in the process of being assigned to a pathotype.
[b] Isolate from Denmark with the same genotype as the Polish pathotype 2(Ch1).
[c] Isolate from Denmark with the same genotype as the German pathotype 8(F1).
[d] The German pathotype 18(T1) used for the genome and transcriptome shotgun sequencing.

Sequence assembly and annotation of the contigs. The non-Solanaceae sequence reads and those mapped to *B. dendrobatidis* were assembled using CLC Genomics Workbench 7.5. The default assembly parameters were used, with the exception that the similarity fraction was adjusted to 90%. The assembly was performed for the selected cDNA and genomic DNA separately. The assembly was also performed for the whole trimmed raw reads of the cDNA and genomic DNA separately.

Automatic annotation of the assembled cDNA contigs was performed by a BLAST search of the National Center for Biotechnology Information (NCBI) database (Altschul et al. 1997) with the Blast2GO software (Götz et al. 2008) using a BLAST threshold value of 0.1. Gene ontology (GO) term analysis and mapping to the Enzyme Commission (EC) classification databases were also performed using the Blast2GO software (Götz et al. 2008). Further selection and refinement of *S. endobioticum* sequences were performed based on the BLAST hits.

Verification of selected *S. endobioticum* contigs by PCR. Primer sets were designed for 96 randomly selected putative *S. endobioticum* transcriptome contig sequences and verified by PCR using DNA isolated from four German pathotypes—1(D1), 2(G1), 8(F1), 18(T1)—and uninfected Tomensa leaves. PCR mixtures were prepared using 20 ng of genomic DNA in a total volume of 25 µl containing 0.5 µM each of the forward and reverse primers, 1 U of Taq polymerase, and 0.12 mM dNTP in 1× buffer provided by the manufacturer (Bioline, Luckenwalde, Germany). The PCR conditions were as follows: initial denaturation for 4 min at 94°C; followed by 35 cycles of 45 s at 94°C, 1 min at 64°C, and 1.5 min at 72°C; and a final extension of 10 min at 72°C. The PCR products were mixed with 10% Orange G loading dye (0.25% Orange G, 30% glycerine, and 1 mM EDTA) and separated on a 1.5% agarose gel in 1× Tris-acetate-EDTA buffer.

Sequence-characterized amplified region marker development. Of the 96 primer sets that were verified by PCR above, 1 amplified a polymorphic fragment specific to pathotype 1(D1) and an additional fragment that was common to all five pathotypes (Supplementary Fig. S4A). The PCR product specific for the single pathotype 1(D1) was excised from the agarose gel, purified using a QIAquick PCR Purification Kit (Qiagen), and completely sequenced by Sanger sequencing. Using the sequence information of this pathotype 1(D1)-specific fragment, a sequence-characterized amplified region (SCAR) marker, called 14425P1, was developed. The sequences of the SCAR marker 14425P1 primers were as follows: forward, 5′ GGTTG GCAGCGAGCTAGATA 3′ and reverse, 5′ TGAGCTCGTCACT ATCATGG 3′. PCR mixtures were prepared using 20 ng of genomic DNA in a total volume of 25 µl containing 0.25 µM each of the forward and reverse primers, 1 U of Taq polymerase, and 0.12 mM dNTP in 1× buffer provided by the manufacturer (Bioline). The PCR conditions were as follows: initial denaturation for 1 min at 94°C; followed by 30 cycles of 30 s at 94°C, 30 s at 62°C, and 45 s at 72°C; and a final extension of 2 min at 72°C. The PCR products were mixed with 10% Orange G loading dye and separated on a 1.5% agarose gels in 1× Tris-acetate-EDTA buffer. In the PCR mixtures of the SCAR marker 14425P1, a primer pair from the sporulation protein RMD1 (forward, 5′ GTTCTTGATGCACCCGAACT 3′ and reverse, 5′ TC TCATCGTGACTGCTCGTC 3′) was included as an internal control.

Additional resequencing and single-stranded confirmation polymorphism analyses. Sixty of the genes that were verified by PCR and showed a single banding pattern were amplified from the DNA of the five German *S. endobioticum* pathotypes 1(D1), 2(G1), 6(O1), 8(F1), and 18(T1). The PCR was performed with an annealing temperature of 64°C using proofreading PrimeSTAR HS DNA polymerase (TAKARA BIO Inc., Saint-Germain-en-Laye, France) following the procedures described by the manufacturer. PCR products from 33 of the 60 genes from all five German pathotypes were purified using a QIAquick PCR Purification Kit (Qiagen) and completely sequenced by Sanger sequencing. Comparative sequence analyses among the five pathotypes were made for all 33 genes using CLC Genomics Workbench 7.5. PCR products of the remaining 27 genes from the five German pathotypes were analyzed by single-stranded confirmation polymorphism (SSCP) (Orita et al. 1989). The 27 genes were selected based on their smaller fragment size appropriate for an SSCP analysis. For the SSCP analyses, the PCR products were separated using nondenaturing polyacrylamide gel electrophoresis on 0.5 × mutation detection enhancement gel (Biozym, Hessisch Oldendorf, Germany).

Selection of *S. endobioticum* genomic contigs. *S. endobioticum* expressed sequence tags (EST) were used as a reference to capture the corresponding genomic sequences. First, the larger genomic contigs were split into 1,000-bp fragments with 100-bp overlaps using splitter (http://emboss.bioinformatics.nl/cgi-bin/emboss/splitter). The genomic contigs were then mapped to the *S. endobioticum* EST. The mapped *S. endobioticum* genomic contigs were then extracted and

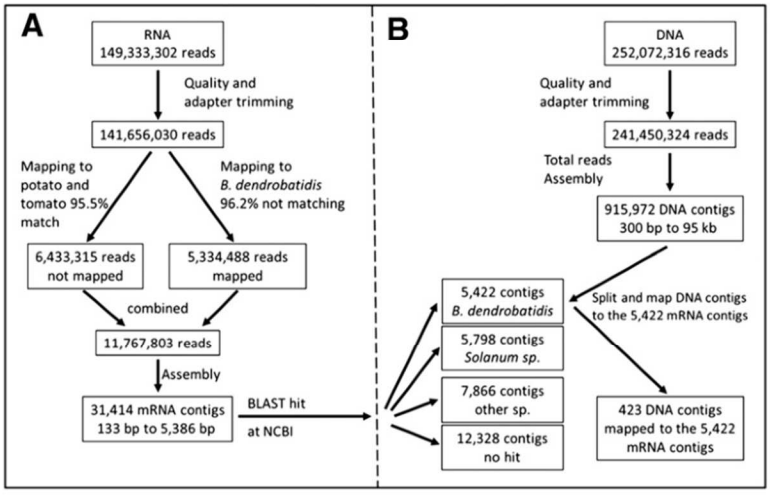

Fig. 1. Schematic illustration of **A,** transcriptome sequence analyses steps for the preliminary enrichment of *Synchytrium endobioticum* cDNA sequences and **B,** steps to obtain the 423 DNA contigs corresponding to the 5,422 *S. endobioticum* mRNA contigs.

the corresponding larger contigs were obtained from the original assembly by using CLC Genomics Workbench 7.5.

Identification of *S. endobioticum* simple sequence repeat motifs. The selected *S. endobioticum* pathotype 18(T1) genomic contig sequences were screened for hexa-, penta-, tetra-, tri-, and dinucleotide simple sequence repeat (SSR) motifs using the "SSR locator" software (da Maia et al. 2008). Primer sets corresponding to 41 randomly selected SSR motifs were designed, and each of the forward primers was end-labeled with infrared fluorescent dye 700. PCR mixtures were prepared using 20 ng of genomic DNA in a total volume of 25 µl containing 0.25 µM each of the forward and reverse primers, 1 U of Taq polymerase, and 0.12 mM dNTP in 1× buffer provided by the manufacturer (Bioline). The PCR conditions were as follows: initial denaturation for 1 min at 94°C; followed by 30 cycles of 30 s at 94°C, 30 s at 64°C, and 45 s at 72°C; and a final extension of 2 min at 72°C. The PCR products were separated on 6% polyacrylamide gel (Sequagel XR; National Diagnostics Inc., Charlotte, NC) using a DNA Analyzer (LI-COR Biosciences, Lincoln, NE).

RESULTS

Whole-transcriptome shotgun sequencing and selection of *S. endobioticum* sequences. In total, 149,333,302 reads were obtained from the whole-transcriptome shotgun sequencing of proliferated tissue from Tomensa potato infected with the German *S. endobioticum* pathotype 18(T1). After quality and adapter trimming, 141,656,030 reads remained (Fig. 1A).

The mapping of these reads to the potato and tomato genomic and transcriptomic sequences revealed 95.5% matching sequences. The 4.5% (6,433,315 reads) sequences that were not mapped to these sequences were selected as putative *S. endobioticum* reads. In parallel, the 141,656,030 trimmed reads were compared using BLAST to the *B. dendrobatidis* genome and transcriptome, and 3.8% (5,334,488 reads) produced a significant match. These 5,334,488 reads were also selected as putative *S. endobioticum* reads. Although the majority of the reads that were mapped to *B. dendrobatidis* could have already been represented in the 6,433,315 nonpotato and nontomato reads, some additional reads were obtained (Fig. 1A).

The 6,433,315 nonpotato and nontomato reads and the 5,334,488 reads that matched *B. dendrobatidis* were combined and assembled into 31,414 contigs, which ranged in length from 133 to 5,386 bp (Fig. 1A) and had an N50 (an assembled contig size above which the size of 50% of the whole assembled contigs are represented) of 328 bp.

Annotation of the contigs through a sequence similarity search of the NCBI database. A BLAST similarity search identified 5,422 contigs with hits to *B. dendrobatidis*, 5,798 contigs with hits to *Solanum* spp., 12,328 contigs without any hits and 7,866 contigs with hits to other species.

The 5,422 mRNA contigs with a BLAST hit to *B. dendrobatidis* sequences are hereafter referred to as *S. endobioticum* EST. The sizes of these EST ranged from 200 to 2,979 bp, and 3,849 EST were

TABLE 2. Descriptions of the 41 simple sequence repeat motifs and the corresponding primer sets evaluated using DNA from the five German *Synchytrium endobioticum* pathotypes 1(D1), 2(G1), 6(O1), 8(F1), and 18(T1)

Primer set	Forward	Reverse	Motif	Product size (bp)
2978	5′ ACGTATGCACGGATCAAACA 3′	5′ TACAACCCCACCTTTTCCTG 3′	(TA)11	323/330
11176	5′ TGCACTTTGTACCAGCCAAG 3′	5′ TATACCGACCTGTCCAAGCC 3′	(AT)7	384/390
4865	5′ CTACAAGCCAGTCACCGTCA 3′	5′ CCACCGAATATCCCATCATC 3′	(GGAT)8	392/402
16161	5′ GGCTTCGTCTTCAGCGATAC 3′	5′ CCATCTTCCGGTCTGTTCAT 3′	(GGTGTC)4	369/377
818598	5′ GGCCAGACGTCGATATTTCT 3′	5′ GTACGACCTCCCAGCATTGT 3′	(ATAGC)6	294/304
4761	5′ TGTGCACACTTTGAGAAGGG 3′	5′ GGTTCCAGCACGTTGGTAGT 3′	(CT)4-(AT)4	383
7711a	5′ GACCTTGTGTCCCGGAGTAA 3′	5′ TAGCTTCGCGGATAGGAAGA 3′	(GT)11	196
7711b	5′ GCCAGTAACGCGACCATATT 3′	5′ AATAAGCGAGGAATCAGCGA 3′	(TA)11	286
459	5′ AAAGCTAATGGCATGGTTGG 3′	5′ AGACGCCATCACTATGCCTC 3′	(TC)5-(GA)5	364
t322060	5′ GATCAACATGTGCTGGATCG 3′	5′ ATGATTATGACACGGTGCGA 3′	(TC)6-(GA)4	217
3714	5′ AGAACGTCTCTGCTGCCAAT 3′	5′ TTTGGTGTTGCCCTTGTGTA 3′	(AT)11	350
492	5′ ACGATGAGGATAATGCTGCC 3′	5′ TGATGGATGATGATCGTGCT 3′	(TA)8	384
4846	5′ AGTATGGGACGTCAGAACCG 3′	5′ TAACCGTGGGATATCATGGC 3′	(AC)4-(AC)5	223
9030	5′ GGACGGATCCTGCCATAGTA 3′	5′ GTCATGACTTAGGGGGCAAA 3′	(AT)4-(TA)4	315
3881	5′ TCGTAGATGTCGTGGTCGAG 3′	5′ CAGTAGGACAGGGGACCAGA 3′	(ACA)7	287
t107447a	5′ AACAAGAAAGCTGGGTGTGG 3′	5′ ATCAGTGGAGAGCCCACAAG 3′	(TAT)8	393
t107447b	5′ GTATTCGGAGGTGTCGCTGT 3′	5′ TTGTGTCCACCGAGTATCCA 3′	(CGT)9	267
t107447c	5′ AAGGCGTGCACGAGATAGTT 3′	5′ TAATCTGACTTGCCTGTGCG 3′	(GCGT)7	297
639	5′ TGGTCTTCCAATGGCCTAAC 3′	5′ AAATGCCAACCACGTAGGAG 3′	(CAT)7	327
3129	5′ ACTGCACCTTCTCCCACATC 3′	5′ AATTCCAAACATGGTAGGCG 3′	(AAG)8	358
2826	5′ GAATACGCCCGAACTAACCA 3′	5′ TTTCTGCACCTTGTCGACTG 3′	(TACGGA)4	369
2035	5′ GTCGCCCTTGATTCATGTTT 3′	5′ AACTGTCGCTGAAGAGGGAA 3′	(CCCA)7	259
2035A	5′ TTCCCTCTTCAGCGACAGTT 3′	5′ ATTTACACTGGGGGTAGGGG 3′	(CTA)11	328
2035B	5′ CATGTGTTTGACCGAGGATG 3′	5′ TGCAATGAAAGCTATGCTGG 3′	(GCAAAA)5	317
3244	5′ ATCTATCATGCATCCCGTCC 3′	5′ CACTCCACTTGTCGGACTGA 3′	(CGGT)6	361
5274	5′ TTGCAATGCGTTACTTGCTC 3′	5′ GTTGCGCGATGATCACTAGA 3′	(GGCGTG)4	262
2733	5′ ATTGGTGTGAAGATGAGGGC 3′	5′ ATCCATGACGTCGAAACCTC 3′	(GGTGTG)4	106
2894	5′ CAACCACACGAGTGGACATC 3′	5′ ATGTGTAAATGGTGTGCGGA 3′	(CAAAC)5	313
2785	5′ GAGGTCGAGTCTTTGCGTTC 3′	5′ AGGTTCCTCTGTCCATCGTG 3′	(CAGCCG)5	276
2723	5′ TCGTTGTCTGCACCTACCTG 3′	5′ GTTTCGATTGGGCATCAACT 3′	(TGCT)6	307
693	5′ ATTATTTTGGCCAAGCAACG 3′	5′ CCTCTATAGGAATGCGCAGC 3′	(TGTCGC)6	371
705	5′ TGCAAGGAATGCGACTATTG 3′	5′ CTCGTGCTGCTGATGATTGT 3′	(ACAGG)5	348
345443	5′ TGGACTTCCCTACGTTGGTC 3′	5′ CCATGATGGATGTGGATGAA 3′	(TGGTGC)7	399
442158	5′ AATCGGTACAGCTTGGGATG 3′	5′ GACGAGGAGTTTGGATGGAA 3′	(GTGCC)5	263
213091	5′ TCGAGCTCCTCCTCTAGTGC 3′	5′ TTAATGGGGTTCTCGTCAGG 3′	(CTTCCT)4	248
4884	5′ CGCCATCATGATCCTTCTTT 3′	5′ CACTGTTCGGGCTCTTTTTC 3′	(CATCG)5	384
t397081	5′ CGGGAGTCGTATGGCAGTAT 3′	5′ GGTGTGTGATGCAGGTATGC 3′	(TA)12	315
29983	5′ TCTTGCATGGCCAGTAGACA 3′	5′ TATAGTCATCCTCCCTGGCG 3′	(TA)13	203
2671	5′ TGAGAAGAGCCGAAGATTCC 3′	5′ CATCCATCCATCCATCCATC 3′	(TAGA)6	100
24845	5′ AAACTTACCGTGAGCCCCTT 3′	5′ CCATGCATTTGCATCTCTCT 3′	(AT)14	261
626	5′ TGTTGTTGTTCCTGCTGCTC 3′	5′ GGAGGCTTTGATATTTCCCC 3′	(CTA)7-(CTA)13	291

longer than 300 bp. This Transcriptome Shotgun Assembly project has been deposited at DDBJ/EMBL/GenBank under the accession GDBM00000000. The version described in this article is the first version, GDBM01000000.

Blast2Go analysis retrieved GO terms for 3,583 of the 5,422 EST. Accordingly, 434 EST were grouped into GO terms for metabolic and oxidation-reduction processes within the biological process category (Supplementary Table S1). Within the molecular function category, 1,010 EST were grouped into GO terms for ATP and protein binding and, within the cellular component category, 452 EST were grouped into GO terms for nucleus and cytoplasm. Among the EST for which GO term descriptions were assigned, 1,007 EST were effectively grouped into the six EC classifications. The majority of the EC-annotated EST belonged to EC 3 (hydrolases), followed by EC 2 (transferases), EC 1 (oxidoreductases), and EC 6 (ligases) (Supplementary Fig. S1).

PCR verification of some of the 5,422 putative *S. endobioticum* EST. Primer sets corresponding to 95 of the 96 EST produced the desired PCR fragments specifically from the DNA isolated from proliferating Tomensa tissue infected with *S. endobioticum* pathotypes but not from the uninfected Tomensa DNA (Supplementary Fig. S2). Primer sets from 94 of the 95 EST produced fragments of identical size in all five pathotypes: 1(D1), 2(G1), 6(O1), 8(F1), and 18(T1). However, 1 of the 95 primer sets (14425) amplified a 772-bp fragment specific to only pathotype 1(D1) and a 446-bp fragment common to the five German pathotypes 1(D1), 2(G1), 6(O1), 8(F1), and 18(T1) (Supplementary Fig. S4A). Sixty of the verified genes showed a single banding pattern in each of the pathotypes.

Screening for polymorphism between *S. endobioticum* pathotypes. Sequencing of the pathotype 1(D1)-specific 772-bp fragment that was obtained using the primer set 14425 revealed a 326-bp insertion into the 446-bp sequence. The 446-bp sequence was common to all five pathotypes: 1(D1), 2(G1), 6(O1), 8(F1), and 18(T1) (Supplementary Fig. S3). Interestingly, the 326-bp sequence was characterized by a 7- and 65-bp direct repeat. The EST detected by this primer set has a sequence similarity to a transmembrane segment (TMS) membrane protein/tumor differentially expressed gene.

PCR using the SCAR marker 14425P1 confirmed a 367-bp fragment in pathotype 1(D1) which was absent in all other German pathotypes: 2(G1), 6(O1), 8(F1), and 18(T1) (Supplementary Fig. S4B). Further tests using DNA from 11 additional pathotypes and isolates obtained from Denmark, Greece, Northern Ireland, and Poland indicated the presence of the 367-bp fragment in pathotypes 39 (P1) and 2(Ch1) and four isolates. The internal control primer from the sporulation protein RMD1 amplified a 175-bp fragment in all 16 isolates, indicating the intactness of all DNA and the optimum performance of the PCR procedures.

Sequencing 33 of 60 EST showing a single banding pattern resulted in sequences with a length ranging from 285 to 4,615 bp. However, no sequence variation was detected between the five pathotypes for all 33 EST (Supplementary Table S2). The sequence from these 33 EST covered a region with a size of 37,127 bp. SSCP analysis of the remaining 27 of 60 EST showing a single banding pattern and with less than 690 bp also showed no polymorphisms among the five German *S. endobioticum* pathotypes.

Whole-genome shotgun sequencing and assembly. Whole-genome shotgun sequencing of tissue from Tomensa potato infected with the German *S. endobioticum* pathotype 18(T1) resulted in 252,072,316 reads. After quality and adapter trimming, 241,450,324 reads remained. The assembly of the 241,450,324 reads resulted in 915,972 contigs longer than 300 bp, with a maximum contig size of 95 kb.

Selection of *S. endobioticum* genomic contigs corresponding to the 5,422 EST sequences. In total, 256,210 contigs were split into 1,000-bp fragments with a 100-bp overlapping sequence. Mapping to the 5,422 EST identified 423 *S. endobioticum* pathotype 18(T1) genomic contigs (Fig. 1B). The maximum size of the genomic contigs identified was 43,140 bp and, together, the 423 contigs covered a size of 2,081,121 bp with a GC content of 47%. This Whole Genome Shotgun project has been deposited at DDBJ/EMBL/GenBank under the accession LDOR00000000. The version described in this article is version LDOR01000000.

Identification of *S. endobioticum* SSR motifs. Screening of the 423 putative *S. endobioticum* pathotype 18(T1) genomic contig sequences resulted in 389 motifs with 3 to 26 repeats (Supplementary Table S3). Forty-one primer sets corresponding to 41 randomly selected SSR motifs were successfully evaluated using the five German *S. endobioticum* pathotypes (Table 2). Of the 41 SSR primer sets, 5 sets (2978, 11176, 4865, 16161, and 818598) revealed polymorphisms (Supplementary Fig. S5). These five primer sets also resulted in two different banding patterns on the 11 additional pathotypes and isolates obtained from Denmark, Greece, Northern Ireland, and Poland. The first banding pattern was specific to *S. endobioticum* pathotypes 1(D1), 2(G1), 6 (O1), 2(Ch1), 39(P1), and 3(M1) and the four isolates D12, D14, D22, and P2/15, whereas the second banding pattern was specific to *S. endobioticum* pathotypes 8(F1) and 18(T1) and isolate D25.

DISCUSSION

Identification of *S. endobioticum* EST and genomic contigs. In the study presented here, a small-scale next-generation transcriptome and genome sequencing approach was used for the first time on *S. endobioticum*, a quarantine organism for which only

TABLE 3. Genotypes and grouping of the 16 isolates of *Synchytrium endobioticum* based on the six polymorphic markers

Number	Pathotypes, isolates	14425P1[a]	2978[b]	11176[b]	4865[b]	16161[b]	818598[b]	Group
SE1	1(D1)	+	I	I	I	I	I	1
SE2	1(D1)	+	I	I	I	I	I	1
SE3	39(P1)	+	I	I	I	I	I	1
SE4	D12	+	I	I	I	I	I	1
SE5	D14	+	I	I	I	I	I	1
SE6	P2/15	+	I	I	I	I	I	1
SE7	D22	+	I	I	I	I	I	1
SE8	2(Ch1)	+	I	I	I	I	I	1
SE9	3(M1)	–	I	I	I	I	I	2
SE10	2(G1)	–	I	I	I	I	I	2
SE11	2(G1)	–	I	I	I	I	I	2
SE12	6(O1)	–	I	I	I	I	I	2
SE13	D25	–	II	II	II	II	II	3
SE14	8(F1)	–	II	II	II	II	II	3
SE15	18(T1)	–	II	II	II	II	II	3
SE16	18(T1)	–	II	II	II	II	II	3

[a] Symbols + or – indicate the presence or absence, respectively, of the 367-bp fragment for the sequence-characterized amplified region marker 14425P1.
[b] Presence of the banding pattern "I" or "II" is indicated for the five polymorphic simple sequence repeat loci.

eight 18S and 28S ribosomal gene sequences were available thus far in the whole public sequence databases. Of course, the isolation of sequence information from *S. endobioticum* is complicated by the fact that producing a pure culture based on single-spore isolates is not possible because the fungus is an obligate biotroph and impossible to grow on artificial media. *S. endobioticum* produces sporangia carrying motile spores but no hyphae or other fruiting structures (Anonymous 2004; Webster and Weber 2007). Therefore, we decided to isolate the total RNA and DNA from freshly infected tissue and to discriminate *S. endobioticum* sequences from potato sequences through bioinformatics methods. To verify the identified sequences, we designed PCR primer sets for 96 EST sequences and tested them by PCR on four German *S. endobioticum* pathotypes. All except one primer set amplified the expected product from all four German pathotypes.

The lack of an *S. endobioticum* reference genome and the fact that the reference species *B. dendrobatidis* used in this study belongs to a different order than *S. endobioticum* led to fewer BLAST hits compared with, for example, the RNAseq data from *Botrytis cinerea*-infected lettuce tissue (de Cremer et al. 2013). However, the identification of 5,422 EST and the additional 423 genomic contigs of *S. endobioticum* substantially extends the limited information available to date. Many of the remaining 12,328 mRNA contigs without any BLAST hits may also belong to *S. endobioticum*, even though they cannot be identified as such due to the lack of a reference genome that is sufficiently taxonomically close to reveal sequence similarity.

Polymorphic marker-based grouping of *S. endobioticum* pathotypes. More than 40 *S. endobioticum* pathotypes are currently known (Baayen et al. 2006; Çakır et al. 2009; Przetakiewicz 2015a), and they are distinguished based on their infection patterns on differential sets of host genotypes. More than half of these pathotypes are either extinct or of minor relevance (Baayen et al. 2006). Pathotype 1(D1) is the oldest pathotype to which most European potato cultivars are resistant (Anonymous 2004; Flath et al. 2014). Pathotypes 8(F1) and 18(T1) seem more virulent than the other common pathotypes. For instance, 7 of the 10 differential cultivars were susceptible to pathotype 18(T1), compared with only 3 and 5 being susceptible to pathotypes 1(D1) and 6(O1), respectively (Anonymous 2004). An independent test showed that pathotypes 8(F1) and 18(T1) are more aggressive than the other pathotypes (K. Flath, personal communication). The SCAR primer set 14425P1 and the five SSR markers from different, nonoverlapping genomic contigs classified the 16 studied *S. endobioticum* collections into three groups (Table 3). The more virulent pathotypes 8(F1) and 18(T1) and isolate D25 are identified in the same group distinct from all the other used pathotypes and isolates. Based on the SCAR and SSR markers as well as by using differential cultivars (J. Przetakiewicz, personal communication), isolate D25 from Denmark is found to be the same genotype as the German pathotype 8(F1) and the same pathotype. Hence, isolate D25 can be represented by pathotype 8(F1). The markers of the pathotype 8(F1) and 18(T1) group may be used in a preevaluation of previously uncharacterized *S. endobioticum* isolates.

Low levels of diversity between *S. endobioticum* pathotypes. A comparative sequence analysis of 33 contigs covering a region with a size of 37,127 bp and an SSCP analysis of 27 EST covering a size of 10,059 bp revealed no sequence polymorphisms between the five German *S. endobioticum* pathotypes 1(D1), 2(G1), 6(O1), 8(F1), and 18(T1), indicating that *S. endobioticum* pathotypes may be exceptionally similar to each other. It should be noted that the method used to select amplicons for sequencing was extremely conservative to ensure that all sequences originated from *S. endobioticum*, and this may result in bias toward the sequencing of conserved regions. However, our observation was corroborated by a recent report, in which extremely low levels of diversity were detected among eight isolates by using complexity reduction of polymorphic sequences analyses (Bonants et al. 2015). This result is astonishing given the clear differentiation of pathotypes based on infection assays. The low sequence diversity is probably due to the development of new *S. endobioticum* pathotypes by very limited changes in avirulence factors rather than extensive genetic recombination between divergent genotypes.

CONCLUSIONS

For the first time, a large number of *S. endobioticum* genome and transcriptome sequences are presented in this study. Compared with the very limited information available to date, the large set of EST sequences and more than two million bases of genomic sequences reported here will assist future studies in increasing the understanding of the mechanism of tumor formation and in elucidating the nature of the interaction with the potato host. A simple SCAR marker, 14425P1, is presented that distinguishes the German *S. endobioticum* pathotype 1(D1) from the other pathotypes commonly present in Germany. Five SSR primer sets are identified that clearly distinguish the more virulent pathotypes 8(F1) and 18(T1) from the other European pathotypes and isolates. We hope that the data presented here will help to narrow the current gaps in scientific knowledge of *S. endobioticum*.

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3. General discussion

For the genetic analysis of resistance to potato wart disease three manuscripts are presented, focusing on different aspects: the establishment of a dihaploid potato population to facilitate genetic analyses in potato (manuscript 1), marker development for and genetic mapping of resistance to potato wart disease (manuscript 2), and the analysis of different pathotypes of *Synchytrium endobioticum* for the detection of genetic differences between the pathotypes (manuscript 3). The main results that were obtained in the three studies presented in this thesis are already discussed in the respective manuscripts. More general aspects are discussed in this chapter to connect the findings of the different manuscripts, to draw conclusions on possible implications for potato breeding regarding resistance to potato wart disease and to give an outlook on future research objectives.

3.1. Using a dihaploid population to facilitate tetraploid genetics and to identify resistance loci for potato wart disease

The cultivated potato is a tetraploid species that displays tetrasomic inheritance. Four homologous chromosomes for each of the twelve potato chromosomes pair randomly in two bivalents during meiosis, with the two cellular divisions proceeding normally, resulting in diploid gametes, which again unite randomly and result in tetraploid progeny. Low levels of quadrivalents, trivalents and univalents have been reported for potato (Bradshaw, 2007b). Segregation ratios are corresponding to Mendelian genetics with a 1:1 segregation ratio for a *simplex x nulliplex* cross (Aaaa x aaaa), 3:1 for a *simplex x simplex* cross (Aaaa x Aaaa) and 5:1 for a *duplex x nulliplex* cross (AAaa x aaaa), but considerably higher for *duplex x simplex* crosses (11:1 segregation ratio) and up to 35:1 for *duplex x duplex* crosses, with different frequencies of occurrences of the respective heterozygous genotypes. Inter-locus interactions and double reduction events may occur and the high heterozygosity of potato add considerably to the complexity in potato breeding.

Reducing genomic complexity of a tetraploid cultivar by generating a dihaploid population is a convenient approach to facilitate genetic analysis and allows circumventing problems occurring regarding tetrasomic inheritance in tetraploids. In general, a gene present in simplex allele configuration in the original tetraploid genotype (Aaaa) would segregate in a 1:1 segregation ratio in the dihaploid progeny (Aa and aa). A gene present in duplex allele dosage in the tetraploid genotype (AAaa) would segregate in a 5:1 ratio in the dihaploid genotypes. Dihaploids display much simpler segregation ratios than the tetraploid counterparts. Additionally, heterozygous genotypes only occur with one allele configuration (Aa) in contrast to various possible allele configurations in tetraploid heterozygous genotypes (Aaaa, AAaa and AAAa). Different allele dosages can further complicate

genetic analysis and possible dosage effects are omitted in dihaploid genotypes. Additionally, compared to a conventional cross between two tetraploid cultivars, more markers are segregating from the elite parent which in turn allows mapping of more simplex markers (manuscript 1).

One disadvantage in using dihaploid genotypes for genetic analysis is the rather time-consuming generation of the dihaploid population. Selection of dihaploid genotypes within the progeny, which can also contain triploid and tetraploid hybrids, prolonged the construction of the progeny for genetic analysis, although the chosen approach in this study, with the phenotypic selection of true dihaploid progeny and subsequent ploidy determination via flow cytometry is a straightforward procedure. Dihaploid genotypes were germinated and multiplied *in vitro*. Reduced plant vigor was observed, with slower growth and reduced root development in some genotypes, when compared to tetraploid genotypes. Transferring the dihaploid genotypes into the greenhouse for phenotyping and tuberization revealed additional problems, as acclimatizing was difficult for some genotypes which died after a short time or showed reduced vigor and growth. One reason could be the inheritance of deleterious alleles in the dihaploid progeny which are usually recessive and stay unnoticed in the tetraploid parent.

Resistance phenotyping of the dihaploid genotypes for potato wart disease resulted in a much clearer classification of resistant and susceptible genotypes as compared to the results in a tetraploid population derived from a cross with the same resistant cultivar as resistant parent (Obidiegwu et al., 2015). In our study, only 26 genotypes showed intermediate phenotypes with mean resistance scores between 2.5 and 3.5, while in the tetraploid population of Obidiegwu et al. (2015) a majority of the genotypes showed intermediate resistance scores and had to be excluded for genetic analysis. Occurrence of a multitude of intermediate genotypes could be due to interfering alleles of the second tetraploid parental genotype in the tetraploids, which is not the case in the monoparental dihaploid population. Also, dosage effects in the tetraploid genotypes could result in a broader variation regarding the resistance phenotype. Mapping of the major resistance locus resulted in a better resolution in the dihaploid than in the tetraploid population (manuscript 2). Although one has to keep in mind that the genetics of complex traits may not exactly reflect the tetraploid situation, the obtained results point out the usefulness of dihaploid genotypes as a convenient tool for genetic analysis of a single tetraploid potato cultivar, circumventing the problems associated with tetraploid genetics.

3.2. Narrowing down the major resistance locus for wart resistance on chromosome 11 and the identification of potential underlying resistance genes

In our study, we were able to narrow the major wart resistance locus on potato chromosome 11 to approximately 800 kbp (manuscript 2). This *Sen1* locus has been reported in almost all the genetic studies on potato wart resistance and is conferring resistance to the most important pathotypes 1, 2 6 and 18 (Hehl et al., 1999; Gebhardt et al., 2006; Ballvora et al., 2011; Groth et al., 2013; Obidiegwu et al., 2015). The achieved resolution of the resistance locus in our study is the highest reported so far and allows the analysis of candidate genes in the future. The number of underlying genes in this genomic region in the potato reference genome is quite low, with 87 genes, of which 13 are resistance genes or resistance gene analogs (RGAs). These 13 RGAs represent the most promising candidate genes. Resequencing of resistant and susceptible genotypes could be helpful to identify polymorphisms within the genes to further narrow down possible candidate genes. However, structural variations may occur when comparing newly generated sequences with the potato reference genome. Generation of *de novo* sequence data for two haplotypes of potato chromosome 5 and comparison with the assembly of the reference genome, revealed large sized structural variation between the different assemblies, ranging from homozygous regions, well-aligned heterozygous regions, interruptions by inserts and absence of sequence homology (de Boer et al., 2015). Structural variations have also been reported for the euchromatin in potato (Iovene et al., 2013) and the extent in potato is much higher than it has been reported for other plant species like maize, rice and soybean (Springer et al., 2009; Yu et al., 2011; McHale et al., 2012). In most of the cases, transposable elements are responsible for the variation in intergenic and genic regions (Morgante et al., 2007). Thus, the reference genome should only be used as support in a resequencing approach and *de novo* assembly is to be preferred to mapping of *de novo* sequences to the reference genome sequence to narrow down the number of potential candidate genes for wart resistance in the 800 kbp genomic region. A subsequent functional analysis of these selected genes could help in identifying the responsible resistance gene or genes. Genetic transformation by complementing a susceptible cultivar like `Tomensa´ with a sense construction of the candidate gene or genes could identify the gene of interest, if expression of the transgene restores the resistant phenotype. Transformation of resistant genotypes where expression of the functional gene is inhibited by e.g. transposon or T-DNA insertion is another approach (Feldmann et al., 1989). However, genetic transformation is very laborious and time-consuming because stable transformation takes several months and a high number of transformants must be analyzed. Also, in case of polygenic traits, partial effects of QTLs represent a major limitation. To circumvent the problems that occur with conventional genetic engineering, new genome editing tools have been developed recently, such as transcription activator-like effector nucleases (TALEN) and clustered regularly interspaced short palindromic repeats (CRISPR)/CRISPR associated (Cas) proteins.

Both methods introduce a double-strand break in the target DNA. TALENs are composed of a DNA-binding domain and the catalytic domain of the restriction endonuclease *Fok*I, which can be easily engineered to target any DNA sequence. Therefore, a genetic construct consisting of an artificial DNA-binding domain, a nuclear localization signal and the *Fok*I catalytic domain are generated. TALENs work as pairs with binding sites chosen to be located on opposite DNA strands and are separated by a small spacer sequence of 12-25 base pairs. In the nucleus, the artificial nuclease binds to the target site, the *Fok*I domains dimerize and cause a double-strand break in the spacer sequence (Christian et al., 2010). Target site recognition using the CRISPR/Cas system is achieved by binding of a non-coding guide RNA which is complementary to the DNA target site. Cas9 is composed of two endonuclease domains which each cut one strand of DNA upon binding of the guide RNA, resulting in a double strand break, which occurs three base pairs upstream the protospacer adjacent motif (Jinek et al., 2012). Both methods have been used extensively for plant genome editing and can also be applied for gene replacement or gene stacking (Malzahn et al., 2017). Wang et al. (2014) generated a powdery mildew resistant wheat line by knocking out three *MLO* homologs. For a functional analysis both techniques could be used to knock-out candidate genes for resistance to potato wart disease in resistant cultivars or to introduce genes in susceptible ones.

3.3. Discriminating resistance loci for *S. endobioticum* pathotypes 6 and 18

Correlations of resistances to *S. endobioticum* pathotypes 2, 6, 8 and 18 have been reported repeatedly in different studies carried out in mapping populations of different genetic backgrounds (Ballvora et al., 2011; Groth et al., 2013; Obidiegwu et al., 2015). In our study, we could also show that resistance to pathotypes 6 and 18 was highly correlated with a correlation coefficient of r = 0.8 (manuscript 2). Qualitative resistance mapping allowed positioning of the resistance loci for both pathotypes in the same chromosomal interval on chromosome 11. These findings imply that the same underlying resistance genes are responsible for wart resistance to the different pathotypes. However, one genotype of our dihaploid population showed differentiating resistance values for the two pathotypes. For this genotype the mean resistance score for pathotype 18 was 3.75 and 1.67 for pathotype 6, classifying the genotype as susceptible to pathotype 18 and resistant to pathotype 6, which indicates that two different genes might be responsible for resistance to pathotype 6 and 18 which are both located in the same genomic region. However, only four tubers were successfully inoculated with pathotype 18, leading to an exclusion of this genotype from further resistance analysis in our study. Repeat experiments would be necessary before a real recombination between the two resistance specificities can be postulated.

3.4. Diagnostic markers for wart resistance

Diagnostic markers to detect wart resistance to the different pathotypes of *S. endobioticum* would greatly facilitate the selection of resistant genotypes early in the breeding process and would speed up the cultivar development immensely. Phenotypic resistance assessment in most countries of the European Union are carried out according to the guidelines provided by the European and Mediterranean Plant Protection Organization (EPPO, 2004) using the Spieckermann (Spieckermann & Kothoff, 1924) or the Glynne-Lemmerzahl method (Glynne, 1925; Lemmerzahl, 1930). For a reliable resistance assessment at least 20 tubers per genotype and pathotype have to be inoculated, and nonetheless, the resistance screening oftentimes remains ambiguous. Additionally, it is laborious and time-consuming and therefore costly. Molecular marker based studies to map resistance loci for potato wart, have one common resistance locus: the *Sen1* locus on chromosome 11. Nevertheless, it has been reported that other resistance loci are contributing to the resistance to different pathotypes, depending on the genetic background of the populations used in the respective studies (Hehl et al., 1999; Brugmans et al., 2006; Ballvora et al., 2011; Groth et al., 2013; Obidiegwu et al., 2015). To develop diagnostic molecular markers a genome-wide association mapping approach could be conducted. Association mapping or linkage disequilibrium (LD) mapping is used to investigate correlations between genotypes and phenotypes of unrelated individuals (Myles et al., 2009). In contrast to traditional linkage mapping where two individuals are crossed to generate a mapping population, a collection of individuals with unknown relatedness, derived from wild species, germplasm collections or breeding germplasms, is used in association mapping (Rafalski, 2010). While in linkage mapping with biparental crosses the number of segregating alleles is limited and therefore recombination events occur rarely (Zhu et al., 2008), association mapping utilizes the natural genetic diversity within the association panel and its historical and evolutionary recombination to dissect complex traits in a large number of species (Yu & Buckler, 2006). This results in an increased mapping resolution (Hall et al., 2010) and less research time because the laborious and time-consuming establishment of a mapping population is circumvented (Myles et al., 2009). Association mapping enables the identification of QTLs based on the strength of the correlation between genetic markers and traits (Mackay & Powell, 2007). Linkage disequilibrium, the non-random association of alleles at different loci, plays a central role in association mapping (Flint-García et al., 2003). Its extent is strongly dependent on the population structure, it is increased by population bottlenecks, genetic drift and inbreeding (Rafalski, 2002), whereas admixture decreases linkage disequilibrium in outcrossing species (Flint-García et al., 2003). LD generally decreases with the distance between the marker loci and is very non-uniform across the genome, with more LD in centromeric regions (Rafalski, 2010). The degree and distribution of LD determines the association mapping methodology to identify QTLs. In the candidate gene approach, alleles at a few selected genes are tested for association with a certain trait, and in

genome-wide association studies, the whole genome is scanned to identify regions that are associated with a phenotype (Myles et al., 2009). The number of loci that have to be scanned within the genome is dependent on the extent of the LD. Where it declines slowly with increasing distance from the gene that is responsible for the phenotype, a low marker density is sufficient to identify associated markers, whereas where LD declines rapidly around the causal gene, a higher density of markers is needed to identify an associated marker (Rafalski, 2002). In general, LD decreases faster in outbred species than in inbreds (Rafalski, 2010). However, resistance sources for potato wart disease may vary between different cultivars, making it impossible to develop a molecular marker with true diagnostic value over a wide range of varieties. Markers with diagnostic values specific for the different resistance sources would be needed in order to identify resistant cultivars using multiplexed marker-assisted selection. Another difficulty is the localization of *R* genes in the potato genome: resistance genes or RGAs are oftentimes located in so-called resistance "hot-spots" in the telomeric regions of the respective chromosomes (Jupe et al., 2012; 2013), which generally show higher recombination rates than centromeric regions, so that diagnostic markers have to be tightly linked to the respective resistance gene or be located within the resistance gene itself. Most suitable markers for association mapping are SSR and SNP markers, due to their high occurrences in the potato genome and the relatively easy handling and detection methods. However, more varieties would need to be evaluated phenotypically for resistance to potato wart disease and the different pathotypes of *S. endobioticum* to develop molecular markers with true diagnostic value.

3.5. Pathotype identification using molecular markers and possible alternatives to improve resistance phenotyping for potato wart disease

Pathotype identification of *S. endobioticum* is based on a differential set of potato cultivars with different resistance properties to the respective pathotypes. Therefore, the identification of pathotypes remains as laborious and time-consuming as the general resistance assessment. Molecular markers that are specific for the respective pathotypes would greatly facilitate and fasten control measures and descheduling of infected fields. Markers for the detection of *S. endobioticum* in soil samples are available (Niepold & Stachewicz, 2004; Abdullahi et al., 2005; van den Boogert et al., 2005; van Gent-Pelzer et al., 2010), but differentiation between the respective pathotypes using molecular markers has not been reported yet. Bonants et al. (2015) were the first to report a PCR-assay to discriminate between pathotype 1 and the other pathotypes 2, 6, 8 and 18. Markers presented in this study (manuscript 3) allowed to discriminate pathotypes 8 and 18 from pathotypes 1, 2 and 6. Virulence of pathotype 18 includes that of pathotype 8, which might explain the indistinguishability between the two pathotypes, but differences to the remaining pathotypes. In general, genetic

polymorphisms between the pathotypes turned out to be very low, which is contrasting to the observed specificities of the respective pathotypes regarding the infection of the differential cultivars. Further sequencing approaches of the different pathotype genomes are needed to develop pathotype-specific markers. This would also help in giving insights into the evolution of the fungus and the development of different pathotypes. Homologous genomes of related fungi are scarce with *Batrachochytrium dendrobatidis* being the only other chytridiomycete with available sequence data.

Sequencing and annotation of the fungus would also help to identify *S. endobioticum* specific and at best pathotype specific genes, which in turn would help understanding the biology of the fungus. Although the infection cycle of the fungus is well described, relatively little is known about the host-pathogen interaction and the mechanisms underlying tumor generation on the one hand, and the resistance response on the other hand. Resistant cultivars show a hypersensitive response upon infection which is a common reaction to biotic stress in plants (Jones & Dangl, 2006). Dissecting the underlying signal transduction pathways could help in understanding the pathogenicity of *S. endobioticum*. Other tumor inducing pathosystems like that of *Agrobacterium tumefaciens* are much better understood (Escobar & Dandekar, 2003). The obligate biotrophic and soil-borne nature of *S. endobioticum* further complicates analysis of the pathosystem. Although, the predominant infections occur on tubers, symptoms are sometimes also observed on other parts of the plants, e.g. lower stems that were below ground upon infection. Given that any meristematic tissue can be infected with the fungus, artificial infections could also be carried out on above ground parts of the plants, e.g. nodes of seedlings. Injection of zoospore-suspensions or inoculation with wart tissue in a moist environment to allow zoospore mobility could result in tumor-like outgrowths in the nodes or any other susceptibility-specific symptoms that would allow distinguishing between resistant and susceptible cultivars. However, contaminations with other pathogens would have to be considered in the experimental design. Any resistance assessment that would be independent from tuber availability, would greatly improve resistance breeding. Inoculation of micro-tubers that are induced *in vitro* is not representing a promising alternative to fasten the resistance phenotyping process. Micro-tubers are oftentimes too small to be successfully inoculated with *S. endobioticum*, and their induction *in vitro* is almost as time-consuming as the tuberization of plants in the greenhouse.

3.6. Future prospects of potato wart resistance breeding

The overall goal of the collaboration project is the establishment of precision breeding for starch potatoes. Diagnostic molecular markers for potato wart resistance in combination with markers for nematode resistances and resistance to potato virus Y, for which diagnostic markers are already available (Sattarzadeh et al., 2006; Kasai et al., 2000; Flis et al., 2005; Witek et al., 2006; Sato et al.,

2006) will lead to marker-assisted breeding of high-yielding starch potatoes. Markers for starch properties have also been developed (Li et al., 2005; 2008). As an important renewable raw material for industrial applications, breeding of starch potatoes will become more and more important. Only a few crop plants, like maize, tapioca and potato, are covering the increasing demand for industrial starch (Batchelor et al., 1996). Potato starch is easily obtained and the amount of contamination with fat and protein is relatively low (von Tucher, 1995).

The molecular markers developed in this thesis with potential diagnostic value could represent the first step towards potato wart resistance breeding. The abovementioned ideas on future research objectives regarding wart resistance together with the developed markers could help to accelerate the detection of resistant cultivars early in the breeding process, which in turn could improve sustainable and profitable cultivation of starch as an important industrial raw material.

As an alternative to marker-assisted selection in plant breeding, genomic selection has become more important in the last years. Rather than looking for single loci that are significantly associated with a trait of interest, genomic selection uses all available marker data to calculate predictions on the performance of a given genotype, exploiting high-throughput genotyping methodologies (Heffner et al., 2009; Jannik et al., 2010). Superior genotypes can be selected early on in the breeding process showing resistance specificities for multiple diseases or other valuable traits with economic importance and genotypes showing specific SNP haplotypes could be selected and used for the development of new potato cultivars. Alternative solutions for potato wart resistance breeding is gene stacking by pyramiding resistance genes from resistant potato species or cultivars. However, it can be inefficient for quantitative traits that are controlled by multiple loci, of which some might have only a small effect on the phenotype (Heffner et al., 2009). Additionally, the establishment of new resistance sources in potato cultivars will decelerate the duration of the breeding process and only very few varieties are resistant to multiple pathotypes of *S. endobioticum* (K. Flath, personal communication). Developing new potato cultivars takes up to ten years if two elite tetraploid cultivars are crossed to generate a breeding population (Jansky, 2009). If resistances of mostly diploid wild potato species are crossed into existing tetraploids which are then used to generate new cultivars the breeding process can last three to five times as long (E. Tacke, personal communication). The high heterozygosity and the tetrasomic inheritance of potato further complicate resistance breeding. To detect new resistance sources in wild relatives of the potato, wild potato accessions would have to be phenotyped for resistance to different pathotypes of *S. endobioticum*.

3.7. Verification of thesis objectives

The main objectives of this thesis were previously described in chapter 1.3.1. To which extent the thesis objectives were achieved is summarized below.

i. Determination whether genetic analysis of a dihaploid potato population is a convenient tool to analyze the genetics of potato wart resistance and additional phenotypic traits of a tetraploid potato cultivar.

Genetic analysis of resistance of potato to potato wart disease in this study was carried out in a dihaploid potato population derived from a resistant tetraploid cultivar. The construction of the population was achieved by prickle pollination with dihaploid inducer clones of the diploid wild potato species *S. phureja*, namely clones IVP101 and IVP35. Although the construction is more laborious than that of a conventional tetraploid population of a biparental cross, selection of dihaploid genotypes in the progeny was straightforward. Due to the incorporated seed-marker which also leads to an anthocyanin pigmentation in the nodes of seedlings of triploid and tetraploid progeny, dihaploid genotypes can easily be distinguished phenotypically. Confirmation of the diploid ploidy using flow cytometry allowed a high throughput screening of large numbers of samples. Frequencies of dihaploids obtained from the crosses were quite low with approximately 11 – 18 %. Preselection for an embryo spot on the seed increased the number of dihaploid genotypes in the germinating seedlings to more than 50 %. Genetic mapping in the dihaploid population using SNP marker data allowed the construction of 45 linkage maps. Only two of the twelve potato chromosomes were represented by less than four linkage groups. Comparison of available marker data with that of a cross between two tetraploid cultivars, revealed a much higher amount of usable simplex SNP markers in our dihaploid population. QTL mapping was possible for several phenotypic traits in our population, revealing already known as well as new QTLs. Mapping of resistance to potato wart disease improved the mapping resolution and showed a much clearer segregation of resistance, indicating the presence of a major dominant factor responsible for the resistance segregation in the dihaploid population.

ii. Determination of the extent of introgression of the dihaploid inducer genome of the wild potato species *Solanum phureja* in the dihaploid population and whether it does disturb genetic analyses of dihaploid potato genotypes.

Introgression of the pollinator genome of *S. phureja* was analyzed in two dihaploid populations. SSR markers as well as SNP marker data was successfully applied. SSR markers revealed one and two genotypes in the two populations, respectively, that showed significant introgression of *S. phureja*, which indicates that elimination of *S. phureja* chromosomes takes place after fertilization. SNP marker data additionally revealed putative introgressions of the pollinator genome in nearly every genotype and on almost every chromosome. Introgressions occurred as individual events on the respective chromosomes, with one exception on chromosome 8 where two adjacent SNP markers showed high consistency in occurrence in the same genotypes, indicating a true introgression event. In general, the overall percentages of introgression of the pollinator genome are very low in the dihaploid populations and could also be due to genotyping errors. In general, the detected pollinator introgressions do not disturb analyses on the diploid level.

iii. Identification of molecular markers for resistance against *S. endobioticum* pathotypes 6 and 18 that are linked to resistance to potato wart disease.

Resistance loci for pathotypes 6 and 18 could be identified using the SNP marker data. For both pathotypes the major resistance locus could be mapped in the *Sen1* region of chromosome 11, which is known to be conferring resistance to various pathotypes. Additionally developed molecular markers allowed improvement of the mapping resolution in comparison to previously published data, by reducing the interval of the locus to less than 800 kb. Eight markers were segregating without recombination to resistance. Altogether, 99 SNP markers were identified to be significantly linked to resistance to pathotype 18. These markers were located on chromosomes 10 and 11. Eighty-seven markers on chromosome 11 were identified to be significantly linked to resistance to pathotype 6.

iv. Analysis whether resistances for the *S. endobioticum* pathotypes 6 and 18 are conferred by different resistance loci.

The number of identical SNP markers, significantly linked to resistance to both pathotypes was quite high with approximately 93 % identical markers. The twenty markers with the lowest p-value, thus being most significantly linked to resistance, were identical for both pathotypes. In general, resistance to both pathotypes was highly correlated. An additional minor QTL for resistance to pathotype 18 could be identified on potato chromosome 10, which was not detected for pathotype 6. One genotype

showed an interesting phenotype, being the only genotype with differentiating resistances to the two pathotypes. This genotype was resistant to pathotype 6 and susceptible to pathotype 18, indicating that resistances to the two pathotypes could be controlled by different genes. However, the genotype had to be excluded from further analysis, because the number of successfully inoculated tubers was too low and did not reach the exclusion threshold for our analyses. Additional resistance phenotyping of tubers is needed to show whether a recombination for resistance occurred for this genotype.

v. Development of molecular markers with diagnostic value that can be applied in populations derived from cultivars with different genetic backgrounds.

Molecular markers that were developed for the fine mapping of the major resistance locus on chromosome 11 were tested in a small association panel, consisting of 38 and 45 tetraploid cultivars with known resistance properties to pathotype 18 and pathotype 6, respectively. Two markers showed promising results, showing the expected genotypes in 89.5 % and 86.6 % of the tested cultivars for pathotype 18 and pathotype 6 with four and six varieties showing non-matching genotypes for the two markers. To the best of our knowledge, these two markers show the highest diagnostic value for resistance to pathotypes 18 and 6 to date. The non-matching varieties may be explained with possible different underlying resistance sources in these cultivars, which can stay undetected with the molecular markers developed in the genetic background of our resistant cultivar. Nevertheless, our markers are the most promising to detect resistance in a wide range of different cultivars and are the first markers described with potential diagnostic value for the pathotypes 6 and 18. To further substantiate the diagnostic value of our markers, additional tetraploid cultivars have to be tested for resistance to different pathotypes of *S. endobioticum*.

vi. Identification of genetic differences between the different pathotypes of *S. endobioticum* and development of molecular markers specific for the respective pathotypes of *S. endobioticum* that can be used for pathotype identification.

Sequencing of different pathotypes of *Synchytrium endobioticum* revealed a very low polymorphism rate between the pathotypes. Markers developed in this study, allowed the distinction between pathotypes 1, 2 and 6 and pathotypes 8 and 18. Additional sequencing approaches are needed to distinguish genetic differences between the other pathotypes. Sequencing data of other chytridiomycetes is scarce, impeding the annotation of *S. endobioticum* specific genes.

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Description of the electronic appendices

In addition to the supplementary data provided for each of the three manuscripts, the following files are also provided as electronic appendix:

- Fine_mapping_Sen1.xls contains marker data for the fine mapping of the major resistance locus on chromosome 11.
- JoinMap_DHs.xls contains the SNP marker data used for the genetic linkage mapping in the dihaploid population.
- Markers_K-W.csv contains the marker data used for the statistical marker-trait association analysis using a Kruskal-Wallis test.
- Phenotypes_P6.xls contains the data of the phenotypic resistance assessment for the dihaploid population inoculated with pathotype 6 used for the statistical marker-trait association analysis using a Kruskal-Wallis test.
- Phenotypes_P18.xls contains the data of the phenotypic resistance assessment for the dihaploid population inoculated with pathotype 18 used for the statistical marker-trait association analysis using a Kruskal-Wallis test.
- R_code_K-W.txt contains the R code used for the statistical marker-trait association analysis using a Kruskal-Wallis test.
- Traits_QTL_DHs_BCX contains the Box-Cox-transformed trait data for the QTL mapping in the dihaploid population.

Curriculum vitae

Name:	Annette Bartkiewicz
Date of birth:	30.04.1988
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Academic education

Since 07/2013	PhD student at the Institute of Plant Genetics in the Department of Molecular Plant Breeding at the Leibniz Universität Hannover Member of the Research Training Group GRK1798 “Signaling at the Plant-Soil Interface”
10/2009-12/2011	Master of Science in Plant Biotechnology at the Leibniz Universität Hannover Master thesis at the Institute of Plant Genetics in the Department of Molecular Plant Breeding
10/2006-10/2009	Bachelor of Science in Plant Biotechnology at the Leibniz Universität Hannover Bachelor thesis at the former Institute of Ornamental and Woody Plant Science in the Department of Floriculture

Employment history

03/2013-09/2017	research assistant at the Institute of Plant Genetics in the Department of Molecular Plant Breeding at the Leibniz Universität Hannover
01/2013-02/2013	internship at the Böhm-Nordkartoffel-Agrarproduktion OHG
12/2012	student assistant at the Institute of Plant Genetics in the Department of Molecular Plant Breeding at the Leibniz Universität Hannover

School education

2000-2006	secondary school (Gymnasium) St. Ursula-Schule in Hannover with acquisition of the general qualification for university entrance (allgemeine Hochschulreife)
1998-2000	middle school (Orientierungsstufe) Ludwig-Windthorst-Schule in Hannover
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Publications

Bartkiewicz, A., Chilla, F., Terefe-Ayana, D., Lübeck, J., Strahwald, J., Tacke, E., Hofferbert, H.-R., Linde, M. and Debener, T. (2018): Maximization of Markers Linked in Coupling for Tetraploid Potatoes via Monoparental Dihaploids. Submitted to Frontiers in Plant Science.

Bartkiewicz, A., Chilla, F., Terefe-Ayana, D., Lübeck, J., Strahwald, J., Tacke, E., Hofferbert, H.-R., Flath, K., Linde, M. and Debener, T. (2018): Improved genetic resolution for linkage mapping of resistance to potato wart in monoparental dihaploids with potential diagnostic value in tetraploid potato varieties. Submitted to Theoretical and Applied Genetics.

Busse, F., Bartkiewicz, A., Terefe-Ayana, D., Niepold, F., Schleusner, Y., Flath, K., Sommerfeldt-Impe, N., Lübeck, J., Strahwald, J., Tacke, E., Hofferbert, H.-R., Linde, M., Przetakiewicz, J. and Debener, T. (2017): Genomic and Transcriptomic Resources for Marker Development in *Synchytrium endobioticum*, an Elusive but Severe Potato Pathogen. Phytopathology, Vol. 107, No. 3: 322-328.

Conference poster contributions

2016 "Identification of molecular markers linked to resistance to different pathotypes of *Synchytrium endobioticum* in a dihaploid potato population", Plant & Animal Genome Conference, San Diego, USA

2016 "Fine mapping of resistance against potato wart disease in a dihaploid potato population", 3rd Doctoral Researchers Conference of the GRK1798, Goslar, Germany

2015 "Identification of potential resistance genes against potato wart disease in a dihaploid potato population", 2nd Doctoral Researchers Conference of the GRK1798, Bad Salzdetfurth, Germany

2014 "Resistance mapping against potato wart disease in a dihaploid potato population", main conference of the GPZ (Gesellschaft für Pflanzenzüchtung e. V.), Kiel, Germany

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