R gene stacking by trans- and cisgenesis to achieve durable late blight resistance in potato

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CHAPTER 1

General introduction

Potato

Potato (*Solanum tuberosum* L.) is the third food crop in the world after wheat and rice. There are more than 4300 edible varieties and more than 180 related wild species from the Andes mountains of South America. These wild species are the ancestors of today's cultivated potato, and they possess important genetic variation against biotic and abiotic stresses. It is believed that the wild potato was first domesticated from the high mountain slopes of the Bolivian and Peruvian border area, and brought to Europe in the late 16th century (http://cipotato.org/potato/facts). Potato is a specialized stem (stolon) growing underground, which serves as a sink for carbohydrates. A freshly harvested potato tuber contains 80% water and 20% of dry matter, which is mainly starch. A good diet can be achieved with potato, including (pulse) vegetables and whole grain foods (http://www.potato2008.org). There are more than one billion people eating potatoes worldwide. Globally 25 Mha of potatoes are grown with an average yield of 16 tons/ha, which represents an annual economic value of about 32 billion euro (Haverkort et al., 2008). The highest potato production is in China, followed by Russia and India. From 1991 till 2007, the world potato production increased, mainly due to an increasing contribution from the developing countries (Fig. 1). In the developed countries the production is slightly decreasing.

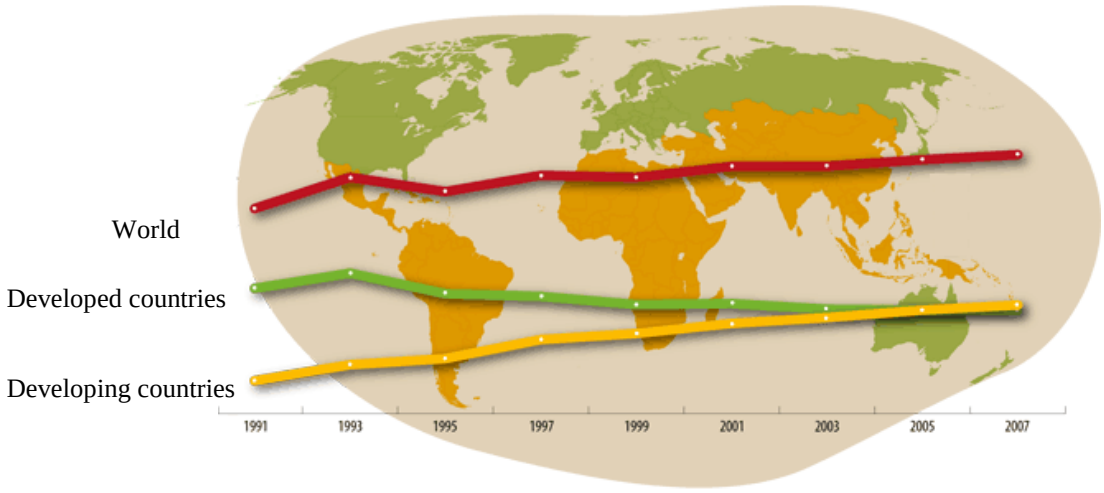

Figure 1. Trend of world potato production between 1991-2007 (Source: FAOSTAT)

Potato is a vegetatively propagated crop, and can be grown from a potato tuber called "seed potato". The new plant can produce 5-20 tubers, with the same genotype as the mother plant. Except the seed tubers, true seeds can be obtained from berries after flowering and pollination. The seedlings are genetically different from the mother plant, due to the high genetic heterozygosity of potato (http://cipotato.org/potato/facts). Most cultivated potato plants are tetraploid (2n=4X=48), but other ploidy levels, such as diploid, pentaploid and hexaploid do also exist. Recently, two genomes from *S. tuberosum* groups Phureja (DM; a doubled monoploid) and Tuberosum (RH; diploid), of around 844 Mbp, were sequenced and they harbored around 39,000 genes, mostly located within the 570 Mbp of euchromatin (Xu et al., 2011).

Potato late blight

Late blight is one of the most serious diseases of potato and tomato, caused by the oomycete pathogen *Phythophthora infestans* (Mont.) de Bary, originating from Central America (Mexico) or South America (Andean region) (Grunwald and Flier, 2005, Gomez-Alpizar et al., 2007, Pule et al., 2013). Worldwide, on average 15% yearly loss of potato is caused by late blight (Haverkort et al., 2008). *P. infestans* is a hemibiotrophic pathogen, with biotrophic and necrotrophic phases. *P. infestans* obtains nutrients through specialized haustoria structures in the living host cells during the asymptomatic biotrophic phase, which was followed by a necrotrophic stage that is characterized by extensive sporulation and necrosis of host tissue (Lee and Rose, 2010). *P. infestans* isolates can infect potato leaves, tubers (Fig. 2) and stems (Fry, 2008), and it develops fast under humid and cool (~15 ^{o}C, above 80% relative humidity) environmental conditions (Fig. 2). The easy asexual reproduction of *P. infestans* isolates can cause a wide spread of the disease in a relatively short time (Judelson and Blanco, 2005). Next to an asexual life cycle, *P. infestans* has a sexual reproduction cycle in which oospores are formed by the fusion of antheridia and oogonia from A1 and A2 mating types (Fig. 3). The sexual cycle contributes to the complexity of the late blight problem, because on the one hand oospores can survive in the soil and form a source for epidemic development in the next season (Brurberg et al., 2011). On the other hand, sexual reproduction can increase the genetic variation and evolution rate of this pathogen (Yuen and Andersson, 2012), which can enlarge the chance of overcoming the existing resistances (Fry, 2008).

Figure 2. *Phytophthora infestans* infection of foliage and tubers

Between 1845-1850, the “Great Famine” occurred in Ireland, which was caused by late blight (Goodwin et al., 1994). It caused the death of approximately one million people and the fleeing of more than one million people from Ireland. This was due to the fact that the potato cultivars planted at that moment were all highly susceptible for the fast spreading potato late blight pathogen (McDonald and Linde, 2002). In the sixties and seventies of the last century associations have been observed between human birth defects like Anencephaly and Spina Bifida (ASB) and consumption of high amounts of blighted potatoes during overwintering (Renwick, 1973). There seemed to be a rough regional concordance between the birth incidence rates of ASB and the prevalence of potato blight. This was the

main reason that chemical control and improved storage during winter time were stimulated. Recently, the whole genome from one of the *P. infestans* isolates, T30-4, was sequenced (Haas et al., 2009). Comparative genome analysis revealed a special characteristic of *P. infestans* that supports the quick adaptation of *P. infestans* isolates to the host plant. The virulence genes are localized in repeat-rich and gene-sparse regions, referred to as the peripheral genome, which makes up 74% of the total genome. Genes from these regions can evolve fast and adapt to the capacity of the pathogen (Haas et al., 2009). In contrast, housekeeping genes are mostly located in the repeat-poor and gene-dense regions, referred to as the core genome (Vleeshouwers et al., 2011, Raffaele et al., 2010).

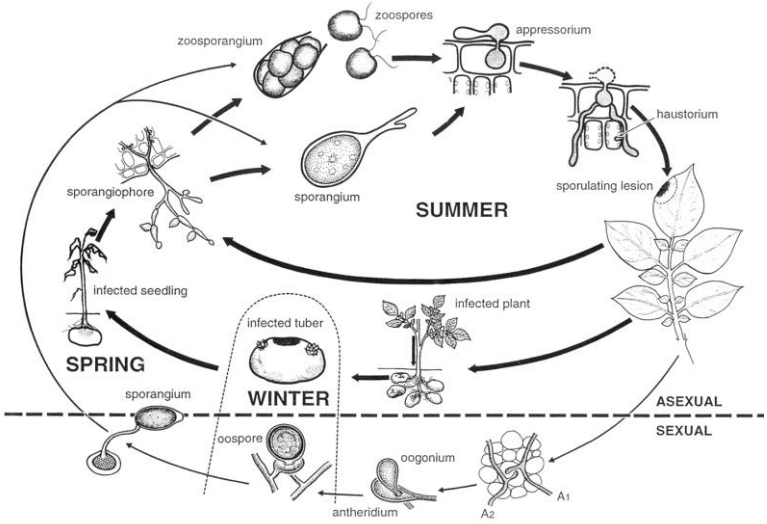

Figure 3. (A)sexual life cycles of *Phytophthora infestans*
Laboratory of Phytopathology, Wageningen University
(http://www.plantenziektekunde.nl/DVDPhytophthora)

Resistance mechanism and potato differential set

P. infestans isolates infect the plants by secreting pathogen associated molecular patterns (PAMPs) to induce PAMP triggered immunity (PTI). This immunity can be suppressed by the pathogen using virulence effectors to promote effector triggered susceptibility (ETS). Resistance (*R*) genes, encoding intracellular *R* proteins serve as a second layer of defense to directly or indirectly detect an effector protein from *P. infestans*, resulting in effector triggered immunity (ETI). Hence effectors that are recognized by *R* proteins are referred to as a-virulence (*Avr*) effectors. The resistance reaction that is triggered involves a programmed cell death mechanism and is called hypersensitive response (HR) (Jones and Dangl, 2006). If the pathogen evolves new effectors to suppress this ETI, a resistance is broken and successively a second *R* gene, recognizing the suppressing effector, could be involved in this arms race. This is the zig-zag(-zig) model for plant - pathogen interaction (Jones and Dangl, 2006, Hein et al., 2009a).

Study of the interaction between *R* genes and *Avr* effectors can bring information about the resistance spectrum of the *R* genes to be employed in a resistance breeding strategy. A collection of

differential plants in which different *R* genes are present alone or in combination are highly instrumental for these studies. Half way the previous century, differential sets were obtained by crossing with *Solanum demissum* and backcrossing with cultivated potato (Black et al., 1953). The Mastenbroek differential set consisted of eleven plants (Ma*R1*-Ma*R11*) and was postulated to harbor eleven different *R* genes *R1-R11*, (Black et al., 1953; Malcolmson and Black, 1966). However, the deployment of these genes, even in combination did not bring durable resistance. Potato cultivar Pentland Dell harboring *R1*, *R2* and *R3* was released in 1961, but extensive infection by new *P. infestans* races occurred only six years later (Hein et al., 2009b). It is indicated that the wide cultivation of cv Pentland Dell imposed the selection pressure for the *P. infestans* population and increased the frequency of more complex isolates (Shattock et al., 1977). The detection of *P. infestans* A2 mating type in 1980s in Europe, presumably originating from Mexico (Drenth et al., 1993), tremendously increased the virulence diversity of the *P. infestans* population (Drenth et al., 1994). A remarkably increased frequency of isolates with A2 mating type was reported in the Netherlands, Ireland (Drenth et al., 1993, Cooke et al., 1995), and other European countries. This increase of A2 mating type was dominated by one clonal lineage in Europe, called "blue 13", from which the frequency was determined to vary from 12% to 70% during the 2005-2007 growing seasons (Cooke et al., 2007). The emergence and population increase of particular clonal lineages was associated with resistance to anti-fungal crop protection agents (Cooke et al. 2007). These cases sparked the researchers to investigate how *P. infestans* populations respond to the deployment of resistant cultivars and the management of resistance durability. The above mentioned differential set of eleven plants was considered to harbor eleven different single *R* genes in each individual plant and they were used to characterize the race spectrum for *P. infestans* isolates (Guo et al., 2009, Wang et al., 2012).

Many *R* genes have been cloned recently. *R1*, cloned from Ma*R1*, can provide resistance both in leaves and tubers (Ballvora et al., 2002, Pel, 2010), and is involved in the recognition of *Avr1* (Vleeshouwers et al., 2011). *R2*, *R2-like*, *Rpi-abpt1* and *Rpi-blb3* were cloned as homologous genes, which are all functioning in leaves, and they are involved in the recognition of the *Avr2* effector family (Vleeshouwers et al., 2011). *Rpi-abpt1* was reported not to confer resistance in tubers (Park et al., 2005). Whereas unexpectedly, *Rpi-blb3* (a homolog of *Rpi-abpt1*) containing transformants in cv Desiree was shown to confer resistance in tubers (Pel, 2010). Ma*R3* harbored two *R* genes: *R3a* (Huang et al., 2005) and *R3b* (Li et al., 2011), corresponding to *Avr3a* (Armstrong et al., 2005) and *Avr3b* (Li et al., 2011), respectively. *R3a* could confer resistance only in leaves, whereas *R3b* might also show resistance in tubers (Lapwood and Mckee, 1961; Park et al., 2005). Late blight resistance in Ma*R4* was determined to be caused by a single dominant gene *R4* (van Poppel et al., 2009), which is involved in the recognition of *Avr4* (van Poppel et al., 2008). At the moment, other *R* genes (*R4-R11*) from this differential set are being mapped or cloned (Bradshaw et al., 2006, Hein et al., 2009b, Verzaux, 2010, Jo et al., 2011).

An important observation is that extra *R* genes were detected recently in several differential plants. For example, Ma*R3* harbored *R3a* (Huang et al., 2005) and *R3b* (Li et al., 2011). Ma*R8* and Ma*R9*

were shown to harbor also more *R* genes (Kim et al., 2012). A differential set is more useful when the exact nature and number of *R* genes is known in each individual member. Such plants with individual *R* genes can also be used more accurately as trap plants in the field to investigate and monitor virulence towards *R* genes under local, and natural circumstances.

Potato breeding towards durable late blight resistance

Potato production largely relies on application of chemicals such as Mancozeb with fungicide activity (Grunwald et al., 2002). In the past, metalaxyl was used. However, resistance to metalaxyl was easily established in the *P. infestans* populations after its introduction (Matuszak et al., 1994) and was associated with the emergence of clonal lineage blue 13 (Cooke et al. 2007). An environmental friendly solution for potato late blight would be to breed varieties with genetically encoded resistance. Breeding for late blight resistance started already in the mid-19th century. Since then eleven *R* genes (*R1*-*R11*) from the differential set mentioned above had been introgressed and domesticated from hexaploid *S. demissum* into cultivated potato by conventional breeding. This was especially the case for *R1*, *R2*, *R3*, *R4* and *R10*. *R1* (Ballvora et al., 2002) was not only found in the differential plants Craig's Snow White and Ma*R1* (CEBECO-43154-5), but also in Ma*R5* (Black 3053–18), Ma*R6* (Black XD2–21) and Ma*R9* (Black 2573(2)); except these, *R1* was also present in Austrian cultivar Linzer Delikatess and German cultivar Laura (Trognitz and Trognitz, 2007). French cultivars Naturella, Eden were reported to contain *R2*, and French cultivars Fresco and Rector contained *R1*, *R2* and *R3* (Pilet et al., 2005). Cultivar Pentland Dell was also reported to contain *R1*, *R2* and *R3* (Malcolmson, 1969). Furthermore, Dutch cultivar Escort harbored *R1*, *R2*, *R3* and *R10* (Bormann et al., 2004); and cultivars Estima and Premiere carried *R10* (Turkensteen, 1987). Except Ma*R4* (Cebeco44-31-5), and Bl*R4* (1563 c), cultivars Avondale, Epoka, Gelda, Greta, Isola, Pentland Squire, and Tylva also harbored *R4* (van Poppel et al., 2009). However, all these *R* genes, which conferred race-specific resistance, had already been overcome quickly by the fast evolving *P. infestans* isolates (Wang et al., 2008). The observation in the fifties of the last century, that resistance from single major *R* gene containing plants was easily overcome, induced breeding research to focus on horizontal resistance with quantitative and polygenic resistance genes (e.g. quantitative trait loci, QTL) in the absence of major *R* genes. A few QTLs were identified based on different populations made after intraspecific crosses, crosses between cultivated species and crosses between cultivated and different wild species (Nowicki et al., 2012). Similar to major resistance traits, QTLs vary with different pathogen isolate combinations, and are rather related to the method of evaluation, mapping, and different environments (Korol et al., 1995). Also in many cases, the introduction of QTLs in breeding lines carried linkage drag, such as lateness in maturity. Furthermore, the performance of QTLs was often not consistent across the populations, which limited their potential application (Sliwka et al., 2007). A well-known example is the QTL located in the vicinity of gene *R1* from *S. demissum* on chromosome 5 (Ballvora et al., 2002). This QTL seems to be consistent across several studies. However, there was also a correlation with late maturity (Gebhardt et al., 2004, Visker et

al., 2005, Beketova et al., 2006). Altogether, this approach of quantitative resistance was not providing sufficient levels of resistance to combat potato late blight.

For decades the research of major *R* genes from wild species was pursued only by a few researchers. Hermsen and Ramanna (1973) investigated the presence of *R* genes in highly resistant but distantly related species like *S. bulbocastanum*. Double bridge crosses with *S. acaule* and *S. phureja* were needed to transfer these *R* genes from *S. bulbocastanum* into cultivated potato. Only in the beginning of this century the first resistant varieties from this research were released (notably the cultivars Toluca in 2004 and Bionica in 2005). The main difficulty in the domestication of the *R* gene(s) was the linkage drag of alleles from the wild species coding for undesired traits (Jacobsen and Schouten, 2007, Park et al., 2009, Hein et al., 2009b). Removal of the linkage drag requires meiotic recombination, which occurs only at low frequency between non-sister chromatids from different species (VIB, 2013).

Intensive exposure of resistant cultivars to pathogen populations increases the frequency by which the resistance is overcome. In the disease management, many plant breeders consider major *R* genes as a potentially non-renewable resource; once a pathogen has overcome the resistance of a *R* gene, the *R* gene has lost its value (Lo Iacono et al., 2013). Durability of disease resistance is the phenomenon that the resistance remains effective for a long time during wide cultivation. The achievement of durable disease resistance remains a major issue. Past experience showed that employment of a single *R* gene is hardly ever a durable solution, especially not in the case of *P. infestans*. Durability of the resistance in cultivar Sarpo Mira was recently found to be associated with the presence of multiple (at least five) *R* genes (Rietman et al., 2012). Therefore, stacking of *R* genes is expected to bring durable resistance, especially with multiple but different broad spectrum *R* genes.

Several broad spectrum *R* genes have been cloned. Examples are *Rpi-sto1*, *Rpi-pta1* and *Rpi-blb1* which are homologs, sharing the same a-virulence effector *Avrblb1* (Vleeshouwers et al., 2008), *Rpi-blb2* (van der Vossen et al., 2005), 3), and *Rpi-vnt1.1* (Pel et al., 2009). These *R* genes all encode proteins with a central nucleotide binding site and a C terminal leucine rich repeat (NBS-LRR) structure. The *R* proteins respond to *P. infestans* effectors which all harbor the conserved amino acid sequence RXLR that facilitates their translocation into the host cell (Vleeshouwers et al., 2011). Except the above mentioned effectors *Avr1* (Guo, 2008), *Avr2* (Saunders et al., 2012), *Avr3a* (Armstrong et al., 2005), *Avr3b* (Li et al., 2011), *Avr4* (van Poppel et al., 2008), and *Avrblb1* (Vleeshouwers et al., 2008), other effectors such as *Avr8* (Jo, 2013), *Avr10* (Rietman, 2011), *Avrchc1* (J. Vossen, unpublished data), *Avrblb2* (Oh et al., 2009) and *Avrvnt1* (Pel, 2010) were also cloned. The availability of these *Avr* genes is instrumental to test the functionality of their corresponding *R* genes, especially in plants with *R* gene stacks, where no differential isolate for each individual stacked *R* gene is available.

Genetic transformation

Another approach to increase the resistance level against potato late blight in a breeding program is genetic transformation, which does not have the above mentioned problem of the longtime breeding process and the existence of linkage drag from conventional introgression breeding. Genetic transformation or genetic modification of plants indicates the process by which genetic material is transferred and integrated into a recipient genome. Genetic transformation is distinct from introgression breeding which uses the plants reproductive system to achieve the same goal. In this case, a good variety with desired economic traits is needed to be used as the recipient. Roughly, three classes of genetic transformation or genetic modification can be distinguished: transgenesis, intragenesis and cisgenesis. Transgenesis introduces transgenes which are synthetic genes or genes that entirely/partly originate from non-crossable species. The bacterial selection marker *nptII*, coding for kanamycin resistance, is an example of a transgene. Intragenesis refers to the introduction of intragenes which are originating from the breeders' gene pool but often with slight modifications. For instance chimeric genes can consist of coding sequences combined with regulatory elements from other genes (Molesini et al., 2012). Cisgenesis refers to the introduction of cisgenes, which are unmodified, natural genes from the breeders' gene pool. Only the identical copy of a gene in a sense direction under the control of its own promoter and terminator is allowed to be used as a cisgene (Schouten et al., 2006). The resulting plants that contain only the acquired cisgenes, without a selection marker gene or vector backbone sequences, are proposed to be named cisformants (Chapter 4 of this thesis).

There are generally four methods of performing transformation: particle bombardment, *Agrobacterium* mediated transformation, electroporation and viral transformation, from which the first two methods are widely used. The "particle bombardment" device is also known as "gene gun", it employs high-velocity micro-projectiles to deliver foreign DNA into cells and tissues. This method can be applied to all plant species which can be regenerated, but its transformation efficiency is generally lower than *Agrobacterium* mediated transformation (Kikkert et al., 2013). *Agrobacterium* mediated transformation is a natural process that makes use of a Tumor inducing (Ti) plasmid. Part of the Ti plasmid, called T-DNA, is transferred and integrated into the genome of recipient cells, causing the well-known crown gall disease (Gelvin, 2003). Scientists replaced the original genes from the T-DNA that induce crown gall disease with other gene(s), coding for different agronomic traits and a selection marker (Yi et al., 2011, Veale et al., 2012, Breitenbucher et al., 2012). This method can produce more frequently a lower copy number of T-DNA integration in the recipient plant genome as compared to particle bombardment. For this reason and also for reasons of efficiency, *Agrobacterium* mediated transformation is the most commonly used method for plant transformation. However, its application is more easy on dicots than on monocot species (Gahakwa et al., 2000, Leelavathi et al., 2004, Harrison et al., 2006, Dutt et al., 2008). Many studies have been performed on transformation efficiency of different *Agrobacterium* strains on different crops. Also various transformation vectors were used, among which binary vector pBINPLUS is widely used (van Engelen et al., 1995). Chetty et al. (2013) evaluated the transformation rate of the *nptII* gene in vector pBI121 from four *Agrobacterium* strains AGL1, EHA105, GV3101, and

MP90. Flachowsky et al. (2008) inserted a gene against apple fire blight, which coded for an extracellular polysaccharide (EPS)-depolymerase in the binary vector pBinARdpo in *Agrobacterium* strains EHA105 and LBA4404. Wolters et al. (1998) transformed an anti-sense granule-bound starch synthase gene in the vector pKGBA50 in *Agrobacterium* strain LBA4404, to result in amylose-free starch in potato. Furthermore, transformation efficiency was widely shown to be genotype dependent (Iser et al., 1999, Heeres et al., 2002, Harwood, 2012).

In many plant transformation systems, the use of a selection marker, eg. *nptII*, coding for kanamycin resistance, can guarantee sufficiently high numbers of transgenic plants with good expression of transgenes. This is due to the pre-selection for kanamycin resistance, while non-transformed cells were killed (Bevan et al., 1983). However, when the transformation is accomplished, the presence of a marker gene becomes useless and nowadays even undesirable. More and more researchers are trying to obtain transformants, harboring only the desired traits with good expression, due to the safety concern that antibiotic resistance, eg. *nptII*, might spread into the ecosystem (Fuchs et al., 1993). In order to obtain this kind of desired transformants without transgenic selection marker, researchers have been developing various strategies, such as 1). Performing the transformation in the absence of a selection marker. 2). Marker gene excision by genetic segregation or recombination (Zhou et al., 2003, Holme et al., 2012). 3). Use of cisgenic markers from plants (Espley et al., 2007).

The first method is also called marker free transformation. De Vetten et al., (2003) transformed potato cultivars with a potato granule-bound starch synthase (GBSSI) antisense gene without using the *nptII* selection marker. This intragenic plant indeed showed the desired amylose-free phenotype. However, PCR-selection of marker free transformants is different from kanamycin selection. It has to be seen whether PCR-positive plants are sufficiently expressing the introduced cisgene(s) and whether epistatic effects will be observed or not (Cordell, 2002).

As an example of marker gene excision by genetic segregation, Holme et al. (2012) used co-transformation with two vectors (one containing a marker gene and the other one containing the gene of interest) in one *Agrobacterium* strain for the transformation of barley. In this case, the marker gene and the gene of interest are expected to integrate in different loci of the recipient genome. Sexual crossing would allow them to separate both inserts in order to produce cisgenic plants. Another way of marker gene excision is based on the induced recombination. This method employs a gene coding for a site-specific recombinase with two recombination sites (Rs) flanking the marker gene. The recombinase was placed under the control of a glucocorticoid inducible promoter. Treatment with the glucocortoid dexamethasone (Dex) allows the expression of the recombinase at any desired moment (Krens et al., 2011). The resulting plants no longer contain the marker gene and only one copy of the recombination site is left behind in the recipient genome. This system was tested in strawberry (Schaart et al., 2004) and very recently applied in apple to obtain cisgenic apple plants which are resistant to apple scab (Schouten,

pers. comm.). Recently, researchers have developed also plant specific selection markers. For example, the *Arabidopsis thaliana* ATP binding cassette (ABC) transporter (*Atwbc19*) gene was used to replace the bacterial *nptII* gene for kanamycin resistance in the plant transformation process. Overexpression of *Atwbc19* gene in transgenic tobacco yielded similar degrees of kanamycin resistance (Mentewab and Stewart, 2005). Other promising examples are the dominantly mutated *MdMYB10* transcription factor from apple (Espley et al., 2007, Kortstee et al., 2011) and MYB-related Rosea1 (Ros1) transcription factor from garden snapdragon (Bedoya et al., 2012). These two genes induce the anthocyanin accumulation which is visible by the red color in transformed tissue and can be used as dominant cisgenic selection markers in the plant transformation.

Due to the generally acknowledged safety of cisgenesis (EFSA, 2012), researchers switch focus towards cisgenesis (Holme et al., 2013), from which the product is very similar to that from the conventional breeding (Jacobsen and Schouten, 2008), which was also indicated to have a higher consumer acceptance (Eurobarometer, 2010). The possibility to develop cisformants and the availability of more and more cloned cisgenic *R* genes with broad spectrum resistance will be helpful to achieve durable resistance against late blight by *R* gene stacking. These developments could improve the acceptance of GM-plant technology in Europe and the rest of the world. In order to achieve durable resistance via stacked *R* genes using genetic transformation, it is essential that more knowledge is obtained about the transformation efficiency, the functionality of the stacked *R* genes, duration and stability of this functionality in the obtained plants (transformants or cisformants) with multiple *R* genes. Thus studies to select broad spectrum *R* genes, the construction of vectors containing multiple *R* genes, as well as to the best ways of obtaining plants with these stacks need to be conducted. In case of cisformants, the question has to be answered, how to obtain plants harboring only the cisgenes, but lacking the integration of backbone sequences. Furthermore, besides foliar resistance, resistance in tubers from plants harboring stacked *R* genes needs further investigation. Functional stacking of *R* genes by transformation, as such, is a new field of research. In practice, it means that not only the cisgene has to be cloned, but also the cognate *Avr* genes need to be available to determine the biological functionality of the inserted individual *R* genes. Finally as already outlined, a reliable differential set with known *R* gene(s) in each individual differential plant would provide great help in monitoring the virulence of (local) *P. infestans* populations towards individual *R* genes. Such a set gains quality when the genetic backgrounds of the plants are highly similar. Using genetic transformation of individual cloned *R* genes (or different combinations of *R* genes), this background can even be identical (Chapter 5 of this thesis).

Scope of this thesis

The objectives of this thesis are 1, investigating the possibility of gene stacking towards achieving durable resistance against potato late blight by transgenesis and cisgenesis; 2, a. characterization and

improvement of the existing differential set by adding and/or replacing plants containing single *R* genes and new *R* genes, and b. setting up a new GM based differential set.

In Chapter 2, the introduction of three *R* genes through transgenesis is described. First, three *R* genes were selected based on the resistance spectrum of their transformants towards 44 isolates from the Netherlands and China. Single *R* gene (*Rpi-sto1*, *Rpi-vnt1.1*, *Rpi-blb3*, *R3a*) containing transformants in cv Desiree were used as a differential set (part of the GM differential set in chapter 5). The selected broad spectrum *R* genes (*Rpi-sto1*, *Rpi-vnt1.1*, *Rpi-blb3*) were combined into one binary vector, pBINPLUS, which was used to transform cv Desiree. Transformants containing these *R* genes showed the stacked resistance spectrum of all three *R* genes. The individual functionality of each *R* gene was confirmed by agro-infiltration using the corresponding *Avr* genes.

In Chapter 3 an extended set of triple *R* gene transformants, obtained with the construct from Chapter 2, was produced in order to gain a better insight in the genomic integration of the different parts of the transformation vector (T-DNA, vector borders and vector backbone sequences). Furthermore, the efficiency by which all genes from the T-DNA, in vector backbone free events, are functionally expressed, was studied in this transformation experiment. Besides the study of the primary transformants, also the segregation of the triple *R* genes and late blight resistance in the sexual progeny was tested. A Mendelian segregation of the entire T-DNA was observed after crossing three triple *R* gene containing transformants with cv Katahdin. Functional resistance was maintained in the leaves and tubers of progeny plants containing these triple *R* genes.

In Chapter 4, three different approaches were investigated to produce cisformants (transformed plants containing only cisgenes) with multiple *R* genes: 1, two *R* genes were combined into one transformation vector; 2, co-transformation with two cisgenes in separate vectors; 3, co-transformation with a vector containing a selection marker and a marker free vector harboring only three cisgenic *R* genes. Most important observation was that in approaches 1 and 2 after PCR selection for the presence of *R* genes level of resistance in such transformed plants was not always sufficient. Strategy 3 proved to be the best by considering the resistance level of inserted *R* genes. Resistance of *R* gene containing sexual offspring plants was not always as high as in the parental plants and furthermore resistance levels in tubers were more frequently sufficient in cisformants with higher copy number integration.

Chapter 5 aims to provide materials for late blight virulence typing. The *R* gene content of the individuals from the currently used Mastenbroek differential set was investigated using agro-infiltration with available *Avr* genes *Avr1*, *Avr2*, *Avr3a*, *Avr3b*, *Avr4*, *Avr8* and *Avr10*. The *R* gene content was found to be polygenic in many cases and the Mastenbroek set was therefore expanded with plants containing a reduced number of *R* genes, such as seedlings SW8540-025 (containing only *R3a*) and SW8540-325 (containing only *R3b*). Also the differential set was extended with plants harboring new *R* genes, like blb8005 (containing *Rpi-blb1*), blb2002 (containing *Rpi-blb2*), vnt367-1 (containing *Rpi-vnt1.1*), etc. To

overcome the difficulty with the different genetic backgrounds of this extended conventional differential set, a GM differential set in cv Desiree background was created, which consisted of ten transformants, each harboring a single cloned *R* gene. This GM differential set is expected to provide a more accurate characterization of the fast evolving *P. infestans* population.

In the general discussion, an integrated view on the results from different chapters is provided and discussed such as the observed differences between cisgenesis and transgenesis. Furthermore, various aspects towards achieving durable resistance against potato late blight are indicated.

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CHAPTER 2

Functional stacking of three resistance genes against *Phytophthora infestans* in potato

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Abstract

Functional stacking of broad spectrum resistance (*R*) genes could potentially be an effective strategy for more durable disease resistance, for example, to potato late blight caused by *Phytophthora infestans*. For this reason, three broad spectrum potato *R* genes (*Rpi*), *Rpi-sto1* (*Solanum stoloniferum*), *Rpi-vnt1.1* (*S. venturii*) and *Rpi-blb3* (*S. bulbocastanum*) were selected, combined into a single binary vector pBINPLUS and transformed into the susceptible cultivar Desiree.

Among the 550 kanamycin resistant regenerants, 28 were further investigated by gene specific PCRs. All regenerants were positive for the *nptII* gene and 23 of them contained the three *Rpi* genes, referred to as triple *Rpi* gene transformants. Detached leaf assay and agro-infiltration of a-virulence (*Avr*) genes showed that the 23 triple *Rpi* gene transformants were resistant to the selected isolates and showed HR with the three *Avr* effectors indicating functional stacking of all the three *Rpi* genes. It is concluded that *Avr* genes, corresponding to the *R* genes to be stacked, must be available in order to assay for the functionality of each stack component. No indications were found for silencing or any other negative effects affecting the function of the inserted *Rpi* genes. The resistance spectrum of these 23 triple *Rpi* gene transformants was, as expected, a sum of the spectra from the three individual *Rpi* genes. This is the first example of a one-step approach for the simultaneous domestication of three natural *R* genes against a single disease by genetic transformation.

Key words potato · gene stacking · durable resistance · functionality

Introduction

Many devastating diseases are threatening plants, such as potato late blight, apple scab and wheat yellow rust. In contrast to higher animals, which have an advanced adaptive immune system, plants mainly rely on innate immunity to resist invading pathogens (Staskawicz et al. 1995; Jones and Dangl 2006). As a consequence of competition, plant resistance and pathogen virulence have been co-evolving up till now. Co-evolution, on the one hand, is diversifying the species; on the other hand, it is also making the competitive species more adapted to the required environment (Rosenthal et al. 1976; Rausher 2001). A good example, is the late blight pathogen *Phytophthora infestans*, whose genome has been recently sequenced (Haas et al. 2009). This pathogen evolved by mutation of effectors frequently and rapidly. Consequently, individual resistance genes (*Rpi*) were overcome relatively fast after their introduction into the potato crop (Black et al. 1953).

A promising strategy for breeding more durable resistance is to stack multiple, broad spectrum, resistance genes in one genotype (Halpin 2005; Douglas and Halpin 2010), especially with resistance genes originating from different gene clusters, representing different HR interactions between *R* genes and their cognate *Avr* effectors. In comparison to conventional breeding with the increasing problem of linkage drag during stacking, genetic transformation is a fast and efficient way to introduce and

domesticate multiple *R* genes into an existing cultivar. For potato, three different transformation methods can be used for *R* gene stacking: 1. transformation of one or two *R* genes followed by re-transformation of a selected, well performing, resistant transformant with additional *R* genes; 2. in one step via co-transformation, by using mixed *Agrobacterium* strains containing unlinked *R* genes in two or more vectors; 3. with multiple *R* genes in one vector (Douglas and Halpin 2010).

Multiple transgene introduction has been practiced to combine different traits using various methods, such as particle bombardment or *Agrobacterium* mediated transformation (Altpeter et al. 2005; Sharma et al. 2005; Halpin 2005; Lacorte 2006). Until now, they were transgenes or pathway genes, corresponding to different agronomic traits, and the individual functionality of which could be easily detected in the transformed plants (Campbell et al. 2000; Beyer et al. 2002; Anand et al. 2003; Agrawal et al. 2005; Cao et al. 2005; Chan et al. 2005; Schmidt et al. 2008). The application of gene stacking using GM technology is increasing. In 2010, worldwide 148.1 million hectare of GMO crops were grown, of which 32.3 million hectares with two or three stacked traits. Double traits stacking was mainly restricted to insect and herbicide resistance. Triple traits stacking was restricted to herbicide resistance and two different insect pests (ISAAA, 2010). Our approach differs from these examples as we are introducing three natural *R* genes to render immunity to one disease, potato late blight.

To date, several *R* genes against *P. infestans*, originating from different *Rpi* gene clusters and various wild species, have been cloned. For example, *R1*, *R2*, and *R3a* from *Solanum demissum* (Ballvora et al. 2002; Lokossou et al. 2009; Huang et al. 2005), *Rpi-blb1*, *Rpi-blb2* and *Rpi-blb3* from *S. bulbocastanum* (Song et al. 2003; van der Vossen et al. 2003; van der Vossen et al. 2005; Lokossou et al. 2009); and *Rpi-vnt1.1* from *S. venturii* (Foster et al. 2009; Pel et al. 2009). Some of them showed a broader resistance spectrum than others with a selected set of late blight isolates. It is expected that stacking of at least three broad spectrum *Rpi* genes is needed to obtain durable resistance in the field. Important reasons are the observation that multiple *Rpi* genes are frequently found in individual resistant plants in nature (Huang et al. 2005; Lokossou et al. 2010; Verzaux 2010). The *Rpi* gene differentials *R8* and *R9*, which have been reported to exhibit durable resistance (Zhang et al. 2007; Lehtinen et al. 2008; Dr. G. Kessel, Plant Research International, Wageningen, The Netherlands, unpublished; YL, unpublished), are known to contain multiple *R* genes (Trognitz and Trognitz 2004; JV, unpublished). These observations show that stacking of broad spectrum *Rpi* genes in plants could contribute to the durability of resistance for potato in the field.

Therefore, it is important to know how to stack three or more *Rpi* genes into a potato variety efficiently and how to test the biological functionality of each gene individually. One way to answer this question is to investigate the relationship between the resistance reaction of individual *Rpi* genes and the HR reactions in response to cognate *Avr* effectors (Vleeshouwers et al. 2008). The consensus RXLR pattern of *Avr* genes and the recent genomic sequence of *Phytophthora infestans* promoted the research

on *Avr* gene(s) considerably. *Avr1* (Dr. F. Govers, Phytopathology, Wageningen University and Research Center, Wageningen, The Netherlands), *Avr2* (Dr. P. Birch, Plant Pathology, SCRI, United Kingdom), *Avr3a* (Armstrong et al. 2005), *Avr4* (Poppel et al. 2008), *Avrblb2* (Oh et al. 2009), *Avrvnt1* (Pel 2010), and *Avrsto1* (Pieterse et al. 1994; Vleeshouwers et al. 2008) were cloned, and they are recognized by *Rpi* genes *R1*, *Rpi-blb3*, *R3a*, *R4*, *Rpi-blb2*, *Rpi-vnt1.1*, and *Rpi-sto1*, respectively.

In this paper, three broad spectrum resistance genes, *Rpi-sto1* (Vleeshouwers et al. 2008), *Rpi-vnt1.1* (Pel et al. 2009) and *Rpi-blb3* (Lokossou et al. 2009), were selected and constructed into a binary vector pBINPLUS, which was used to transfer these three *Rpi* genes simultaneously into the susceptible potato cultivar Desiree by *Agrobacterium tumefaciens* mediated transformation. Co-integration and co-functioning were confirmed by molecular analyses, disease tests and agro-infiltration using corresponding a-virulence (*Avr*) effectors. This approach provides a new foundation to distinguish the functionality of each stack component. Finally, it is concluded that the expected broadened resistance spectrum based on the functional combination of the three individual *Rpi* genes, was indeed achieved in the triple *Rpi* gene transformants.

Materials and Methods

Materials

Susceptible potato cultivar Desiree (*Solanum tuberosum*) was used for *Rpi* gene transformation. Single *Rpi* genes were transformed earlier in house and resulted in Desiree transformants harboring *R3a* (A04-22), *Rpi-blb3* (A03-142), *Rpi-vnt1.1* (SF3) and *Rpi-sto1* (A09-6), respectively. These single *Rpi* gene transformants were tested for their resistance spectrum. Three plasmids, pBINPLUS:*Rpi-blb3*, pBIN19:*Rpi-vnt1.1* and pBINPLUS:*Rpi-sto1* were used as a basis for designing the triple *Rpi* gene construct. Supplementary Table 1 lists the effectors or a-virulence genes used in the agro-infiltration. Sixteen late blight isolates, derived from multiple locations in Europe and America, used for *Rpi* gene spectrum analysis, were maintained at Wageningen UR Plant Breeding, Wageningen. This in house set of isolates was partly selected based on their compatibility reaction to particular *Rpi* genes containing plants. For example, isolate EC1 showed a compatible interaction with *Rpi-vnt1.1* containing plants; isolates IPO-0, PIC99177, PIC99189 and PIC99183 are compatible with *Rpi-sto1* containing plants; and isolate USA618 is compatible with *Rpi-blb3* containing plants. Twenty-eight additional isolates (Table 1), from four different provinces in China, were provided by CAAS in Beijing, China.

Construction of triple *Rpi* gene vector

Three *Rpi* genes *Rpi-sto1* (Vleeshouwers et al. 2008), *Rpi-vnt1.1* (Pel et al. 2009) and *Rpi-blb3* (Lokossou et al. 2009) had been cloned. In order to design the triple *Rpi* gene vector, at first the two *Rpi* gene construct pBINPLUS:*Rpi-blb3*:*Rpi-sto1* was created by using the unique enzyme sites *Sma*I and *Sbf*I flanking *Rpi-blb3* in the donor vector pBINPLUS:*Rpi-blb3* and thus *Rpi-blb3* was transferred into

the recipient vector pBINPLUS:*Rpi-sto1*. *Rpi-vnt1.1* was inserted into vector pBINPLUS:*Rpi-blb3*:*Rpi-sto1*, using the unique enzyme site *Sbf*I, located between *Rpi-blb3* and *Rpi-sto1*. Since the *Sbf*I site was not available in pBIN19:*Rpi-vnt1.1*, the *Rpi-vnt1.1* fragment flanked by *Eco*RI was cloned into a modified pGEM-T vector, with *Sbf*I-*Eco*RI-*Sbf*I enzyme sites in the multiple cloning site (MCS). Subsequently, the *Rpi-vnt1.1* fragment flanked by *Sbf*I was subcloned into pBINPLUS:*Rpi-blb3*:*Rpi-sto1*. Both anticipated orientations of the *Rpi-vnt1.1* gene were found and the clone with the orientation as depicted in Figure 1 was selected. The resulting pBINPLUS:*Rpi-blb3:Rpi-vnt1.1:Rpi-sto1* construct had a total size of 31.8 Kb and harbored a 22 Kb T-DNA insert (Figure 1).

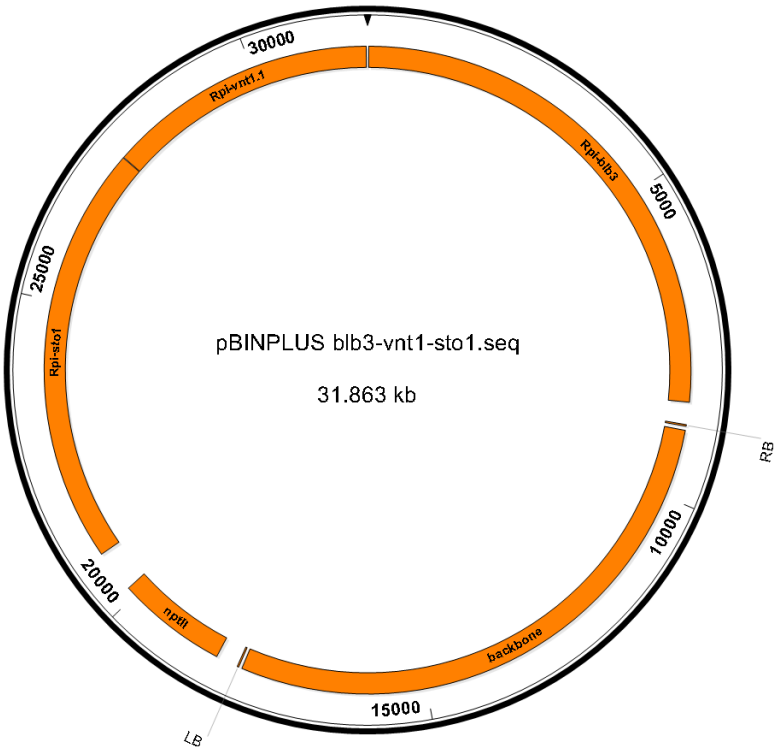

Fig. 1 Graphical view of binary vector pBINPLUS:*Rpi-blb3*:*Rpi-vnt1.1*:*Rpi-sto1*.

Restriction enzyme cleavage sites, used for the cloning of the *Rpi* genes into pBINPLUS have been indicated.

Stability and functionality tests of triple Rpi *gene vector*

After the vector had been made, we tested if it could replicate stably in *Agrobacterium* strains. Therefore, the stability of this construct was tested.

DNA was extracted from *E. coli* and transformed into three *Agrobacterium* strains (AGL1, AGL1+VirG and Cor308) using electroporation, and transformants were cultured in three different media: LB (LB Broth: 20 g/L), LB-selected (Pepton-select: Gibco 10 g, yeast extract select Gibco 5 g, NaCl 5 g and agar 8 g per liter) and YEB (5 g beef extract, 5 g bacteriological peptone, 5 g sucrose, 1 g yeast extract and 2 ml 1M MgSO4 per liter). Two colonies obtained from each of nine combinations (three *Agrobacterium* strains-three culture media) were cultured for DNA isolation. These 18 DNA samples

were transformed back into *E. coli*. Two colonies from each transformation were selected and DNA from 36 samples (two *E. coli* colonies were picked up from each of 18 DNA transformations) was extracted and digested with *Hin*dIII. The products were loaded on 1% agarose gel to check whether the digestion pattern was the same with the original DNA isolated from *E. coli*.

Agrobacteria with stable plasmid insertions were used to check the functionality of the genes in the construct. Vector pBINPLUS:*Rpi-blb3*:*Rpi-vnt1.1*:*Rpi-sto1* was co-infiltrated with *Avr* effectors *Avr2*, *Avrvnt1* and *Avrsto1*, corresponding to *Rpi* gene *Rpi-blb3*, *Rpi-vnt1.1* and *Rpi-sto1*, respectively. Three clones (clone 2-2: vector in COR308 cultured in LB-select media, 5-1: vector in AGL1 cultured in LB-select, and 7-2: vector in AGL1+virG cultured in LB) showed positive results in both stability and functionality tests, and they could be used for the transformation experiments.

Plant transformation

Stable insertion of the triple *Rpi* genes into susceptible potato cultivar Desiree was carried out using *Agrobacterium* mediated transformation (Filati et al. 1987; Hoekema et al. 1989), as adapted by Pel et al. (2009). Bacterial colony 5-1, selected from the stability and functionality tests, was used for this transformation. The transformation efficiency was calculated as the number of rooted shoots regenerated on kanamycin containing medium divided by the number of explants used within the time course of the experiment.

Molecular analysis of transformants

Presence or absence of specific genes in the regenerated plants was screened by PCR. Primers were listed in supplementary Table 2. PCR was performed in a 25 µl reaction, including 0.5 µl of forward primer (10 µM), 0.5 µl of reverse primer (10 µM), 0.5 µl of dNTPs (5 mM each), 2.5 µl of 10× buffer, and 2.5 units of Dream Taq polymerase (Fermentas).

Functional analysis of transformants

Rpi gene transformants were characterized using a detached leaf assay (DLA), in which eight 10 µl droplets of a zoospore suspension (50000 spores/ml) of a specific *P. infestans* isolate (Vleeshouwers et al. 1999) were applied to the abaxial side of the leaf. Two complex leaves, each with three single leaves close to the leaf tip, were tested for each clone. Criteria for scoring (DLA): R, all of the inoculation spots on a detached leaf showed resistance; RQ, 6-7 out of eight spots on a leaf showed resistance; Q, 3-5 out of eight spots on a leaf showed resistance; SQ, 1-2 out of eight spots on a leaf showed resistance; S, all spots on a leaf showed susceptibility.

Agro-infiltration, which is referred as *Agrobacterium tumefaciens* transient assay (ATTA), was used to assay for hypersensitive response (HR) to the cognate *Avr* effectors (Bendahmane et al. 2000;

Vleeshouwers et al. 2008). Nine replicates were performed (three plants per clone, with infiltration of three leaves per plant) using each effector at three different concentrations, with optical density at wavelength 600nm: 0.2, 0.05 and 0.0125 (Figure 2). OD600nm is successively used throughout this paper. The functionality of each inserted gene was tested by isolates test and agro-infiltration using the scheme in the supplementary Table 3.

Results

Selection of cloned Rpi *genes for broad spectrum resistance*

In order to achieve more durable resistance to potato late blight by stacking genes with different resistances, the individual resistance spectra of four cloned *Rpi* genes (*Rpi-sto1*, *Rpi-blb3*, *R3a* and *Rpi-vnt1.1*) were compared with transformants of cv. Desiree using a set of 44 isolates, consisting of 16 in house and 28 Chinese isolates.

Table 1. DLA on transgenic Desiree plants harboring single *Rpi* genes using 44 *P. infestans* isolates

Isolate Plant	IPO-0	PIC99177	PIC99189	PIC99183	EC1	USA618	NL00228	VK98014	F95573	89148-09	NO01096	IPO-C	Set 1	Set 2	Set 3	Set 4
Desiree:*Rpi-sto1**	S	S	SQ	SQ	R	R	R	R	R	R	R	Q	4R	10R	12R	6 R
Desiree:*Rpi-blb3**	R	Q	S	R	R	S	Q	RQ	R	R	R	S	4R	9R;1S	12R	3R;3S
Desiree:*R3a**	R	S	R	S	S	S	R	RQ	S	R	Q	S	4S	10S	12S	6S
Desiree:*Rpi-vnt1.1*$^{\#*}$	R	R	R	R	S	R	R	R	R	R	R	R	4R	10R	12R	6R
Desiree	S	S	S	S	S	S	S	S	SQ	S	S	S	S	S	S	S

Set 1: In house isolates 88069, H3PO4, 90128 and 428-2.
Set 2: Ten isolates from province Inner Mongolia of China.
Set 3: Eight isolates from province Hei Longjiang and four isolates from province Fu Jian of China.
Set 4: Six isolates from province Yunnan of China.
* Desiree:*Rpi-xxx*: cv. Desiree transformant harboring *Rpi-xxx*.
# Part of the data of *Rpi-vnt1.1* was adopted from Pel (2010).
R: all of the 8 inoculated spots on each leaf showed resistance.
RQ: 6-7 out of 8 spots on each leaf showed resistance.
Q: 3-5 out of 8 spots on each leaf showed resistance.
SQ: 1-2 out of 8 spots on each leaf showed resistance.
S: all of the 8 spots on each leaf showed susceptibility.

Table 1 shows the resistance pattern of the four *Rpi* genes against these 44 *P. infestans* isolates. Forty isolates showed an incompatible interaction with plants containing *Rpi-sto1*, 37 with plants containing *Rpi-blb3*, five with plants containing *R3a* and 43 with plants containing *Rpi-vnt1.1*. It is known that *R3a* is overcome frequently by European isolates; it was therefore not remarkable that also the 28 Chinese isolates were all compatible with *R3a* containing plants. In contrast to this, the *Rpi-vnt1.1* and *Rpi-sto1* containing plants were completely resistant to all Chinese isolates; and *Rpi-blb3* containing plants showed resistance to all isolates from two provinces of China and in a third province to nine out of ten isolates. In Yunnan province, only three out of six isolates were incompatible with *Rpi-blb3*

containing plants. Consequently, *Rpi-sto1*, *Rpi-vnt1.1* and *Rpi-blb3* were selected as broad spectrum *Rpi* genes, and these three genes were combined into a binary vector pBINPLUS, which included the *nptII* transgene as a selection marker (Figure 1).

Transformation efficiency of a triple* Rpi *gene vector

Vector pBINPLUS:*Rpi-blb3*:*Rpi-vnt1.1*:*Rpi-sto1,* containing native promoters and terminators of each *Rpi* gene, was used to transform the late blight susceptible cv. Desiree. After co-cultivation with *A. tumefaciens* using 850 stem explants, 121 and 550 rooting regenerants were produced at the fifth and ninth month after the transformation, which resulted in a transformation efficiency of 14% and 59%, respectively.

Characterization of individual regenerants by PCR, isolates test and agro-infiltration

The first obtained 28 kanamycin resistant regenerants were selected for further characterization. Due to the broad spectrum of *Rpi* genes used for the stacking, no isolates were available, which can directly distinguish the functionality of each *Rpi* gene without the interference from the other *Rpi* genes as outlined in supplementary Table 3. This specific way of functional gene testing is needed for plants harboring multiple broad spectrum *R* genes against the same disease. These regenerants were characterized using *Rpi* gene specific primers (Table 2; Supplementary Table 2), selected isolates (EC1 and PIC99189; Table 2), and the corresponding *Avr* effectors (Figure 2 and Table 2). Both isolates were selected due to the fact that 1. isolate EC1 is compatible with *Rpi-vnt1.1* containing plants but incompatible with *Rpi-sto1* and *Rpi-blb3* containing plants, and 2. isolate PIC99189 is incompatible with *Rpi-vnt1.1* containing plants, whereas, it is compatible with *Rpi-sto1* and *Rpi-blb3* containing plants.

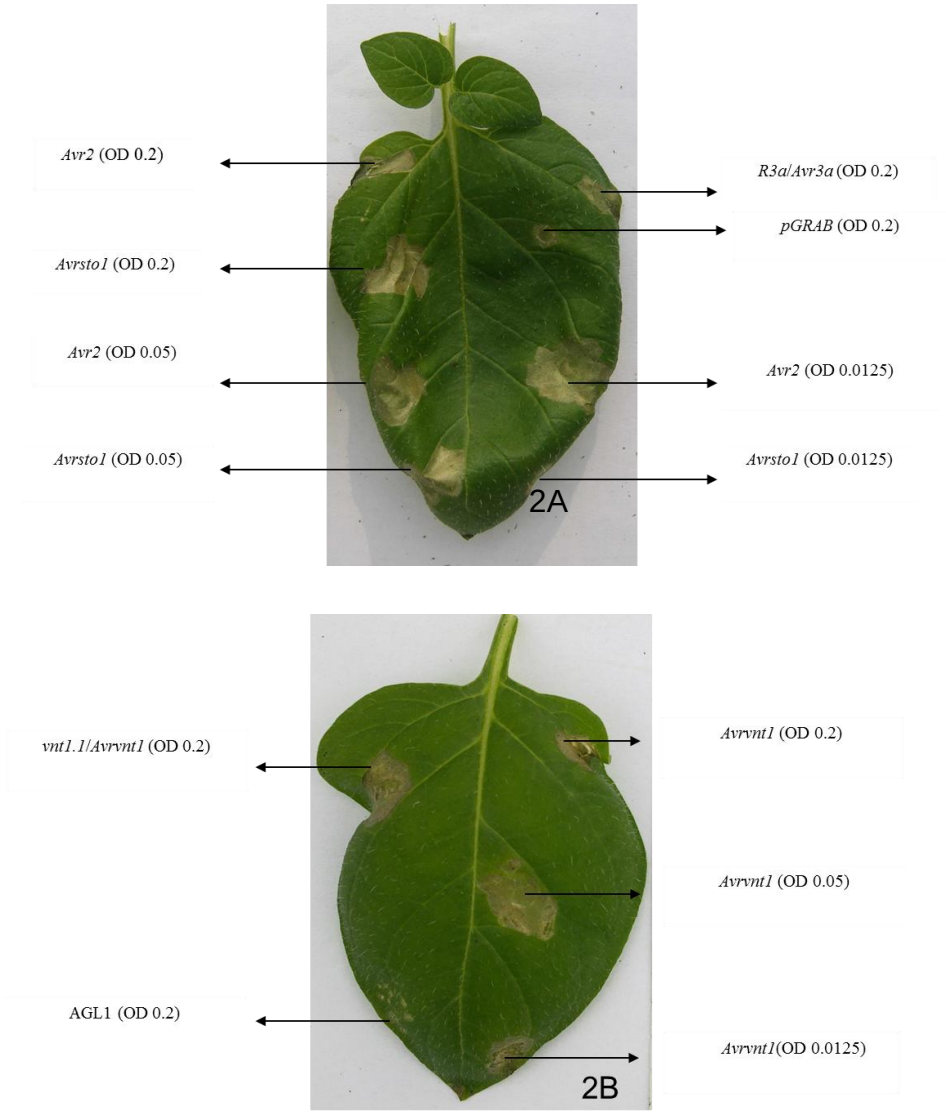

Fig. 2 Agro-infiltration of *Avr* effectors in triple *Rpi* gene regenerant A14 Z-22 using different densities of inoculum. Clear HR reactions were observed at all densities for *Avr2*, *Avrsto1* and *Avrvnt1*. Co-infiltration of *R3a*/*Avr3a*, and *vnt1.1*/*Avrvnt1* served as positive controls. Empty vector pGRAB, and *Agrobacterium* strain AGL1 were negative controls. Figure 2A showed the infiltration with *Avr2* and *Avrsto1*. Figure 2B showed the infiltration with *Avrvnt1*.

Together with cv. Desiree and the single *Rpi* gene transformants harboring *Rpi-sto1*, *Rpi-blb3* or *Rpi-vnt1.1* in cv. Desiree, the 28 kanamycin resistant regenerants were investigated. PCR analysis showed that all 28 regenerants (Table 2) contained *nptII*, among which, 23 harbored all three *Rpi* genes. All five kanamycin resistant plants (18%), without *P. infestans* resistance, turned out to lack the three *Rpi* genes (as evidenced by PCR analysis). DLA and agro-infiltration tests indicated that the 23 triple *Rpi* gene transformants (82%), also functionally expressed all three *Rpi* genes. Remarkably, no silencing or other interfering effects were observed for any of the transformed *Rpi* genes.

Table 2. Presence and activity of *Rpi* genes in transgenic Desiree plants after inoculation with two *P. infestans* isolates and agro-infiltration with three *Avr* genes

	(# of) plant	PCR				*P. infestans* inoculation		effector agro-infiltration		
		nptII	*sto1*	*vnt1.1*	*blb3*	EC1	PIC99189	*Avrsto1*	*Avrvnt1*	*Avr2*
Regenerants (28)	23	+	+	+	+	R	R	HR	HR	HR
	5	+	-	-	-	S	S	-	-	-
Control	Desiree:*Rpi-sto1**	+	+	-	-	R	S	HR	-	-
	Desiree:*Rpi-vnt1.1*	+	-	+	-	S	R	-	HR	-
	Desiree:*Rpi-blb3*	+	-	-	+	R	S	-	-	HR
	Desiree	-	-	-	-	S	S	-	-	-

The presence of the *Rpi* genes was determined by PCR. Biological activity of the *Rpi* genes was determined by *P. infestans* isolates inoculation in detached leaf assays or by agro-infiltration using *Avr* effectors matching the introduced *Rpi* genes.
* Desiree:*Rpi-sto1* stands for one cv. Desiree transformant harboring *Rpi-sto1*.
HR: Hypersensitive response
R: Resistant; S: Susceptible
"-" in PCR means no PCR product using gene specific primer pairs, and "-" in agro-infiltration means no HR symptom.

Discussion

Generally, *R* gene cloning from donor wild species, followed by one-step transformation into cultivated plants can be considered as an efficient method for domestication of natural *R* genes into the agricultural environment. Stacking of several *R* genes is expected to provide more durable and broadened resistance as compared to the introduction of a single *R* gene, which was proven to be ineffective in the past. In this paper, a study was performed to explore the strategy of functional stacking broad spectrum *Rpi* genes. *Rpi-blb3*, *Rpi-vnt1.1* and *Rpi-sto1* were selected as broad spectrum *Rpi* genes based on virulence tests in the laboratory studies. It was observed that *Rpi-sto1* and *Rpi-vnt1.1* were potentially useful in all Chinese provinces and *Rpi-blb3* in three provinces and to a lesser extent in Yunnan province of China. Another observation was that not a single isolate from China was incompatible with *R3a* containing plants. This could be confirmed with the molecular data of an overlapping and bigger set of 49 Chinese isolates (Li et al. 2009). Li et al. detected only the presence of the virulence allele for *avr3a* in these isolates, indicating the general breakage of *R3a*. In contrast to the results from in house isolates, *Rpi-vnt1.1* was still a broad spectrum *Rpi* gene in Europe, and *Rpi-sto1* and *Rpi-blb3* were still applicable in the Netherlands, but the resistance from *R3a* was widely broken.

Recently, a similar experiment with *P. infestans* isolates from Argentina was described. The response of local isolates to ten different cultivars unexpectedly showed that cv. Russet Burbank possessed an *Rpi* gene, which was useful in this country (Andreu et al. 2010). However, in the United

States, this *Rpi* gene is broken because of the general susceptibility of cv. Russet Burbank to *P. infestans* isolates in the USA (Staples 2004). The examples above show that isolates collected from specific geographical areas can predict the value of resistant varieties or help to select broad spectrum *R* genes for gene stacking. Consequently, this information could contribute to resistance breeding for specific geographical areas, where local field experiments have to be confirmed.

The triple *Rpi* gene construct showed a transformation efficiency of 14% and 59% at the fifth and ninth month after the transformation, respectively. These results showed that the transformation efficiency of this triple *Rpi* gene construct can be as high as that from transformations with single *Rpi* gene constructs, according to in house transformation experience (Heeres et al. 2002), but with a delay in regeneration time (data not shown). It is important to be aware of this phenomenon for future transformation experiments with stacked genes in one vector.

The aim of this study was stacking of broad-spectrum *Rpi* genes to achieve durable resistance to *P. infestans*. This approach inherently causes difficulties to select late blight isolates for determining biological functionality of each individual *Rpi* gene within the same plant. In this study, biological functionality could be proven for *Rpi-vnt1.1* by using isolates PIC99189 and EC1, but not for the other two *Rpi* genes. Therefore, agro-infiltration with three *Avr* effectors *Avrsto1*, *Avrvnt1* and *Avr2* (*Avrblb3*) was used to determine the biological functionality of each integrated gene. This not only provides a strategy to detect multiple functional genes in the same plant, but also confirmed the necessity of the screening and cloning of cognate *Avr* effectors of the *R* genes used. Twenty-eight transformants were investigated in detail in this study, among which, 23 harbored all three *Rpi* genes. They gave full resistance in the detached leaf assay, as well as HR after agro-infiltration using corresponding *Avr* effectors. It was observed that there were no silencing effects among these 23 transformants. All the integrated *Rpi* genes functioned properly.

On the other hand, there were five out of 28 transformants only harboring the selection marker gene *nptII*. This phenomenon is not unique. In experiments with rice, transformation was performed with multiple transgenes, *VHb* (*Vitreoscilla* hemoglobin gene), *tzs* (*trans*-zeatin secretion gene) and *EPSP* (modified 5-enolpyruvylshikimate-3-phosphate synthase gene) in addition to the hygromycin-gene as a selection marker, located close to the T-DNA left border (Cao et al. 2005). Cao et al. observed that three out of 113 transformants contained only the *hyg* selection marker gene, but not any target gene (*VHb*, *tzs* or *EPSP*). Also our results indicated that T-DNA transfer might start in a way only resulting in the functional insertion of the *nptII* gene close to the left border of T-DNA, with a much higher frequency observed than by Cao et al. (2005).

Another significant observation is the 100% match between disease resistance, mediated by *Rpi-vnt1.1* and agro-infiltration induced hypersensitivity reaction as all 23 triple *Rpi* gene transformants were resistant to isolate PIC99189 and responded to *Avrvnt1* (Table 2). Similarly, we found that *Rpi-blb3* or

Rpi-sto1 were biologically active from both disease resistance tests with isolate EC1 and agro-infiltration experiments. This shows that agro-infiltration could potentially substitute for DLA in testing multiple *Rpi* gene containing plants. This predictive potential will be tested in the future for more *R-Avr* gene combinations and for different varieties. In addition, a 100% match was observed between the presence of *Rpi* genes indicated by gene specific PCR results and their biological activities after disease test and effector agro-infiltration, respectively. This indicates that, for this construct, PCR on *Rpi* genes can be directly used as an indication not only for the presence but also for the biological functionality of all three integrated *Rpi* genes in cv. Desiree transformed with this construct.

This paper adopted the approach to stack broad spectrum *Rpi* genes by *Agrobacterium* mediated transformation using one transformation vector. This one vector and one-step transformation strategy, in comparison with re-transformation or multiple plasmids transformation, has the advantage of creating an artificial cluster of *Rpi* genes at one locus of the plant genome (Hiei et al. 2007). The plants produced in this paper, harboring these triple *Rpi* genes at presumably one locus, can be efficiently used as a breeding parent to transfer all three *Rpi* genes simultaneously into offspring plants.

The durability of the employed *Rpi* genes is an important phenomenon. The conventional differential set *R1-R11* has been used for many years as trap plants in potato fields for *P. infestans* population studies in order to check the presence or appearance of new (complex) virulent isolates (Dr. G. Kessel, Plant Research International, Wageningen UR, The Netherlands). In this differential set, the plants containing *Rpi-blb3*, which is a functional homolog of *R2*, is already represented by Ma*R2*. This type of field study can now be extended to differential plants harboring *Rpi-sto1* and *Rpi-vnt1.1* and be compared with triple *Rpi* gene transformants harboring *Rpi-sto1*, *Rpi-blb3* and *Rpi-vnt1.1*, in order to study and compare the potential differences in trapping candidate virulent isolates.

In conclusion, this paper showed an important way to stack three resistance genes using one vector in potato. The plants showed an expected broadened resistance spectrum, without the problem of any silencing effects. The use of *Avr* genes matching the *Rpi* genes to be stacked in this approach is crucial.

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Supplementary Table 1. Bacterial lines used in agro-infiltration studies

Vector	Gene	*Agrobacterium* strain
pBINPLUS	*R3a*	AGL1
pBINPLUS	*Rpi-vnt1.1*	AGL1
pBINPLUS	*Rpi-sto1*	AGL1
pBINPLUS	*Rpi-blb3*	AGL1
pK7wG2	*Avrsto1*	AGL1
pGRAB	*Avr2*	AGL1
pGR106	*Avrvnt1*	AGL1+virG
pGRAB	*Avr3a*	AGL1
pGRAB	empty	AGL1

Supplementary Table 2. List of primers used for PCR analysis

Gene	Primer orientation	Primer sequence	Annealing temperature (°C)	PCR product size (bp)
Rpi-sto1	Forward	ACCAAGGCCACAAGATTCTC	65°C	890
	Reverse	CCTGCGGTTCGGTTAATACA		
Rpi-blb3	Forward	AGCTTTTTGAGTGTGTAATTGG	63.5	305
	Reverse	GTAACTACGGACTCGAGGG		
Rpi-vnt1.1	Forward	CCTTCCTCATCCTCACATTTAG	60	302
	Reverse	CTCATCTAATAGATCCTCCAC		
nptII	Forward	TCGGCTATGACTGGGCACAACAGA	55.5	722
	Reverse	AAGAAGGCGATAGAAGGCGATGCG		

Supplementary Table 3. Testing scheme for determining individual *Rpi* genes' functionality in the triple *R* gene regenerants using specific late blight isolates and corresponding *Avr* effectors in disease assay and effector agro-infiltrations, respectively.

Isolate /*Avr* effector	Desiree	Expected reaction of cv. Desiree transformants			
		Rpi-blb3	*Rpi-vnt1.1*	*Rpi-sto1*	*Rpi-blb3*:*Rpi-vnt1.1*:*Rpi-sto1*
EC1	S	R	S	R	R
PIC99189	S	S	R	S	R
Avr2	-	HR	-	-	HR
Avrvnt1	-	-	HR	-	HR
Avrsto1	-	-	-	HR	HR

Cultivar Desiree and the single *Rpi* gene transformants of cv. Desiree containing *Rpi-sto1*, *Rpi-vnt1.1* or *Rpi-blb3*, respectively were used as control plants.
R: resistant; S: susceptible.
HR: hypersensitive response;
-: no HR.

CHAPTER 3

Genomic integration, functional expression and genetic inheritance of three potato late blight resistance genes in cv Desiree provided through a single T-DNA

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Abstract

Genetic transformation with multiple resistance (*R*) genes is expected to enhance resistance durability against pathogens, especially for potato, a vegetatively propagated crop with tetrasomic inheritance and a long-term breeding program. The resistance against *Phytophthora infestans* in potato with inserted *R* genes has been investigated in this study. 128 potato transformants were analyzed for the presence of T-DNA, T-DNA border and vector backbone sequences. The transformants were obtained after transformation using a construct containing *nptII* and three late blight *R* genes (*Rpi-sto1*: *Rpi-vnt1.1*:*Rpi-blb3*). It was shown that 45% of the *R* gene containing transformants harbored one or two T-DNA copies, without the integration of vector backbone and borders.

Transformants harboring three *R* genes showed resistance against isolate IPO-C (*Avrsto1*, *Avrvnt1*, *avrblb3*) under laboratory and field conditions. Two transformants harboring one T-DNA copy were crossed with susceptible cv Katahdin. In the resulting T1 populations, all four genes in the T-DNA showed clustered inheritance and Mendelian segregation. In a T1 population, deriving from a transformant harboring three T-DNA copies, an un-linked segregation pattern was observed for the T-DNA inserts, showing that T-DNAs had integrated in the genetically unlinked parts of the genome. All the *R* genes were functionally expressed in the leaves of the T0 plants as well as in the tested T1 plants. Resistance against two different late blight isolates, EC1 (*Avrsto1*, *avrvnt1*, *Avrblb3*) and PIC99189 (*avrsto1*, *Avrvnt1*, *avrblb3*), was also observed in the tubers of T0 and T1 plants. The finding that resistance was stably inherited through sexual as well as vegetative reproduction has important implications towards achieving durable resistance against late blight in potato.

Key words Potato late blight · *R* gene stacking · Vector integration · Inheritance · Field resistance · Tuber and leaf resistance

Introduction

With the milestone of achieving seven billion people in the world on October 31st, 2011 (ISAAA 2012), the challenge for food security is getting an even more prominent position on the agenda. Plants play an important role in people's daily life, and an effective way to protect crops from diseases, is to introduce beneficial genes by genetic modification. The first genetically modified plants, were described in 1983 for tobacco (Herrera-Estrella et al. 1983) and potato (Ooms et al. 1983). In the Netherlands already in 1995 a GM starch potato (cv Apriori) was approved and grown by the company AVEBE b.a. (Jaccaud et al. 2003). Between 1995 and 2001 Monsanto company produced GM potato in different varieties called "Newleaf/NatureMark" with resistance against Colorado potato beetle and potato leafroll virus (Toevs et al. 2011). Another example of a GM potato is cv Amflora from the company BASF, which was approved for cultivation and processing in 2010 (BASF 2010). So far there has not been any commercial cultivation of GM potato growing widely. However, in 2011 there were more than 160 million hectares

of other GM crops grown globally. In 2012 even the vast majority of corn (88%), soybean (93%) and cotton (94%) in the USA were GM (ISAAA 2012; USDA 2012).

Agrobacterium mediated transformation, relying on the use of a binary vector system, is the most commonly used technique to obtain GM plants. Ideally, the integration process starts from the right border (RB) of the T-DNA plasmid and ends at the left border leaving a single T-DNA integrated into the recipient plant genome (Gelvin 2003). However, many studies showed that upon genetic modification not only the gene(s) from the T-DNA can be integrated, but also sequences from the vector backbone, outside the T-DNA region, can be found in the recipient genome (van der Graaff and Hooykaas 1996; Wolters et al. 1998; Rommens et al. 2004; Lange et al. 2006). Kononov et al. (1997) found that 75% of transgenic tobacco plants contained vector backbone sequences. Abdal-Aziz et al. (2006) showed the presence of the vector backbone gene *trfA* in 66% of their transgenic strawberry plants. Petti et al. (2009) reported that *A. tumefaciens* strain LBA4404 had higher frequency of vector backbone sequence integration into the potato genome than strain AGL1. However, these studies were restricted to only one or a few backbone genes covering only part of the vector backbone sequence. GMO safety regulations, like Directive 2001/18/EC in Europe, require that varieties that are released into the environment are vector backbone free. These observations and rules induced a number of research questions on vector backbone analysis and early prediction on their absence. Thus more studies on vector backbone integration patterns are required. In this study we investigate the T-DNA and vector backbone sequence integration patterns in transgenic potato plants that were transformed with a binary vector harboring three late blight resistance genes.

Late blight is a devastating disease for potato cultivation worldwide and is caused by the oomycete *Phytophthora infestans*. Late blight provides a problem during potato growth in the field, because the foliage of the crop can be completely destroyed resulting in tuber yield losses. Also the stored tubers remain susceptible to late blight resulting in post-harvest losses. Genetic resistance is available among wild relatives of potato but, unfortunately, the introgression of these resistance (*R*) genes is very time consuming. Moreover, resistance based on the presence of a single *R* gene is not durable (Lowe et al. 2011), gene pyramiding or gene stacking is expected to enhance durability of resistance (Cao et al. 2002; Halpin 2005; Que et al. 2010; Revathi et al. 2010; Kim et al. 2012). Recently, introduction of three *R* genes by transformation was proven to be successful (Zhu et al. 2012). Using a single binary plasmid with three late blight *R* genes (*Rpi-sto1*, *Rpi-vnt1.1* and *Rpi-blb3*) and *nptII* as selectable marker, transformants were made and selected in which all three *R* genes were functioning simultaneously. In this study we extend the analysis of these transgenic plants by producing additional transgenic events and by studying their late blight resistance in the foliage under field conditions and in the tubers.

Another important issue for applying transgenic plants in breeding practice is to investigate whether the introduced *R* genes are inherited to the next generation as a cluster, and whether all T-DNA

genes remain their functionality in the offspring plants. Studies in potato (Visser et al. 1989; Wolters et al. 1998), petunia (Wang et al. 2009) and in rice (Song et al. 2012) showed stable Mendelian inheritance of transgenes. However, these investigations were performed with simple T-DNAs, containing one or two relatively small genes. T-DNA complexity rises with the number and size of the genes and with the extent of homology between the genes and repetitiveness of sequences. The segregation of complex T-DNAs after crossing has sparsely been described and requires further investigation.

In this study three selected cv Desiree transformants, harboring complex T-DNAs containing three late blight resistance genes and the *nptII* kanamycin resistance gene were crossed with the susceptible potato cultivar Katahdin. The three *R* genes (*Rpi-sto1*, *Rpi-vnt1.1* and *Rpi-blb3*) were confirmed to be inherited as a single gene cluster and segregated in a tetrasomic pattern. Genomic and functional stability of the introduced *R* genes was confirmed in the next generation, both in leaves and in tubers. Also under field conditions the resistance was stable in two successive years. Furthermore, the three T-DNA insertions in one transformant were demonstrated to be un-linked since they were inherited independently in a T1 population. Additionally, the integration pattern of T-DNA genes, T-DNA border sequences and eight vector backbone genes showed that 45% of 128 investigated transformants were harboring one or two T-DNA copies, without the integration of vector backbone sequences and borders. Backbone gene *tetA* was found to be a good marker for the early prediction of backbone sequence integration.

Materials and methods

Biological materials

Agrobacterium strain AGL1 harboring binary vector pBINPLUS:*Rpi-sto1*:*Rpi-vnt1.1*:*Rpi-blb3* (Zhu et al. 2012) was used to transform the susceptible cultivar Desiree. Cultivar Desiree and T0 plants were maintained *in vitro* in the laboratories of Wageningen UR Plant Breeding. Two late blight isolates EC1 (*Avrsto1*, *avrvnt1*, *Avrblb3*) and PIC99189 (*avrsto1*, *Avrvnt1*, *avrblb3*) were used in the leaf and tuber late blight assays. For late blight field assays the isolate IPO-C (*Avrsto1*, *Avrvnt1*, *avrblb3*) was used. The primary Desiree transformants (T0 plants) were obtained using the construct pBINPLUS:*Rpi-sto1*:*Rpi-vnt1.1*:*Rpi-blb3* (Zhu et al. 2012) and were called A14-(event number). T1 populations were made by crossing susceptible cv Katahdin with three T0 plants, harboring one (A14-207, A14-225) and three (A14-203) T-DNA copies, resulting in T1 populations PBM12-002, PBM12-003 and PBM12-001, respectively. Six single T0 plants containing *Rpi-blb3* (A03-142, A03-515, A03-516, A03-517, A03-520 and A03-521), *Rpi-sto1* (A09-6, A09-252, A09-257, A09-267, A09-268 and A09-277) and *Rpi-vnt1.1* (A13-SF3, A13-8, A13-11, A13-13, A13-14 and A13-21), respectively, were used as controls in the tuber disease tests to assess the resistance activity of the individual *R* genes present in the triple *R* gene containing transformants.

DNA extraction

DNA extraction was performed using two young leaves from *in vitro* plants, with the automated sbeadex_Mini_Plant F1 96PW protocol in the Kingfisher FLEX machine. The quantity and quality of the DNA were checked using a Nanodrop spectrophotometer and by PCR with *EF-1alfa* primer pairs (Supplementary Table 1), respectively.

Investigation of T-DNA border and vector backbone sequences

In order to investigate the presence or absence of left border (LB) and right border (RB) sequences, as well as the possible presence of sequences flanking the LB and RB in the T-DNA region, PCR analysis with four primer pairs was performed. Two primer pairs for each border, towards T-DNA orientation and vector backbone orientation were designed (Supplementary Fig. 1). Two primer pairs for LB (SZ31/SZ32 and SZ33/SZ34) and two primer pairs for RB (SZ29/SZ30 and SZ27/SZ28) were used (Supplementary Table 1). The presence of vector backbone sequences was analyzed by PCR using primer pairs from eight vector backbone genes referred to as *tetR*, *oriv3+5*, *traJ*, *oriV*, *insB*, *nptIII*, *trfA*, and *tetA* (Supplementary Table 1).

Investigation of T-DNA copy number

T-DNA copy number was determined using genomic DNA by quantitative real time PCR (qRT-PCR). PCR reactions were performed using the qRT-PCR kit (Biorad) with the Bio-Rad icycler iQ machine. The reaction mixtures consisted of 5 ng genomic DNA, 5 µl of Sybr green mix, 3 mM forward primer, 3 mM reverse primer in a final volume of 10 µl. An *EF-1alfa* primer pair was used as an internal calibrator for the amount of genomic DNA. Three events with one T-DNA insertion of each *R* gene were used as standards for Ct values of single copy plants. Negative controls were non-transformed cv Atlantic and MQ water. Primer sequences are listed in Supplementary Table 1.

nptII *expression assay*

To test *nptII* expression, transformants were grown in MS medium with kanamycin 100 mg/l (Zhu et al. 2012). Rooting efficiency was scored after one, two and three weeks. Plants harboring roots longer than 1 cm are considered as kanamycin resistant.

Effector responsiveness

Three *Phytophthora infestans* effectors *Avrsto1*, *Avrvnt1* and *Avrblb3* and negative control plasmid pK7WG2 in *Agrobacterium tumefaciens* strain AGL1+VirG were used for agro-infiltrations (Rietman et al. 2012). *Agrobacterium* suspensions of optical densities between 0.1 and 0.2 were infiltrated into the leaves of transformants and seedlings. Four replicates were performed with two plants and two leaves per plant for each genotype.

Detached leaf assay

Detached leaf assays (DLA) to assess late blight resistance were performed according to the protocol described by Vleeshouwers et al. (1999) with isolates EC1 and PIC99189. Two complex leaves, each with three single leaves close to the leaf tip, were tested for each clone. The criteria of scoring are: R, all of the inoculated spots on a leaf showed resistance; RQ, 6-7 out of eight spots showed resistance; Q, 3-5 out of eight spots showed resistance; SQ, 1-2 out of eight spots showed resistance; S, all eight spots showed susceptibility.

Late blight field trials

Late blight resistance assays under field conditions were performed essentially as described previously (Kim et al., 2012) in two years in 2011 and 2012. Potato tubers were planted in the field in the beginning of May. Two replications, each with six plants per genotype, were planted. Inoculation with isolate IPO-C (*Avrsto1*, *Avrvnt1*, *avrblb3*) was performed in the beginning of July. Plants were examined at 10, 19, and 27 days post inoculation (dpi) in 2011, and at 2, 7, 14 and 21 dpi in 2012. The percentage of the leaf area covered by late blight lesions was estimated by visual inspection.

Late blight resistance in tuber slices

Tubers of T0 plants and T1 seedlings were harvested after five months of growing in the clay soil under field conditions with the protection of using fungicide application, and four months of growing in the pots in the wire-screen cage, respectively. The tuber surface was sterilized as described by Pel et al. (2010). Two tubers from each genotype were submerged for 5 minutes in a 5% sodium hypochlorite solution and then rinsed three times with tap water. Four slices were cut from two tubers per genotype and were placed into a tray for the inoculation with one isolate. Each slice was then inoculated with 5 droplets of 10 µl of spore suspension ($5x10^4$ spores ml^{-1}). Inoculated tuber slices were kept at 15ºC and 100% humidity in the dark. Scorings were performed seven days after inoculation by estimating the percentage of the area covered with mycelium.

Results

T-DNA, border and vector backbone integration pattern

Cv Desiree was transformed with vector pBINPLUS:*Rpi-sto1*:*Rpi-vnt1.1*:*Rpi-blb3* containing the *nptII* gene as selectable marker and three late blight resistance genes. Totally, 128 kanamycin resistant transformants were harvested from two independent experiments (73 and 55 respectively). These 128 plants were classified for the presence or absence of *nptII*, *R* genes, vector backbone sequences, and T-DNA borders (Supplementary Table 2).

Table 1. Classification of 128 kanamycin resistant transformants for insertion of T-DNA genes, vector backbone sequences and vector borders

Class	*nptII*	# of *R* genes	Border[$]	Backbone*	Transformants (#)
1	+	3	+	+	21
2	+	3	-	+	3
3	+	3	+	-	1
4	+	3	-	-	73
5	+	0	+	+	4
6	+	0	-	+	0
7	+	0	+	-	0
8	+	0	-	-	24
9	+	1**	-	-	2
Total					*128*

* the presence of at least one of eight vector backbone sequences
[$] either or both borders gave PCR products
** both transformants contained only *Rpi-sto1*

Based on the presence or absence of all three *R* genes, T-DNA borders, and vector backbone, eight classes of transformants were expected of which six were found (Table 1). The classes 6 and 7, without *R* genes but with backbone or with borders, were missing. In addition to these expected eight classes, one additional class of two transformants was found that contained only one *R* gene (*Rpi-sto1*) and no backbone genes or borders (1.5%). Ninety-eight out of 128 transformants (77%) contained all three *R* genes, and 73 of them (57%) showed no backbone sequences or borders. There were 23 transformants (18%), which were positive for both LB-T-DNA and LB-backbone PCR reactions; only eight transformants (6%) harbored RB (both RB-T-DNA and RB-backbone primers produced a product). There were 28 transformants (22%) containing only *nptII*. The integration of vector backbone and border sequences was slightly less frequently found in plants without *R* genes (4/28) than in those with *R* genes (24/98), as the comparison between the classes 5 and 8 or 1 and 4 showed, respectively. Among the 28 transformants with vector backbone (classes 1, 2, and 5), 25 transformants (classes 1 and 5) also showed the integration of border sequences. Remarkably, class 2 with only three representatives, showed the absence of borders but the presence of both T-DNA and vector backbone sequences. Because of the discontinuity as compared to the binary plasmid, it can be concluded that vector backbone sequences can integrate independently from T-DNA sequences. Beside this, the presence of vector backbone sequences close to LB was found in one transformant with the vector border (class 3), while the eight tested backbone sequences were absent.

The T-DNA copy numbers were determined by qRT-PCR using *nptII* primers in 128 transformants. Seventy percent of the transformants had copy numbers varying from 1-3. The two plants, which only contained *nptII* and *Rpi-sto1* harbored two and three copies of *nptII*, respectively. Among the classes of *R* gene containing transformants, all plants containing one copy of *R* gene(s)were backbone free. Similarly, among the plants without *R* genes with one copy of *nptII*, only one out of 14 plants showed the presence of vector backbone. The plants with two or three *nptII* copies showed a higher but still relatively low

frequency of vector backbone sequence integration. This frequency of vector backbone sequence integration increased to more than 50% in plants with more than three T-DNA copies. It can be concluded that vector backbone free plants in combination with harboring a low (1-2) copy number of *R* genes can be obtained at a relatively high frequency.

Table 2. Copy number of *nptII* in relation to *R* gene and vector backbone sequence integration among 128 primary transformants

Copy # of *nptII*[$]	*R*+		*R*-		Total
	bb+	bb-	bb+	bb-	
1	0	21	1	13	35
2	5	19	0	5	29
3	3	22	1	2	28
>3	16	14	2	4	36
Total	24	76	4	24	128

[$] copy number was determined by qRT-PCR. *R*+/*R*-: *R* gene presence or absence; bb: vector backbone sequence

The 28 vector backbone sequence containing plants were divided into two categories, consisting of four plants without *R* genes and 24 plants with *R* genes, respectively (Fig. 1). The four plants with vector backbone but without *R* genes (category 1) contained one, three, or more than four *nptII* copies of incomplete vector backbone sequences. All these vector backbone sequences directly followed LB.

Category 1

Copy number of *nptII*	Vector backbone sequence integration pattern of *R* gene free plants	# of plants
1	LB *tetA* *trfA* *nptIII*	1
3	LB *tetA* *trfA* *nptIII*	1
>4	LB *tetA* *trfA* *nptIII* *insB* *oriV*	1
	LB *tetA* *trfA* *nptIII*	1

Category 2

Copy number of *nptII*	Vector backbone sequence integration pattern of *R* gene containing plants	# of plants
1		0
2	LB *tetA* *trfA* *nptIII* *insB* *oriV* *traJ* *oriv3+5* *tetR* RB	2
	LB *tetA* *trfA* *nptIII* *insB* *oriV* *traJ* *oriv3+5*	1
	LB *tetA* *trfA* *nptIII*	1
	LB *tetA*	1
3	*tetA* *trfA* *nptIII* *insB* *oriV* *traJ* *oriv3+5* *tetR*	1
	tetA *trfA* *nptIII* *insB*	1
	LB *tetA* *trfA* *nptIII*	1
≥4	LB *tetA* *trfA* *nptIII* *insB* *oriV* *traJ* *oriv3+5* *tetR* RB	3
	LB *tetA* *trfA* *nptIII* *insB* *oriV* *traJ* *oriv3+5* *tetR*	7
	LB *tetA* *trfA* *nptIII* *insB* *oriV* *traJ* *oriv3+5*	1
	tetA *trfA* *nptIII* *insB* *oriV* *traJ* *oriv3+5* *tetR*	1
	tetA *trfA* *nptIII* *insB* *oriV* *traJ* *oriv3+5* *tetR* RB	3
	LB *traJ* *oriv3+5* *tetR*	1
Total		24

Figure 1. Copy number of *nptII* in the absence (category 1) or presence (category 2) of three *R* genes in relation with integration of vector backbone sequences and borders in 28 backbone sequence containing transformants LB: plants positive for both LB-T-DNA and LB-backbone PCR. RB: plants positive for both RB-T-DNA and RB-backbone PCR. Backbone and border sequences are ordered according to their positions in the binary vector (LB-*tetA-trfA-nptIII-insB-oriV-traJ-oriv3+5-tetR*-RB)

Category 2 showed the backbone sequence integration of 24 *R* gene containing plants with two or more copies of *nptII*. Remarkably, there was no backbone sequence containing plant with only one copy of *nptII*. Five plants showed the presence of all backbone sequences including both borders. Two of them belonged to the class with two copies. In the class with three *nptII* copies one plant was found with all backbone sequences but without borders. It is clear that longer backbone sequence incidence occurred more frequently in plants with more than three *nptII* copies. Except this, twenty-one out of the 28 backbone sequence containing plants harbored LB. The presence of RB was mostly accompanied by LB and all backbone sequences. The presence of LB was always accompanied by backbone sequences, starting with the direct neighboring gene *tetA*. Among the 28 backbone sequence containing plants, 27 harbored *tetA*. The integration of backbone sequences seems to decrease from LB to RB. In spite of some transformants with backbone sequences or borders, three-quarters of all plants contained all four genes from the T-DNA, in the absence of backbone sequences and borders. These plants will be used for further studies.

Foliar resistance under field conditions in 2011 and 2012

Field analysis of late blight resistance was performed with 28 transformants. Five transformants contained only *nptII* and 23 transformants contained both *nptII* and the three *R* genes. The latter 23 transformants showed the functionality of each *R* gene as determined by DLA and responsiveness to corresponding *Avr* genes (Zhu et al., 2012). In addition, three Desiree transformants that functionally expressed *Rpi-blb3*, *Rpi-sto1* or *Rpi-vnt1.1*, and cvs Desiree, Bintje were used as control plants.

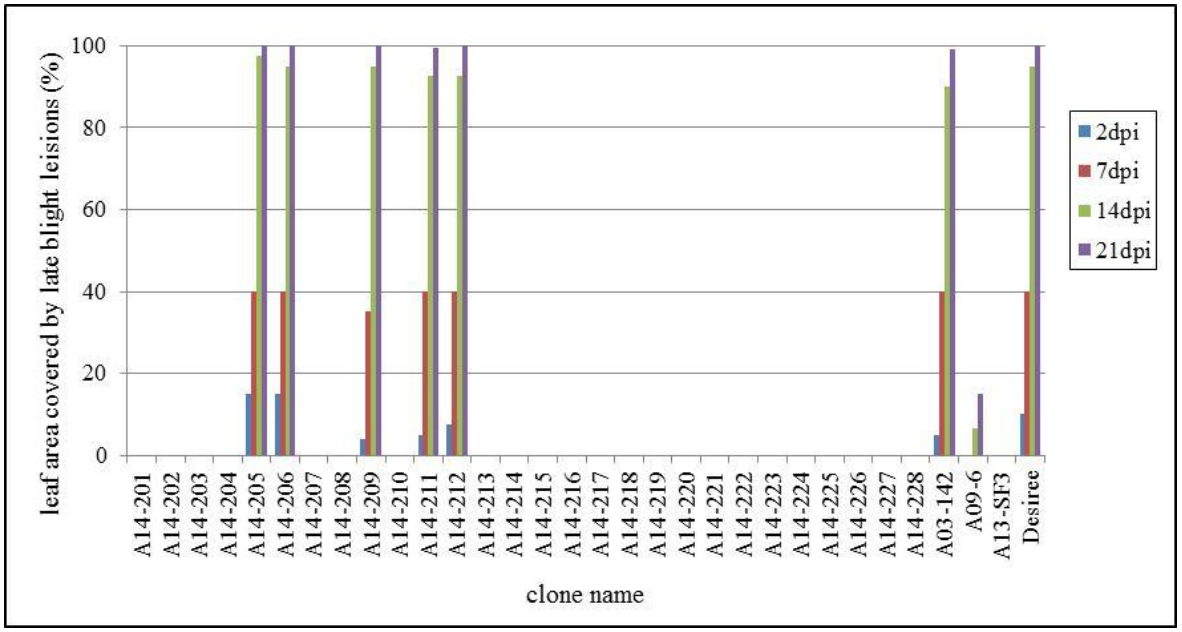

Figure 2. Late blight resistance of *Rpi-blb3*, *Rpi-sto1*, *Rpi-vnt1.1* containing transformants in Desiree (A14) under field conditions in 2012. Two repeats of six plants per event were planted and their average value was shown in the figure. Disease development was measured at 2, 7, 14, 21 days post inoculation in 2012. A03-142, A09-6, A13-SF3 are Desiree transformants containing *Rpi-blb3*, *Rpi-sto1* or *Rpi-vnt1.1*, respectively; A14-xxx: Desiree transformants obtained through vector pBINPLUS:*Rpi-sto1*:*Rpi-vnt1.1*:*Rpi-blb3*. A14-205, 206, 209, 211, 212 harbored only the *nptII* gene.

Results in 2011 and 2012 were very similar for all tested plants. In Fig. 2 the average value from the two repeats for each genotype in 2012 was shown (detailed data for 2012 can be found in Supplementary Table 5). Cvs Bintje and Desiree were fully susceptible, as expected. The triple *R* gene containing transformants, showing resistance in DLA, were also resistant against late blight isolate IPO-C in the field. The five transformants harboring only *nptII* but no *R* genes (A14-205, A14-206, A14-209, A14-211 and A14-212) showed full susceptibility. The Desiree transformant only containing *Rpi-blb3* (A03-142), was susceptible to isolate IPO-C (*Avrsto1*, *Avrvnt1*, *avrblb3*). The transformant containing *Rpi-sto1* (A09-6) showed full resistance at the beginning of the experiment, but 21 days after inoculation around 15% of the foliage was covered by late blight lesions. In contrast, the transformant containing only *Rpi-vnt1.1* (A13-SF3) showed full resistance till the end of the experiment in both years.

Late blight resistance in tubers of* Rpi-sto1:Rpi-vnt1.1:Rpi-blb3 *containing transformants

Tubers from the 28 Desiree T0 plants (Zhu et al. 2012) harvested under field conditions were used for the late blight resistance tests. In addition, six Desiree transformants containing *Rpi-sto1*, six Desiree transformants containing *Rpi-blb3*, and six Desiree transformants with *Rpi-vnt1.1* were planted and the resulting tubers were used for late blight resistance tests with the isolates EC1 (*Avrsto1*, *avrvnt1*, *Avrblb3*) and PIC99189 (*avrsto1*, *Avrvnt1*, *avrblb3*). The resistance in tubers was scored by estimating the area covered with mycelium (Figure 3; Supplementary Table 4). Five out of six *Rpi-sto1* containing transformants showed susceptibility to the isolate EC1 (containing *Avrsto1*), while all transformants were resistant to this isolate in DLA (Table 3). Five out of six *Rpi-blb3* containing transformants showed full resistance against isolate EC1 at the tuber level, similar to the DLA situation (Table 3). EC1 had broken *Rpi-vnt1.1* both in the leaves and the tubers. Four out of six *Rpi-vnt1.1* transformants conferred full resistance against PIC99189 while all six transformants were resistant in DLA (Table 3). Thus, it can be concluded that *Rpi-blb3* and *Rpi-vnt1.1* can confer resistance in tubers although at slightly lower frequencies than in DLA.

Table 3. Late blight resistance test in leaves and tubers of Desiree triple *R* gene transformants compared with transformants containing only one *R* gene

Transgenes in Desiree	# of clones	DLA**		Resistance in tubers			
				EC1		PIC99189	
		Resistant	Susceptible	Resistant	Susceptible	Resistant	Susceptible
nptII:*stol*:*vnt1.1*:*blb3*	23	23	0	23*	0	23	0
nptII	5	0	5	0	5	0	5
nptII:*sto1*	6	6	0	1	5	0	6
nptII:*blb3*	6	6	0	5	1	0	6
nptII:*vnt1.1*	6	6	0	0	6	4	2
not transformed	1	0	1	0	1	0	1

* 7 out of 23 showed slight *P. infestans* growth around the inoculum sites.
** In DLA, isolates EC1 and PIC99189 were used for testing *nptII*:*sto1*:*vnt1.1*:*blb3* containing transformants, all 23 transformants showed resistance against both isolates; isolate 90128 was used for testing *Rpi-blb3* and *Rpi-vnt1.1* containing transformants. Isolate 89148-09 was used to test the *Rpi-sto1* containing transformants.

The 23 transformants containing the three *R* genes (Zhu et al 2012), which showed full resistance in DLA against both isolates PIC99189 and EC1 (Fig. 3), were not all fully resistant against the same isolates at the tuber level. Seven out of these 23 clones showed slight infection only at the inoculated sites with isolate EC1. Four out of these seven transformants harbored one copy of the T-DNA. These results showed that the *Rpi-sto1* and/or *Rpi-blb3* might not confer full resistance in tubers, and that resistance might be related to insertion site(s) and copy number. This notion (resistance to EC1 in the tubers) was supported by the finding that one out of six transformants harboring only *Rpi-sto1* was resistant against isolate EC1 in tubers; and five out of six transformants harboring only *Rpi-blb*3 were resistant against isolate EC1 in tubers. As expected, the five plants with only *nptII* were equally susceptible to both isolates as control cv Desiree, confirming that the resistance observed in these experiments was brought about by the introduced late blight *R* genes.

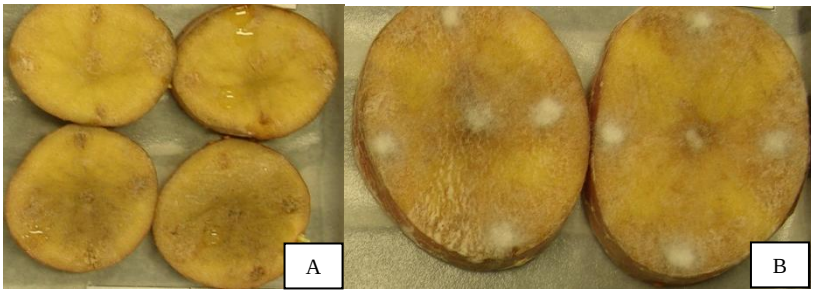

Figure 3. An example of disease resistance and susceptibility against *P. infestans* isolate PIC99189 in tubers.
A: transformant A14-14 (containing *nptII*:*Rpi-sto1*:*Rpi-vnt1.1*:*Rpi-blb3*); B: non transgenic cv Desiree

Inheritance and segregation of the introduced* R *genes in T1 seedlings

In a previous study, 23 plants transformed with the same construct (pBINPLUS:*Rpi-sto1*:*Rpi-vnt1.1*:*Rpi-blb3*) were investigated for the biological functionality of the three inserted *R* genes (Zhu et al. 2012). Detached leaf assay (DLA) as well as agro-infiltration studies using the cognate *Avr* genes showed that resistance and HR reactions were found in all transformants harboring the corresponding *R* genes. It was concluded that all three *R* genes were properly expressed in cv Desiree background, no indications were found for conditions that negatively affected the expression of (one of) these *R* genes.

To investigate if the transgenes remained stable and functionally expressed in different genetic backgrounds, but also to test if transgenes are inherited in a tetrasomic pattern especially when more T-DNA copies are present, three backbone sequence free transformants (A14-203, A14-207 and A14-225) were crossed with cv Katahdin that does not contain these *R* genes (*Rpi-sto1*, *Rpi-vnt1.1* or *Rpi-blb3*). These three transformants harbored three, one and one copy of all four T-DNA genes (*nptII*:*Rpi-sto1*:*Rpi-vnt1.1*:*Rpi-blb3*), respectively. Three T1 populations of around 50 seedlings for each population were investigated for the inheritance of kanamycin resistance, late blight resistance and number of T-DNA genes.

The presence of the inserted T-DNA genes was tested using PCR. Non-transformed Desiree and crossing parents cv Katahdin were negative in this PCR showing that the PCR primers were selective for the transgenes in these genetic backgrounds. In all three offspring populations only two classes of seedlings were observed, those without any additional gene like wild type cv Desiree/Katahdin and those with all four inserted genes (*nptII*, *Rpi-sto1*, *Rpi-vnt1.1* and *Rpi-blb3*). It indicated that, as expected, all four genes from the T-DNA (*nptII*:*Rpi-sto1*:*Rpi-vnt1.1*:*Rpi-blb3*) inherited as a cluster. The transgenes were found to be biologically active in all tested plants, leading to kanamycin resistance, late blight resistance in DLA and HR in *Avr* effector responsiveness (Table 4, Fig. 4).

Table 4. Tetrasomic segregation in T1 populations of cv Katahdin crossed with A14-203, A14-207 and A14-225 with 3 T-DNA copies and 2 times one T-DNA copy, respectively

Population (copy number)	PCR (4 T-DNA genes)	Kanamyc in rooting	DLA[a]	*Avr* response	Number of plants	Expected ratio (χ^2 observed)
PBM12-001 (3) (A14-203*K)	+	+	R[b]	HR[c]	34	7:1 (2.79)
	-	-	S	No HR	9	3:1 (0.38)
PBM12-002 (1) (A14-207*K)	+	+	R[b]	HR[c]	23	1:1 (0.21)
	-	-	S	No HR	20	
PBM12-003 (1) (A14-225*K)	+	+	R	HR[c]	14	1:1 (2.63)
	-	-	S	No HR	24	

+: plants were positive for the PCR tests, or gave roots in kanamycin (100 mg/l) containing media.
-: plants were negative for the PCR tests, or did not give roots in kanamycin (100 mg/l) containing media.
[a]: Two isolates were used in DLA: EC1 (*Avrsto1*, *avrvnt1*, *Avrblb3*) and PIC99189 (*avrsto1*, *Avrvnt1*, *Avrblb3*) for detecting the functionality of *Rpi-sto1*, *Rpi-blb3* and *Rpi-vnt1.1*. R indicates that all of the inoculation spots on a leaf showed resistance; S indicates that all of the inoculation spots on a leaf showed susceptibility.
[b]: Two out of 34 or 23 plants showed RQ (6-7 out of eight spots showed resistance).
[c]: For each population, HR was observed in response to all three *Avr* genes (*Avrsto1*, *Avrvnt1* and *Avrblb3*) in both replicates for around 90% of the PCR plus plants.

All kanamycin resistant T1 plants were found to contain all four genes from the T-DNA, and these plants were also showing disease resistance to the isolates EC1 (*Avrsto1*, *avrvnt1*, *Avrblb3*) and PIC99189 (*avrsto1*, *Avrvnt1*, *avrblb3*). Further evidence that all three *R* genes were actively expressed in the offspring was obtained from agro-infiltrations. HR was observed with all three corresponding *Avr* genes (two OD_{600} concentrations of *Agrobacterium* for each *Avr* gene were used) in both replicates for around 90% of *R* gene containing seedlings in each population; For the other *R* gene containing T1 genotypes, at least one *Avr* gene showing HR was examined (data not shown). This variation in HR reaction might be due to the different genetic backgrounds present in the individual T1 seedlings. In the populations PBM12-002 (A14-207*K) and PBM12-003 (A14-225*K), one insert of all four genes was found. The presence and absence of the T-DNA genes was observed in a ratio of 1:1 by PCR and disease tests, confirming that the mother plants (A14-207 and A14-225) each had one T-DNA insert.

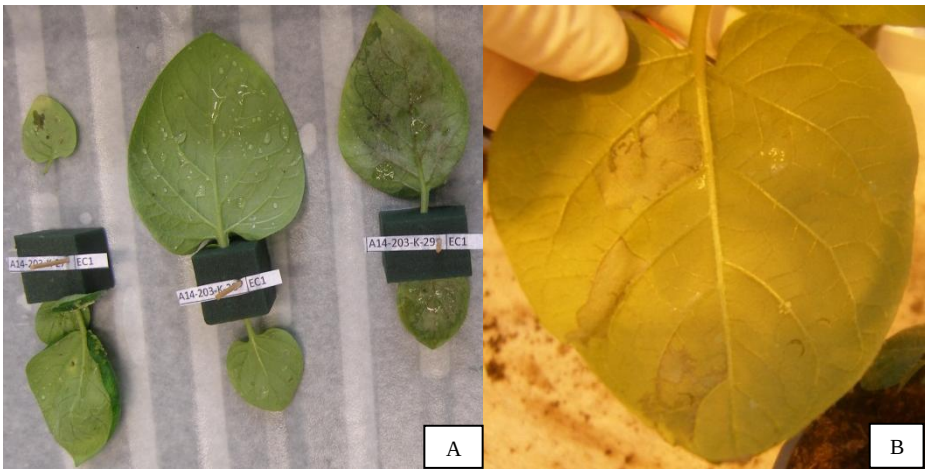

Figure 4. Activity assays for late blight resistance genes. Detached leaf assay using isolate EC1 (A) and agro-infiltration of A14-203*K T1 seedlings with three *Avr*s and *pK7WG2* as a negative control (B).

For a tetraploid crossing population with one parent containing one copy of T-DNA and the other parent harboring no T-DNA, the expected segregation ratio for the number of progenies with/without one copy of T-DNA is 1:1. If the mother plant contains three copies of T-DNA, the segregation ratios for the number of progenies with/without the T-DNA, are dependent on how the T-DNAs were integrated in the mother plant. The three copies of T-DNA can be inserted at the same locus or at two or three different loci with the expected segregation ratios for the number of progenies with/without T-DNA inserts being 1:1, 3:1 and 7:1, respectively. Among T1 offspring from the crossing of A14-203 with cv Katahdin (A14-203*K), a segregation ratio for kanamycin resistance (the number of plants being resistant:susceptible) as well as disease resistance by DLA (the number of plants being resistant:susceptible) fits the ratios of both 3:1 and 7:1 for two and three independent T-DNA inserts, respectively. However, segregation ratio can also be based on inheritance of copy number. In case all three copies of T-DNA are at one locus, a 1:1 segregation ratio is expected for the number of progenies with/without all three T-DNA copies. In case two copies of T-DNA are at one locus and a single copy of T-DNA is located at another locus, a segregation ratio of 1:1:1:1 for the number of progenies containing zero, one, two and three copies of T-DNA is expected. In case of three copies of T-DNA are inserted independently, a 1:3:3:1 segregation ratio for the number of progenies containing zero, one, two and three copies of T-DNA is expected.

Table 5. Segregation analysis of T-DNA inserts in T1 population A14-203 (3 T-DNA inserts) * cv Katahdin

Copy number	Expected (three loci/two loci)	Observed (#)	Expected (#) (three loci/two loci)	χ^2** (three loci/two loci)
0	1/1	9	5.375/10.75	2.95/7.88
1	3/1	15	16.125/10.75	
2	3/1	15	16.125/10.75	
3	1/1	4	5.375/10.75	

Expected ratio of 3 independent inserts is 1:3:3:1
Expected ration of 2 independent inserts (one locus with 2 inserts) is 1:1:1:1
** 3 degrees of freedom, probability levels are shown in Supplementary Table 3

Copy number analysis was performed in T1 population A14-203*K, where the mother plant A14-203 was determined to contain three copies of the T-DNA. All four T-DNA genes from each offspring plant were detected to have the same copy number. Chi-square (χ^2) tests confirmed the expected ratio of 1:3:3:1 for the number progenies containing zero, one, two and three copies of T-DNA, respectively (Table 5). This shows that mother plant A14-203 had three independent complete inserts of the T-DNA which is in agreement with the 7:1 ratio observed for the segregation ratio of the number of plants with/without T-DNA (Table 4).

Late blight resistance in tubers of T1 plants

Previous data showed that all three genes from transformants containing *Rpi-sto1*, *Rpi-vnt1.1* and *Rpi-blb3* could confer resistance in leaves and tubers. Crossings between cv Katahdin and the three A14-transformants (A14-203, A14-207 and A14-225) containing three, one and one copy of the three *R* genes

(*Rpi-sto1*, *Rpi-vnt1.1* and *Rpi-blb3*) in the T-DNA, resulted in populations with the numbers PBM12-001, PBM12-002 and PBM12-003, respectively (Table 6). Here, we would like to investigate whether resistance in tubers is also inherited in these seedlings. Therefore, tubers were harvested and resistance was tested using isolates EC1 and PIC99189, with H14-xian6 (cv Atlantic transformant containing *Rpi-sto1*, *Rpi-vnt1.1* and *Rpi-blb3*), cvs Desiree and Katahdin as controls, together with the three parental transformants A14-203, A14-207 and A14-225.

Table 6. Late blight resistance in tubers of a selection of *R* gene containing T1 seedlings from three populations containing *Rpi-sto1*, *Rpi-vnt1.1* and *Rpi-blb3*

Clone name	Copy number			DLA		% of *P. infestans* infection in tuber disease test	
	sto1	*vnt1.1*	*blb3*	PIC99189	EC1	PIC99189	EC1
PBM12-001-1	1	1	1	R	R	0	0
PBM12-001-3	1	1	1	R	R	0	3
PBM12-001-6	3	3	3	R	R	0	0
PBM12-001-7	3	3	3	R	R	0	0
PBM12-001-28	3	3	3	R	R	0	0
PBM12-001-42	2	2	2	R	R	0	0
PBM12-002-2	1	1	1	R	R	0	0
PBM12-002-3	1	1	1	R	RQ	0	25
PBM12-003-10	1	1	1	R	R	0	3
PBM12-003-13	1	1	1	R	R	0	0
A14-203	3	3	3	R	R	0	0
A14-207	1	1	1	R	R	0	5
A14-225	1	1	1	R	R	3	15
H14-xian6	3	3	3	R	R	0	0
Desiree	0	0	0	S	S	100	100
Katahdin	0	0	0	S	S	100	100

PBM12-001: A14-203*K; PBM12-002: A14-207*K; PBM12-003: A14-225*K.

As shown in Table 6, all triple *R* gene containing seedlings harbored high level of resistance in tubers to both isolates. Seedlings which harbored two or three copies of the T-DNA showed full resistance in DLA and in tubers. A few seedlings containing one copy of the T-DNA showed less resistance against isolate EC1, which is in line with previous observations on primary transformants harboring a low copy number (Table 3).

Discussion

In the binary vector pBINPLUS (van Engelen et al. 1995), which is commonly used for transformation of plants, the *nptII* gene conferring resistance to kanamycin, is located closely to the LB. When genes are inserted into the plant genome during transformation, the whole T-DNA sequence between the LB and RB is expected to be transferred starting at the *RB* and proceeding to the LB. So selection for the gene *nptII* closest to the LB would ensure also the presence of all three *R* genes which are located downstream. In our experiments, there were 28 out of 128 plants only containing *nptII*, and two plants containing *nptII* and *Rpi-sto1*. Therefore, transformation events can also happen in a way that only *nptII* or *nptII* plus *Rpi-*

sto1 were integrated into the plant genome. This might suggest that DNA transfer sometimes starts from the LB. This is in agreement with the report from (Ramanathan and Veluthambi 1996) in which it was shown that DNA transfer may occur at low frequency from the LB. Another possibility could be the wrong recognition of RB due to the similarity of sequences between RB and part of the T-DNA. Alignment of RB sequence and the T-DNA sequence revealed that between *nptII* and the coding sequence (CDS) from *Rpi-sto1*, 8 bp in the 3' transcriptional terminator region (TTR) of *Rpi-sto1*, were 100% identical to the RB sequence (data not shown).

It is proposed that insertion of T-DNA during the transformation process starts sharply at RB, then proceeds into the T-DNA, and stops rather sloppy around the LB sequence (Petti et al. 2009). This also occurred in our study, the insertion process in 28 out of 128 transformants did not stop at LB, but integrated different lengths of vector backbone sequences close to LB into the cv Desiree genome. Two evidences in the current study support this integration pattern: 1) The PCR amplification of eight backbone sequences showed the possible way of backbone sequence integration. The presence frequency of the backbone gene sequences decreased into the direction of RB. Backbone gene *tetA* was the closest backbone gene to LB with an incidence of 96%, then the frequency followed by *trfA* and *nptIII* (93%) till the gene *tetR* (64%) close to RB; and 2) The investigation with four primer pairs for LB and RB (LB-T-DNA, LB-backbone, RB-T-DNA and RB-backbone), indicated that there were 23 plants harboring LB, whereas only eight plants harbored RB. Additionally, there were four plants containing only the RB-T-DNA, but not the RB-backbone region. These four plants contained all four T-DNA genes, also with the integration of LB (both LB-T-DNA and LB-backbone) and the seven or eight backbone gene sequences. This is in agreement with the study from Petti et al. (2009). Petti also found a higher integration of LB than RB using *Agrobacterium* strain AGL1. The integration of backbone sequences during plant transformation is reported to occur frequently in potato (Wolters et al. 1998), tobacco (De Buck et al. 2000), rice (Afolabi et al. 2004), etc. In our study, 28 out of 128 plants were determined to have backbone sequence integration. The integration of backbone sequence was frequently (24/28) accompanied by the presence of border sequences. It was shown that the plants harboring a higher copy number of *nptII* were often harboring backbone sequences; whereas, the length of backbone sequence integration is not associated with the copy number. Since it was found that plants harboring low copy numbers of *nptII* could have the whole vector backbone integration, but plants with higher copy number of *nptII* also harbored shorter backbone sequences. Besides this, in four plants, which only harbored *nptII* but no *R* genes, the integration of backbone sequences was found close to the LB. Also, in 3% of the plants (4 plants without LB and RB integration, Fig. 1), backbone sequences integrated independently of the T-DNA which is similar to a previous report of 2.5% in potato cv Desiree (Petti et al. 2009). Additionally, it was observed that the backbone gene *tetA*, was present in 27 out of 28 backbone containing plants (96%). Therefore, it can be used as an initial indicator to detect most of the plants harboring backbone sequences. From our study, we found that 22% of the plants were harboring backbone sequences. This is lower than the published results in tobacco (75%) (Kononov et al. 1997) and

in strawberry (66%) (Abdal-Aziz et al. 2006). Differences in species, vector and *Agrobacterium* strain used could be responsible for the observed lower frequency of backbone integration. From the copy number analysis of *nptII* and the integration of *R* genes and backbone, it can be concluded that *R* gene containing but backbone free plants can be obtained with very high frequency (45%) among plants with one or two *nptII* copies. It is, however, important to realize that in some other crops, like rice, it is more difficult to produce sufficient numbers of transformants with single T-DNA inserts (Kelly and Kado 2002). In total, 55% of the Desiree transformants did contain all four genes (*nptII*:*Rpi-sto1*:*Rpi-vnt1.1*:*Rpi-blb3*) and no vector backbone sequences and borders. This high frequency of backbone- and border free transformants is useful for performing advanced field trials as required by EU Directive 2001/18/EC. Field experiments of these triple *R* genes containing transformants showed full resistance in both years 2011 and 2012. The next step comes to the selection of the best performing transformants with other traits which are important from an agronomic point of view. This could be used to select an improved Desiree or a so-called "Desiree-plus" variety which has broad spectrum late blight resistance. However, many steps have to be made to fulfill all requirements for market release of such an improved variety, such as the stability of the foreign trait at different trial places, and whether there are adverse effects on the environment, human and animal health related to the cultivation and even the farmer questionnaires (EFSA 2012).

Analysis of the seedlings after crossing Katahdin (susceptible cultivar) with transformants containing three *R* genes showed that the four T-DNA genes always stayed together as one insert, and they also segregated and inherited as a single Mendelian trait. The disease resistance on the offspring plants showed also the functioning of these genes (Table 4). This observation indicates the stability of this construct in the next generation. It has already been described that transgenes are inherited in a Mendelian fashion, for example, in potato (Visser et al. 1989), in barley (Trifonova et al. 2001), and in *Brassica napus* by using two genes (Liu et al. 2011). In other studies, however, 10-50% of the investigated cases did not follow Mendelian segregation (Yin et al. 2004). In Petunia, simple Mendelian fashion was not always observed (Deroles and Gardner 1988), and in maize, the transgene showed a Mendelian segregation only after three generations (Wang et al. 1999). In wheat, a reduced expression of the *bar* gene, coding for phosphinothricin acetyl transferase, was observed from the first to the third generation (Basri 2005). The following examples may explain the reasons. Fedoroff and colleagues found that the non-Mendelian segregation could be due to the nature of the recipient genome having many active transposable elements (Fedoroff et al. 1989). Cannell et al. (1999) showed that the inserted gene was unstable during the inheritance of wheat transformation. Gahakwa showed that non-Mendelian inheritance in rice could be caused by the phenomenon of gamete lethality (Gahakwa et al. 2000). Furthermore, transgene silencing in tobacco (Kunz et al. 1996) may also contribute to non-Mendelian inheritance. In our study of the three T1 populations, no indications for negative expression effects were found in the primary transformants nor in the sexual offspring plants. One of the possibilities could be the

tetraploid (4x) nature of potato providing diploid (2x) gametes, therefore, no homozygosity for the transgenes in the meiotic products occurs. The inserted chromosome is always accompanied by a non-transformed sister-chromosome potentially compensating or buffering negative insertion effects at gamete and offspring level (Field et al. 2012).

Both years of field trials showed that the selected transgenic plants, containing *R* gene stacks not only showed resistance in DLA (Zhu et al. 2012), but also in the field, which is a very important observation for agricultural deployment. Resistance test in tubers of triple *R* gene transformants showed regularly the stacked resistance spectrum in tubers. Single *R* gene transformants containing *Rpi-vnt1.1* and *Rpi-blb3* showed resistance in tubers. This was also found by Pel (2010), who showed that *Rpi-vnt1.1* and *Rpi-blb3* could confer resistance in tubers from Desiree transformants. Our test also showed that resistance in tubers is related to copy number of the insertions, especially for the resistance gene *Rpi-sto1*. Tubers of transformants containing a higher copy number of *Rpi-sto1* conferred higher resistance levels than the plants with lower copy numbers. Furthermore, Park et al. (2005) reported that *R1* could confer resistance in tubers, but not *R3a* (Huang et al. 2005) and *Rpi-abpt1*, which is a functional homolog of *Rpi-blb3* (Lokossou et al., 2009). We also found that *R3a* could neither confer resistance in tubers from the plant SW8540-025, from which *R3a* was cloned, nor in tubers from Desiree *R3a* transformants. Also, we observed that Desiree transformants containing *Rpi-blb1*, a functional homolog of *Rpi-sto1*, could not always confer resistance in tubers (unpublished data). In the current study, we found that *Rpi-sto1* confers resistance in tubers in only one out of six events in a Desiree background. Interestingly, research from Bradeen (2011) showed that Russet Burbank transformants containing *Rpi-blb1* showed resistance in tubers. It remains elusive whether cv Russet Burbank provides a better genetic background for *Rpi-blb1* than cv Desiree or if other factors such as copy number or insertion site play a role. Another example supporting this genetic background dependence for *R* gene activity in tubers, comes from potato cultivars Bionica and Toluca, which both harbor *Rpi-blb2*. However, Bionica showed higher resistance level in tubers than Toluca (Richard Mooijweer, Agrico, pers. communication). We also found that *Rpi-blb2* (Van der Vossen et al 2005) could confer resistance in tubers from transgenic Desiree plants (unpublished data). Altogether, these results indicated that the same gene can show differences in resistance in tubers with various genetic backgrounds. In order to investigate whether a certain *R* gene can confer resistance in tubers, it must be tested in different genetic backgrounds, which can be achieved by transformation or by crossing. However, when the activity of individual *R* genes in stacks needs to be tested, differential isolates are required to distinguish *R* gene activities. Otherwise, response to specific *Avr* effectors would be an efficient solution to detect the functionality of each *R* gene in tubers (Pel 2010), as it was performed in the leaves (Zhu et al 2012).

In conclusion, this study demonstrates that 48% of the potato transformants harbored low numbers of inserts (1-3) of the complete T-DNA with all four genes but without backbone and borders integration. The presence of backbone sequences can be initially monitored by using *tetA* specific PCR.

Furthermore, the four genes in the T-DNA stayed clustered and inherited as one single Mendelian trait after crossing. The T-DNA genes stayed functional in the next generation. Triple *R* gene containing transformants showed as expected full resistance in the field in two years, and resistance in tubers was shown to occur both in parental transformants and their T1 seedlings. Thus selected primary transformants can be used directly as an improved potato variety, and they can also be used as breeding parents for a clustered transfer of three *R* genes into all kinds of offspring in order to breed new potato varieties which are resistant against potato late blight.

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Supplementary Table 1. List of primers

Label of primer pairs	Sequence (5'-3')	Annealing temperature (°C)	Length of product (bp)
nptII	TCGGCTATGACTGGGCACAAC AAGAAGGCGATAGAAGGCGAT	55.5	722
Rpi-sto1	ACCAAGGCCACAAGATTCTC CCTGCGGTTCGGTTAATACA	65	890
Rpi-vnt1.1	CCTTCCTCATCCTCACATTTAG CTCATCTAATAGATCCTCCAC	60	302
Rpi-blb3	AGCTTTTTGAGTGTGTAATTGG GTAACTACGGACTCGAGGG	63.5	305
RB 27/28	GATATATTGGCGGGTAAACCT CATGCACATACAAATGGACG	62.8	105
RB 29/30	CCTGGCGTTACCCAACTTAAT ACCCGCCAATATATCCTGTCA	60	298
traJ	ACGAAGAGCGATTGAGGAAA CAAGCTCGTCCTGCTTCTCT	62.5	260
oriV	ATAAGTGCCCTGCGGTATTG GCAGCCCTGGTTAAAAACAA	60.8	246
insB	GCGCTATCTCTGCTCTCACT AACGGCCTCACCCCAAAAA	62.7	1872
nptIII	GAAAGCTGCCTGTTCCAAAG GAAAGAGCCTGATGCACTCC	60.8	162
trfA	CGTCAACAAGGACGTGAAGA CCTGGCAAAGCTCGTAGAAC	61.5	146
tetA	CTGCTAGGTAGCCCGATACG CCGAGAACATTGGTTCCTGT	61.4	296
tetR	GGGGGAGGGGATGTTGTCTA AGGGGTATGTTGGGTTTCAC	60	843
oriv3+5	TGCGGCGAGCGGTATCAG CTTCTTGATGGAGCGCATGGG	63	1045
LB 31/32	CAACGCTCTGTCATCGTTA TTACACCACAATATATCCTGCC	64	248
LB 33/34	GCAACGCTCTGTCATCGTTA ATTTCGGAACCACCATCAAA	60	442
nptII	CGTTGGCTACCCGTGATATTGC GTCCCGCTCAGAAGAACTCGT	60	qRT-PCR
Rpi-sto1	GCTTGATCAGTTGTGGACATC TTCAATTGTGTTGCGCACTAG	60	
Rpi-vnt1.1	ATGAATTATTGTGTTTACAAGA CAGCCATCTCCTTTAATTTTTC	60	
Rpi-blb3	TGTCGCTGAAAGAGTAGACC CACCTTTTGCCATTGGTTTAG	60	
EF-1 alfa	ATTGGAAACGGATATGCTCCA TCCTTACCTGAACGCCTGTCA	60	

Supplementary Table 2. Molecular analysis of triple *R* gene transformants

Clone	Copy no.	T-DNA PCR				LB		backbone PCR								RB	
	nptII	*blb3*	*vnt1.1*	*sto1*	*nptII*	LB-T-DNA	LB-backbone	*tetA*	*trfA*	*nptIII*	*insB*	*oriV*	*traJ*	*oriv3+5*	*tetR*	RB-backbone	RB-T-DNA
A14-1	1	-	-	-	+	+	+	+	+	+	-	-	-	-	-	-	-
A14-2	1	-	-	-	+	-	-	-	-	-	-	-	-	-	-	-	-
A14-3	1	-	-	-	+	-	-	-	-	-	-	-	-	-	-	-	-
A14-4	1	-	-	-	+	-	-	-	-	-	-	-	-	-	-	-	-
A14-5	1	-	-	-	+	-	-	-	-	-	-	-	-	-	-	-	-
A14-10	1	+	+	+	+	-	-	-	-	-	-	-	-	-	-	-	-
A14-11	1	+	+	+	+	-	-	-	-	-	-	-	-	-	-	-	-
A14-12	2	+	+	+	+	-	-	-	-	-	-	-	-	-	-	-	+
A14-14	2	+	+	+	+	-	-	-	-	-	-	-	-	-	-	-	-
A14-15	5	+	+	+	+	-	-	-	-	-	-	-	-	-	-	-	-
A14-16	2	+	+	+	+	-	-	-	-	-	-	-	-	-	-	-	-
A14-17	3	+	+	+	+	-	-	-	-	-	-	-	-	-	-	-	-
A14-18	3	+	+	+	+	-	-	-	-	-	-	-	-	-	-	-	-
A14-22	4	+	+	+	+	-	-	-	-	-	-	-	-	-	-	-	-
A14-23	5	+	+	+	+	-	-	-	-	-	-	-	-	-	-	-	-
A14-25	639	+	+	+	+	-	-	-	-	-	-	-	-	-	-	-	-
A14-28	1	-	-	-	+	-	-	-	-	-	-	-	-	-	-	-	-
A14-29	1	-	-	-	+	-	-	-	-	-	-	-	-	-	-	-	-
A14-30	4	-	-	-	+	-	-	-	-	-	-	-	-	-	-	-	-
A14-31	3	+	+	+	+	-	-	-	-	-	-	-	-	-	-	-	-
A14-32	1	+	+	+	+	-	-	-	-	-	-	-	-	-	-	-	-
A14-33	3	+	+	+	+	-	-	-	-	-	-	-	-	-	-	-	-
A14-34	2	+	+	+	+	-	-	-	-	-	-	-	-	-	-	-	-
A14-36	1	-	-	-	+	-	-	-	-	-	-	-	-	-	-	-	-
A14-38	1	-	-	-	+	-	-	-	-	-	-	-	-	-	-	-	-
A14-39	2	-	-	-	+	-	-	-	-	-	-	-	-	-	-	-	-
A14-40	2	-	-	-	+	-	-	-	-	-	-	-	-	-	-	-	-
A14-41	1	+	+	+	+	-	-	-	-	-	-	-	-	-	-	-	-
A14-42	3	+	+	+	+	-	-	-	-	-	-	-	-	-	-	-	-
A14-43	4	+	+	+	+	-	-	+	+	+	+	+	+	+	+	-	-
A14-44	2	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
A14-45	3	+	+	+	+	-	-	+	+	+	+	-	-	-	-	-	-
A14-46	5	+	+	+	+	-	-	-	-	-	-	-	-	-	-	-	-
A14-47	2	+	+	+	+	-	-	-	-	-	-	-	-	-	-	-	-
A14-48	2	+	+	+	+	+	+	+	+	+	-	-	-	-	-	-	-
A14-49	6	+	+	+	+	-	-	-	-	-	-	-	-	-	-	-	-
A14-56	2	-	-	-	+	-	-	-	-	-	-	-	-	-	-	-	-
A14-57	2	-	-	-	+	-	-	-	-	-	-	-	-	-	-	-	-
A14-58	8	-	-	-	+	+	+	+	+	+	+	+	-	-	-	-	-
A14-59	2	-	-	-	+	-	-	-	-	-	-	-	-	-	-	-	-
A14-60	1	-	-	-	+	-	-	-	-	-	-	-	-	-	-	-	-
A14-65	2	+	+	+	+	+	+	+	-	-	-	-	-	-	-	-	-
A14-66	2	+	+	+	+	-	-	-	-	-	-	-	-	-	-	-	-
A14-67	2	+	+	+	+	-	-	-	-	-	-	-	-	-	-	-	-
A14-68	2	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
A14-69	3	-	-	+	+	-	-	-	-	-	-	-	-	-	-	-	-
A14-70	3	+	+	+	+	-	-	-	-	-	-	-	-	-	-	-	-
A14-71	2	+	+	+	+	-	-	-	-	-	-	-	-	-	-	-	-
A14-77	3	+	+	+	+	-	-	-	-	-	-	-	-	-	-	-	-
A14-78	1	+	+	+	+	-	-	-	-	-	-	-	-	-	-	-	-
A14-81	1	+	+	+	+	-	-	-	-	-	-	-	-	-	-	-	-
A14-82	1	+	+	+	+	-	-	-	-	-	-	-	-	-	-	-	-
A14-83	1	+	+	+	+	-	-	-	-	-	-	-	-	-	-	-	-
A14-84	1	+	+	+	+	-	-	-	-	-	-	-	-	-	-	-	-
A14-88	3	+	+	+	+	-	-	-	-	-	-	-	-	-	-	-	-
A14-89	2	+	+	+	+	-	-	-	-	-	-	-	-	-	-	-	-
A14-90	5	+	+	+	+	-	-	-	-	-	-	-	-	-	-	-	-
A14-91	8	-	-	-	+	+	+	+	+	+	-	-	-	-	-	-	-
A14-92	5	+	+	+	+	-	-	-	-	-	-	-	-	-	-	-	-
A14-93	2	+	+	+	+	-	-	-	-	-	-	-	-	-	-	-	-
A14-94	3	+	+	+	+	-	-	-	-	-	-	-	-	-	-	-	-
A14-95	4	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
A14-96	1	+	+	+	+	-	-	-	-	-	-	-	-	-	-	-	-
A14-97	1	+	+	+	+	-	-	-	-	-	-	-	-	-	-	-	-
A14-98	6	+	+	+	+	-	-	-	-	-	-	-	-	-	-	-	-
A14-99	2	+	+	+	+	-	-	-	-	-	-	-	-	-	-	-	-
A14-100	2	+	+	+	+	-	-	-	-	-	-	-	-	-	-	-	-
A14-101	1	+	+	+	+	-	-	-	-	-	-	-	-	-	-	-	-
A14-102	3	+	+	+	+	-	-	-	-	-	-	-	-	-	-	-	-
A14-105	6	-	-	-	+	-	-	-	-	-	-	-	-	-	-	-	-
A14-106	5	-	-	-	+	-	-	-	-	-	-	-	-	-	-	-	-
A14-107	3	-	-	-	+	-	-	-	-	-	-	-	-	-	-	-	-
A14-108	3	-	-	-	+	-	-	-	-	-	-	-	-	-	-	-	-
A14-109	3	-	-	-	+	+	+	+	+	+	-	-	-	-	-	-	-
A14-150	3	+	+	+	+	-	-	-	-	-	-	-	-	-	-	-	-

Supplementary Table 2. Molecular analysis of triple *R* gene transformants (continued)

A14-151	1	+	+	+	+	-	-	-	-	-	-	-	-	-	-	-	-
A14-152	1	+	+	+	+	-	-	-	-	-	-	-	-	-	-	-	-
A14-153	8	+	+	+	+	+	+	-	-	-	-	-	-	-	-	-	-
A14-154	3	+	+	+	+	-	-	-	-	-	-	-	-	-	-	-	-
A14-155	7	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
A14-156	2	+	+	+	+	-	-	-	-	-	-	-	-	-	-	-	-
A14-157	4	+	+	+	+	+	+	+	+	+	+	+	+	+	-	-	-
A14-158	1	+	+	+	+	-	-	-	-	-	-	-	-	-	-	-	-
A14-159	2	-	-	+	+	-	-	-	-	-	-	-	-	-	-	-	-
A14-160	1	+	+	+	+	-	-	-	-	-	-	-	-	-	-	-	-
A14-161	3	+	+	+	+	-	-	-	-	-	-	-	-	-	-	-	-
A14-162	3	+	+	+	+	+	-	-	-	-	-	-	-	-	-	-	-
A14-163	4	+	+	+	+	-	-	-	-	-	-	-	-	-	-	-	-
A14-164	3	+	+	+	+	-	-	-	-	-	-	-	-	-	-	-	-
A14-165	5	+	+	+	+	-	-	-	-	-	-	-	-	-	-	-	-
A14-166	3	+	+	+	+	-	-	-	-	-	-	-	-	-	-	-	-
A14-167	2	+	+	+	+	-	-	-	-	-	-	-	-	-	-	-	-
A14-168	8	+	+	+	+	+	+	+	+	+	+	+	+	+	+	-	+
A14-169	6	+	+	+	+	+	+	+	+	+	+	+	+	+	+	-	+
A14-170	7	+	+	+	+	+	+	+	+	+	+	+	+	+	+	-	-
A14-171	1	+	+	+	+	-	-	-	-	-	-	-	-	-	-	-	-
A14-172	3	+	+	+	+	+	+	+	+	+	-	-	-	-	-	-	-
A14-173	1	+	+	+	+	-	-	-	-	-	-	-	-	-	-	-	-
A14-174	3	+	+	+	+	-	-	-	-	-	-	-	-	-	-	-	-
A14-175	3	+	+	+	+	-	+	+	+	+	+	+	+	+	+	+	-
A14-201	1	+	+	+	+	-	-	-	-	-	-	-	-	-	-	-	-
A14-202	2	+	+	+	+	+	+	+	+	+	+	+	+	+	-	-	+
A14-203	3	+	+	+	+	-	-	-	-	-	-	-	-	-	-	-	-
A14-204	3	+	+	+	+	-	-	-	-	-	-	-	-	-	-	-	-
A14-205	1	-	-	-	+	-	-	-	-	-	-	-	-	-	-	-	-
A14-206	1	-	-	-	+	-	-	-	-	-	-	-	-	-	-	-	-
A14-207	2	+	+	+	+	-	-	-	-	-	-	-	-	-	-	-	-
A14-208	5	+	+	+	+	-	-	-	-	-	-	-	-	-	-	-	-
A14-209	1	-	-	-	+	-	-	-	-	-	-	-	-	-	-	-	-
A14-210	6	+	+	+	+	-	-	-	-	-	-	-	-	-	-	-	-
A14-211	4	-	-	-	+	-	-	-	-	-	-	-	-	-	-	-	-
A14-212	1	-	-	-	+	-	-	-	-	-	-	-	-	-	-	-	-
A14-213	8	+	+	+	+	+	+	+	+	+	+	+	+	+	+	-	-
A14-214	2	+	+	+	+	-	-	-	-	-	-	-	-	-	-	-	-
A14-215	2	+	+	+	+	-	-	-	-	-	-	-	-	-	-	-	-
A14-216	2	+	+	+	+	-	-	-	-	-	-	-	-	-	-	-	-
A14-217	7	+	+	+	+	+	+	+	+	+	+	+	+	+	+	-	-
A14-218	7	+	+	+	+	+	+	+	+	+	+	+	+	+	+	-	+
A14-219	8	+	+	+	+	+	+	-	-	-	-	-	+	+	+	-	-
A14-220	4	+	+	+	+	+	+	+	+	+	+	+	+	+	+	-	-
A14-221	4	+	+	+	+	-	-	+	+	+	+	+	+	+	+	+	+
A14-222	4	+	+	+	+	-	-	+	+	+	+	+	+	+	+	+	+
A14-223	4	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
A14-224	4	+	+	+	+	-	-	+	+	+	+	+	+	+	+	+	+
A14-225	1	+	+	+	+	-	-	-	-	-	-	-	-	-	-	-	-
A14-226	2	+	+	+	+	-	-	-	-	-	-	-	-	-	-	-	-
A14-227	4	+	+	+	+	-	-	-	-	-	-	-	-	-	-	-	-
A14-228	1	+	+	+	+	-	-	-	-	-	-	-	-	-	-	-	-

Supplementary Table 3. Chi-square (χ^2)value for different degrees of freedom

	Probability level (alpha)					
Df	**0.5**	**0.1**	**0.05**	**0.02**	**0.01**	**0**
1	0.46	2.706	3.84	5.412	6.635	10.8
2	1.39	4.605	5.99	7.824	9.21	13.8
3	2.37	6.251	7.82	9.837	11.35	16.3

Supplementary Table 4. Scoring disease resistance in tubers using mycelium coverage of *P. infestans* isolates

Clone	EC1	PIC99189
	% of *P. infestans* infection in tuber disease test	% of *P. infestans* infection in tuber disease test
A14-201	5	0
A14-202	5	0
A14-203	0	0
A14-204	0	0
A14-205	100	100
A14-206	100	100
A14-207	5	0
A14-208	0	0
A14-209	72	100
A14-210	12	0
A14-211	15	100
A14-212	25	75
A14-213	0	0
A14-214	15	0
A14-215	0	0
A14-216	3	0
A14-217	0	0
A14-218	0	0
A14-219	0	0
A14-220	0	0
A14-221	0	0
A14-222	0	0
A14-223	0	0
A14-224	0	0
A14-225	15	3
A14-226	12	0
A14-227	0	0
A14-228	0	0
A03-142	5	50
A03-515	0	75
A03-516	25	50
A03-517	0	40
A03-520	0	85
A03-521	0	100
A09-6	3	100
A09-252	5	85
A09-257	5	75
A09-267	15	75
A09-268	5	100
A09-277	30	100
A13-SF3	25	0
A13-8	40	0
A13-11	15	0
A13-13	5	0
A13-14	15	0
A13-21	15	100
Desiree	5	100

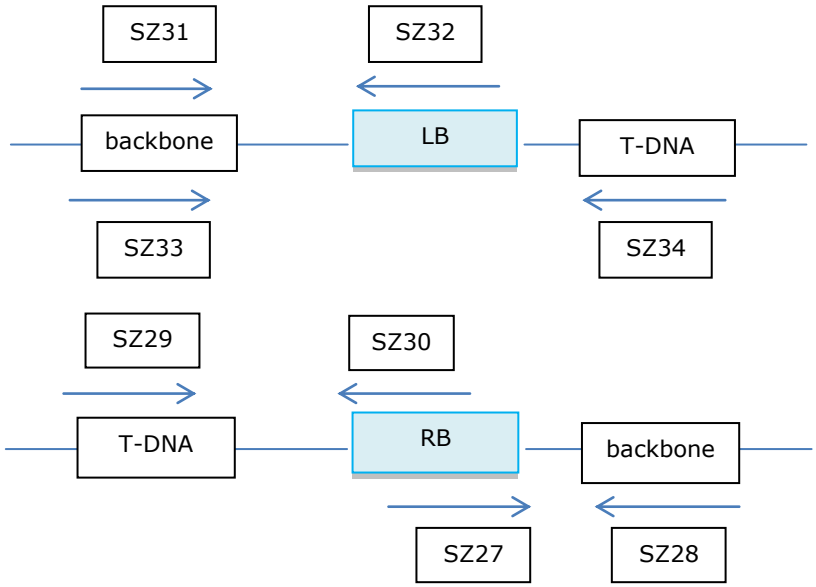
Supplementary Table 5. Foliar resistance under field conditions for A14 transformants and their controls after inoculation with *P. infestans* isolate IPO-C in 2012

days post inoculation	2dpi		7dpi		14dpi		21dpi		average			
replication	1st*	2nd**	1st	2nd	1st	2nd	1st	2nd	2dpi	7dpi	14dpi	21dpi
A14-201***	0	0	0	0	0	0	0	0	0	0	0	0
A14-202	0	0	0	0	0	0	0	0	0	0	0	0
A14-203	0	0	0	0	0	0	0	0	0	0	0	0
A14-204	0	0	0	0	0	0	0	0	0	0	0	0
A14-205	20	10	40	40	100	95	100	100	15	40	97.5	100
A14-206	20	10	40	40	95	95	100	100	15	40	95	100
A14-207	0	0	0	0	0	0	0	0	0	0	0	0
A14-208	0	0	0	0	0	0	0	0	0	0	0	0
A14-209	5	3	40	30	95	95	100	100	4	35	95	100
A14-210	0	0	0	0	0	0	0	0	0	0	0	0
A14-211	5	5	40	40	90	95	100	99	5	40	92.5	99.5
A14-212	5	10	40	40	90	95	100	100	7.5	40	92.5	100
A14-213	0	0	0	0	0	0	0	0	0	0	0	0
A14-214	0	0	0	0	0	0	0	0	0	0	0	0
A14-215	0	0	0	0	0	0	0	0	0	0	0	0
A14-216	0	0	0	0	0	0	0	0	0	0	0	0
A14-217	0	0	0	0	0	0	0	0	0	0	0	0
A14-218	0	0	0	0	0	0	0	0	0	0	0	0
A14-219	0	0	0	0	0	0	0	0	0	0	0	0
A14-220	0	0	0	0	0	0	0	0	0	0	0	0
A14-221	0	0	0	0	0	0	0	0	0	0	0	0
A14-222	0	0	0	0	0	0	0	0	0	0	0	0
A14-223	0	0	0	0	0	0	0	0	0	0	0	0
A14-224	0	0	0	0	0	0	0	0	0	0	0	0
A14-225	0	0	0	0	0	0	0	0	0	0	0	0
A14-226	0	0	0	0	0	0	0	0	0	0	0	0
A14-227	0	0	0	0	0	0	0	0	0	0	0	0
A14-228	0	0	0	0	0	0	0	0	0	0	0	0
A03-142	5	5	40	40	90	90	99	99	5	40	90	99
A09-6	0	0	0	0	3	10	10	20	0	0	6.5	15
A13-SF3	0	0	0	0	0	0	0	0	0	0	0	0
Desiree	10	10	40	40	95	95	100	100	10	40	95	100

Isolate IPO-C (*Avrsto1*, *Avrvnt1*, *avrblb3*) was used for inoculation in the field. A14 transformants: Desiree transformants obtained through vector pBINPLUS:*Rpi-sto1*:*Rpi-vnt1.1*:*Rpi-blb3*. A03-142, A09-6, A13-SF3 are Desiree transformants containing *Rpi-blb3*, *Rpi-sto1* or *Rpi-vnt1.1*, respectively;

* first replication; ** second replication; *** clone name; Clone A14-205 in the first replication showed 20% of leaves infected.

Supplementary Figure 1.



Supplementary Figure 1. Primer pairs around LB or RB for investigating the presence of the T-DNA borders

CHAPTER 4

Strategies of producing potato cisformants with functional resistance genes against late blight

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Abstract

Cisformants are plants obtained by genetic transformation with only cisgenes and no transgenic selection marker. Cisgenes are natural genes cloned from the crop plant itself or from crossable species which are normally used in conventional breeding. Here, multiple cisgenic *R* genes, coding for resistance against *Phytophthora infestans* in potato, have been inserted by *Agrobacterium* mediated transformation (cisgenesis). Three strategies of cisgenesis have been investigated. 1. introduction of two cisgenic *R* genes with one *Agrobacterium* vector, 2. co-transformation with two separate vectors each with one *R* gene, and 3. co-transformation with two vectors, one containing the *nptII* selection marker and the other one with three *R* genes. All three strategies gave rise to useful cisgenic plants with two or three *R* genes, but there were differences. Strategy 1 gave a relatively high frequency of plants with two *R* genes, however, they were not always sufficiently biological active in leaves, tubers, field and in sexual offspring. Selection of well performing plants was needed. Strategy 2 showed many transformants with one of the two *R* genes and a restricted number of plants with both *R* genes which were sufficiently biological active in leaves and tubers. Another bottle neck was the high frequency of vector backbone integration. But a useful cisgenic plant with low inserted T-DNA copy number could be obtained. Strategy 3 showed the advantage of *nptII* selection, because of the high incidence of biological activity of all three *R* genes also in sexual offspring, where the *nptII* gene could easily be separated from the *R* genes. A disadvantage was the instability of the triple *R* gene construct during the transformation process. In strategies 1 and 3, inheritance of *R* genes was as expected but *R* gene containing offspring plants were not all resistant. For improvement of existing varieties strategy 1 is the most efficient and for breeding parent development strategy 3 gives plants with well performing *R* genes also in sexual offspring.

Introduction

Potato late blight is one of the most serious diseases in potato, which is caused by the oomycete *Phytophthora infestans*. More than 20 major late blight resistance (*R*) genes have been cloned so far (Vleeshouwers et al., 2011). However, resistance conferred by single major *R* genes is found to be quickly broken. Recently, cultivar Sarpo Mira, harboring durable resistance, was shown to contain at least five late blight *R* genes (Rietman et al., 2012). Therefore, *R* gene stacking is expected to bring broad spectrum and durable resistance against potato late blight.

Due to the unfriendliness of chemical spraying to the environment and public, breeding strategies, such as introgression breeding and genetic modification/transformation, are pursued to introduce resistance into crops. Introgression breeding of *R* genes requires interspecific crosses followed by backcrosses with cultivated plants and selection for resistance. A major drawback of this breeding strategy is the occurrence of linkage drag with alleles coding for negative traits which cannot always be removed easily by sexual recombination. This is especially the case when multiple *R* genes are introduced from several wild species (Saka, 2006) and in such a case genetic modification (GM) provides

many advantages. Although genetic modification can also be connected with the integration of unwanted vector backbone sequences, this can be easily avoided by selection using molecular tools (Yang et al., 2009, Nandy and Srivastava, 2012). A drawback of GM in Europe is the low acceptance by the public (Schouten et al., 2006, Jacobsen and Schouten, 2009). Based on the origin of the genes to be introduced, genetic modification can roughly be divided into three groups: transgenesis, intragenesis and cisgenesis. Transgenesis is the genetic modification of plants with transgenes, (partly) originating from non-crossable species or synthetic genes. The bacterial selection marker *nptII*, coding for kanamycin resistance is an example of such a transgene. Plants obtained by transgenesis are called transformants. Intragenesis is genetic modification with hybrid genes consisting of functional parts of different genes from the plant itself or from a crossable species. RNAi constructs, targeting an endogenous gene, whose expression is driven by a promoter from the species itself, belong to this category (Rommens et al., 2007). Cisgenesis is the transfer of cisgenes into cultivated plants via genetic modification. A cisgene is a natural gene from the crop species itself or from a sexually compatible plant that can be used in conventional breeding. Plants transformed with only cisgene(s) are called cisformants (Cisgenesis, 2012). Currently a few cisformants have been made in wheat (Gadaleta et al., 2008), apple (Vanblaere et al., 2011) and barley (Holme et al., 2012).

Due to a major drawback of transgenic approaches, being the use of engineered genes, the cisgenesis concept with natural genes from the plant itself (Schaart, 2004) was employed in our study. In order to investigate the possibility of obtaining cisformants with stacked *R* genes towards enhancing durability of late blight resistance in potato, three strategies were pursued: 1. multiple *R* genes in one selection marker free transformation vector. 2. co-transformation with multiple *R* genes in two different marker free transformation vectors. 3. co-transformation with a vector containing a transgenic selection marker and a vector containing only cisgenic *R* genes. Transgenic plants harvested from this co-transformation were crossed with a wild type potato cultivar to select individual offspring plants with only cisgenes. This is called the "marker gene elimination method" (Holme et al., 2012). The success rate of these three strategies paves a way towards achieving potentially durable resistance by stacking resistance genes in a vegetative propagated crop like potato.

Materials and Methods

Plant materials and Agrobacterium tumefaciens *mediated gene transfer*

The potato cultivar Atlantic was used for the transformation experiments, because it showed high *Agrobacterium tumefaciens* mediated gene transfer efficiency (unpublished data). Moreover, Atlantic was indicated to contain a late blight resistance gene (*R1*) from *Solanum demissum* (Grunwald et al., 2001). Starter plants, regenerated shoots, transformants (regenerated shoots that had taken up any transgenes) and cisformants (gene transfer events that only contained cisgenes) were maintained and propagated *in vitro* on MS30 medium at 18-24 degrees, under 16h light and 8h dark conditions. Atlantic

was transformed using vectors pBINAW2:*Rpi-sto1*:*Rpi-blb3* in *A. tumefaciens* strain AGL-1+VirG; pBINAW2:*Rpi-sto1*:*Rpi-vnt1.1* in *A. tumefaciens* strain AGL0+VirG; pBINAW2:*Rpi-sto1* and pBINAW2:*Rpi-vnt1.1*, both in *A. tumefaciens* strain AGL0+VirG; pBINPLUS(*nptII*) and pBINAW2:*Rpi-sto1*:*Rpi-vnt1.1*:*Rpi-blb3* in *A. tumefaciens* strain AGL-1+VirG, respectively. Names of gene transfer events (H14, H43, H44, H48, H62, H63) were given according to the cultivar construct combination, where H indicated recipient cv Atlantic and the subsequent digit indicates the construct or construct combination used for each transformation experiment. Numbers 14, 43, 44, 48, 62, and 63 indicated construct pBINPLUS:*Rpi-sto1*:*Rpi-vnt1.1*:*Rpi-blb3*, pBINAW2:*Rpi-sto1*:*Rpi-vnt1.1*, pBINAW2:*Rpi-sto1*:*Rpi-blb3*, pBINAW2LB4:*Rpi-sto1* & pBINAW2LB4:*Rpi-vnt1.1*, pBINPLUS(*nptII*) & pBINAW2:*Rpi-sto1*:*Rpi-vnt1.1*:*Rpi-blb3*, pBINAW2:*Rpi-sto1* & pBINAW2:*Rpi-vnt1.1*, respectively, where "*&*" means co-transformation, with *R* genes in two separate vectors. The "cultivar construct" combination is followed by "-" and the gene transfer event number (e.g., H44-1). F1 populations from the crossings between susceptible cv Katahdin and trans- or cisformants H62-54, H62-46, H48-KB06-51 and H43-KB05-4 were coded as PBM12-005, PBM12-006, PBM12-007 and PBM12-008, respectively. *Agrobacterium* mediated gene transfer was performed as described before (Pel et al., 2009), kanamycin was not added in the selection medium used from the first two strategies.

In vitro *late blight test*

For the selection of potential regenerants harboring the cisgenic late blight *R* genes, an *in vitro* late blight resistance assay was adopted (Huang et al., 2005). Regenerants were vegetatively propagated and two weeks later the *in vitro* plants were inoculated by pipetting 5 µl isolate suspension ($5*10^4$ zoospores ml^{-1}) on the adaxial side of each leaf using isolate 90128 (*Avrsto1*, *Avrvnt1*, *Avrblb3*). Inoculum preparation and inoculation were performed under sterile conditions to prevent other microorganisms entering the system. Transgenic plants H14-xian6 containing selection marker *nptII* and the three *R* genes (*Rpi-sto1*, *Rpi-vnt1.1* and *Rpi-blb3*) were used as resistant controls (Fig. 1A) and cv Atlantic as susceptible control (Fig. 1B) were prepared with four replicates in one pot (Fig. 1B). Two replicates (two plants) from each regenerated clone were tested (Fig. 1C). The inoculated *in vitro* plants were incubated under 16 hours light per day, at 15 ºC. Three weeks after inoculation disease symptoms were examined. Based on the results, regenerants were divided into three groups: both replicates from one regenerated clone showed resistance (R-R); one replicate showed resistance, the other showed susceptibility (R-S); or both replicates showed susceptibility (S-S). Regenerants with R-R and R-S phenotype were selected and further screened for the presence of *R* genes by PCR.

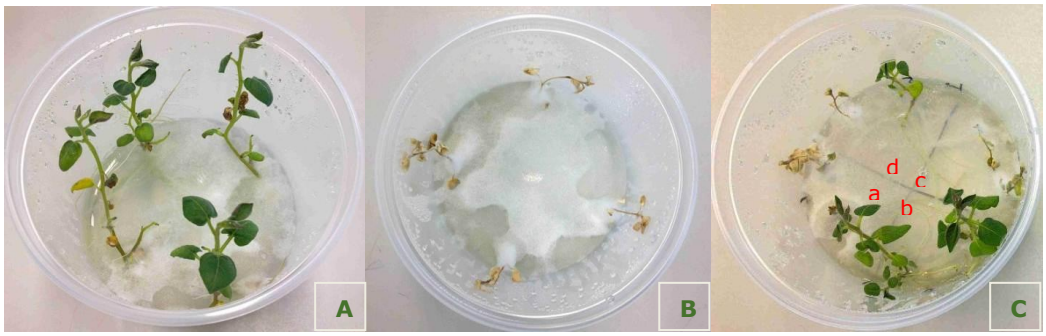

Figure 1. *In vitro* late blight screening at 21 days post inoculation.
In vitro plants were inoculated with late blight isolate 90128 and successively grown for three weeks. A: Resistant control H14-xian6 containing selection marker *nptII* and three *R* genes (*Rpi-sto1*, *Rpi-vnt1.1* and *Rpi-blb3*), B: Non transformed cv Atlantic plants, C: Four Atlantic regenerated clones, each clone with two replicates/plants. Clones a and c showed susceptibility for all two replicates; clone b showed resistance for two replicates; clone d showed resistance for only one out of two replicates.

PCR assays for the detection of T-DNA integration

Specific primer pairs for each gene inside the binary vector (T-DNA and the vector backbone) were used to detect *A. tumefaciens* mediated gene transfer events in regenerated shoots (Supplementary Table 1).

Assessment of T-DNA copy number

T-DNA copy numbers in genomic DNA samples were estimated using quantitative real time PCR (qRT-PCR). The Bio-Rad iCycler iQ machine and the Bio-Rad qRT-PCR kit were used and the reaction was performed in 10 µl volume. Reaction mixtures contained 5 ng of genomic DNA, 5 µl of Sybr green qRT-PCR mix, 3 mM forward primer, 3 mM reverse primer (Table 1). An *EF-1alfa* primer pair was used as an internal calibrator for the amount of genomic DNA. Three events with one T-DNA insertion of each *R* gene were used as standards for Ct values of single copy plants. Negative controls were non-transformed cv Atlantic and MQ water.

Late blight resistance in the foliage under laboratory and field conditions

To estimate the late blight resistance under laboratory conditions, detached leaf assays (DLA) were performed as previously described (Vleeshouwers et al., 1999). Isolates 90128 (*Avrsto1*, *Avrvnt1*, *Avrblb3*), USA618 (*Avrsto1*, *Avrvnt1*, *avrblb3*), PIC99183 (*avrsto1*, *Avrvnt1*, *Avrblb3*), IPO-C (*Avrsto1*, *Avrvnt1*, *avrblb3*), PIC99189 (*avrsto1*, *Avrvnt1*, *avrblb3*) and EC1 (*Avrsto1*, *avrvnt1*, *Avrblb3*) were used in the late blight tests. Two complex leaves, each with three single leaves close to the leaf tip, were tested for each clone. Six days after inoculation late blight resistance was assessed. Leaves were considered fully resistant (R), when 8 out of 8 inoculated spots in a DLA were devoid of symptoms or showed a hypersensitive response. Leaves with intermediate levels of resistance were assigned (RQ) when 6-7 out of 8 inoculated spots showed hypersensitive response; Q, when 3-5 out of 8 inoculated spots showed hypersensitive response; SQ, when 1-2 out of 8 inoculated spots showed hypersensitive response while

the remaining spots showed late blight lesions; and leaves were considered fully susceptible (S) when 8 out of 8 spots inoculated spots showed late blight lesions.

Late blight resistance assays under field conditions were performed essentially as described previously (Kim et al., 2012). Twelve potato tubers per event (Two replications per genotype, each with six plants) were planted in the field, distributed over two plots of six tubers, in the beginning of May. Inoculation with isolate IPO-C (*Avrsto1, Avrvnt1, avrblb3*) was performed in the beginning of July in 2011 and 2012. Plants were examined on day 10, 19, 27 post inoculation (dpi) in 2011, and on day 2, 7, 14 and 21 dpi in 2012. The percentage of the leaf area covered by late blight lesions was estimated by visual inspection.

Agro-infiltration in the foliage

Agro-infiltration with *Avr* genes followed earlier described methods (Vleeshouwers et al., 2008). Six replicates (two plants, three leaves per plant) were agro-infiltrated with *Avrsto1*, *Avrblb3* and *Avrvnt1* in AGL-1+VirG at OD_{600} 0.2, 0.1, 0.05 and 0.0125. HR symptoms were observed five days after infiltration. A transgenic plant H14-xian6 containing the *nptII* gene and three *R* genes (*Rpi-sto1, Rpi-vnt1.1* and *Rpi-blb3*) was used as a positive control, cv Atlantic was used as a negative control. Efficiency of agro-infiltration was tested by co-infiltration of *R3a* and *Avr3a* in *A. tumefaciens* strain AGL-1.

Late blight resistance tests in tuber slices

Tubers were harvested after four months of growth in pots with peat soil in a screen cage or after three months of growth in the clay soil in the field. Field harvested tubers were used in case of transformation with pBINAW2:*Rpi-sto1*:*Rpi-vnt1.1*. Tubers from other experiments were from the screen cage. The sterilizing method of tuber surface was adopted from the wounded tuber assay (Pel, 2010). Two tubers from each genotype were surface sterilised with 5% sodium hypochlorite for 5 minutes and then rinsed three times with tap water. Four slices were cut from each surface sterilized tuber and were placed into a tray. Each slice was then inoculated with 5 droplets of 10 µl of spore suspension ($5x10^4$ spores ml^{-1}) for slices of tubers harvested from the field (5 µl for screen cage harvested tuber slices). Inoculated tubers were kept at 15ºC and 100% humidity in the dark. Scorings were performed seven days after inoculation by estimating the percentage of mycelium coverage on the tuber slice surface. Four slices from two tubers (each two slices from one tuber) were used for the inoculation of one isolate.

Results

Marker free transformation using two *R* genes in one vector

Screening of regenerants by PCR or by in vitro *resistance assays*

Two experiments were performed to transform cv Atlantic with two different *R* genes in one binary vector. In the first experiment, 776 internode stem explants were used for the co-cultivation with *A. tumefaciens* harboring vector pBINAW2:*Rpi-sto1*:*Rpi-blb3*. Totally the experiment yielded 4283 regenerants within nine months. These regenerants were divided into two batches to screen for cisgene integration events. The first batch of 3101 regenerants, harvested in the first six months after co-cultivation, was screened using *in vitro* late blight tests followed by PCR analysis. The second batch of 1182 regenerants harvested from 7-9 months after co-cultivation were directly screened by PCR analysis.

Table 1. Comparison of *in vitro* disease test and direct PCR to screen for *R* gene integration in regenerating shoots after marker free transformation with pBINAW2:*sto1:blb3* or pBINAW2:*sto1:vnt1.1*

Vector	Explants	Harvesting period	Regenerants	*In vitro* disease test		PCR positive #		Gene transfer efficiency (%)[1]
				R-R	R-S	R-R	R-S	
pBINAW2: *sto1*:*blb3* (H44)	776	M1-6 (Batch 1)	3101	83	276	15	2	0.55
		M7-9 (Batch 2)	1182	ND		9		0.76
		M1-9 (Batch 1+2)	4283	ND		26		0.61
pBINAW2: *sto1*:*vnt1.1* (H43)	303	M1-3	315	ND		2		0.64

All regenerants harvested in the first six months after transformation (M1-6) were investigated in duplicate in the *in vitro* late blight test. Regenerants that were susceptible in both duplicates were discarded. When both duplicates showed resistance (R-R), or when one of both duplicates was resistant (R-S), the regenerants were further screened by PCR with *Rpi* gene specific primers. Regenerants obtained from the 7th till 9th month (M7-9) were directly screened by PCR.
[1]percentage of PCR positive regenerants among all regenerants investigated.
ND: not done

Among 3101 regenerants from the first batch, 83 plants were resistant in both duplicates of the *in vitro* disease test. Among these 83 regenerants, 15 (18%) were also positive in the PCR using *Rpi* gene specific primers. Two out of 276 regenerants (0.72%) that were resistant in only one of both duplicates appeared to be PCR positive. There were two PCR positive events containing only *Rpi-blb3*. One of them came from the R-R group, and the other one was from the R-S group. Other 15 PCR positive plants contained both *Rpi-sto1* and *Rpi-blb3*. The strategy to first screen in the *in vitro* disease test resulted in a gene transfer efficiency of 0.55%. When the 1182 regenerants from the second batch were directly screened by PCR, an efficiency of 0.76% was obtained. It can be concluded that among the 26 gene transfer events selected from 4283 regenerants, 24 events contained both *Rpi-sto1* and *Rpi-blb3*, and two events harbored only *Rpi-blb3*, no plants were obtained containing only *Rpi-sto1*.

In a second independent experiment with another construct containing two *R* genes (pBINAW2:*Rpi-sto1*:*Rpi-vnt1.1*), 303 explants were used. Two gene transfer events were obtained after screening 315

regenerants directly by PCR, resulting in an efficiency of 0.64%, which was similar to the efficiency obtained by direct PCR screening in the first experiment.

Selection of cisformants among gene transfer events obtained using two R *genes in one vector.*

The undesired integration of vector backbone sequences was tested using five primer pairs matching the *tetR, oriV, nptIII, trfA* and *tetA* genes. Among the 26 gene transfer events obtained using pBINAW2:*Rpi-sto1*:*Rpi-blb3*, seven contained vector backbone. Consequently, 19 cisformants were obtained out of 26 gene transfer events. Among the two events obtained with pBINAW2:*Rpi-sto1*:*Rpi-vnt1.1*, one plant contained backbone sequences and the other one was considered to be a cisformant.

From the 26 gene transfer events, harvested from construct pBINAW2:*Rpi-sto1*:*Rpi-blb3*, 24 PCR positive plants containing both *R* genes, thirteen, ten and one plants were containing 1, 2 and 3 copies of both *Rpi* genes, respectively. The two plants, which only harbored *Rpi-blb3* possessed one T-DNA copy. For the two plants from construct pBINAW2:*Rpi-sto1*:*Rpi-vnt1.1*, the cisformant H43-KB05-4 harbored two copies of both genes, the other plant was not tested for copy number (Table 2).

Foliar late blight resistance of gene transfer events in the lab and field

The 26 gene transfer events harvested from construct pBINAW2:*Rpi-sto1*:*Rpi-blb3* were tested for late blight resistance using Detached Leaf Assay (DLA) with isolates USA618 (*Avrsto1, avrblb3*) and PIC99183 (*avrsto1, Avrblb3*). Among the 17 (15+2) plants obtained after *in vitro* selection, eleven out of 15 plants, harboring both *R* genes, showed resistance against both isolates, indicating the functionality of both *R* genes. This means that 65% of the preselected resistant plants from the *in vitro* disease test were also resistant at DLA level. However, no single plant could confer resistance to both isolates among the nine plants selected only by PCR analysis (Supplementary Table 2). Among the 24 plants containing both *R* genes (Table 2), 20 plants conferred full resistance to PIC99183 caused by *Rpi-blb3* and eleven plants showed full resistance to USA618, caused by *Rpi-sto1*. These eleven plants, resistant to USA618, were also resistant to PIC99183, showing that both *R* genes are functionally expressed in these plants. Among the two plants only containing *Rpi-blb3*, one plant was resistant to PIC99183 (*avrsto1, Avrblb3*) and the other one was susceptible. Plants containing one T-DNA copy showed more frequently partial resistance or susceptibility in DLA. All the plants with two or three T-DNA copies and five plants with one T-DNA copy showed full resistance in DLA to both USA618 and PIC99183 indicating that both *Rpi-sto1* and *Rpi-blb3* were functional (Table 2).

Among the two gene transfer events harvested from the experiment using vector pBINAW2:*Rpi-sto1*:*Rpi-vnt1.1*, both plants conferred resistance to isolates PIC99189 (*avrsto1, Avrvnt1*) and EC1 (*Avrsto1, avrvnt1*), indicating the functionality of both *Rpi-sto1* and *Rpi-vnt1.1*.

Table 2. Foliar resistance (DLA and field) and resistance in tuber for gene transfer events with pBINAW2:*sto1*:*blb3* or pBINAW2:*sto1*:*vnt1.1* and the control pBINPLUS:*nptII*:*sto1*:*vnt1.1*:*blb3*

Plant :: construct combination	# of clones	# of total plants/ # of plants with vector backbone			# of plants showing resistance in DLA[1]		% of infection in tuber disease test[2] (# of plants)		% of infection in field[3] (# of plants)
		1 copy	2 copies	3 copies	*sto1*	*blb3 or vnt1.1*	*sto1*	*blb3* or *vnt1.1*	Isolate IPO-C
Atlantic:: pBINAW2:*sto1*:*blb3* (H44)	24	13/7	10/0	1/0	11	20	100(7) 87(1) **75(1)** **50(1)**	100(2); 87(2); 75(3); 62(1); **25(1); 0(1)**	80(3) 90(2) **3(1)** **0(1)**
	2**	2/0	0	0	0	1	100(1)	50(1)	ND
Atlantic:: pBINAW2:*sto1*:*vnt1.1* (H43)	2	0	1 (0)	0	2	2	5(1)	0(1)	0 (1)
Atlantic:: *nptII*:*sto1*:*vnt1.1*:*blb3**	1	0	0	1	1	1	0(1)	0(1)	0
Atlantic	1	0	0	0	0	0	100(1)	100(1)	100(1)

ND: not done.
Two transformants from pBINAW2:*sto1*:*vnt1.1*, one plant, without backbone was detected to harbor two copies of *Rpi-sto1* and two copies of *Rpi-vnt1.1*; The copy number of the vector backbone containing plant was not tested.
Foliage late blight resistance was tested using isolates EC1 and PIC99189 for the events deriving from pBINAW2:*sto1*:*vnt1.1* gene transfer, and isolates USA618 and PIC99183 for events deriving from pBINAW2:*sto1*:*blb3* gene transfer.
* (H14-xian6)
** These two plants contained only *Rpi-blb3*.
[1] only plants with full leaf resistance are shown.
[2] Values depicted in bold are the results from clones with 2 and 3 T-DNA copies.
[3] Percentage of foliage area with late blight symptoms. The plants in the field were inoculated with isolate IPO-C (*Avrsto1*, *Avrvnt1*, *avrblb3*).

The foliar resistance of seven H44 gene transfer events from construct pBINAW2:*Rpi-sto1*:*Rpi-blb3* containing both *Rpi-sto1* and *Rpi-blb3* (five plants with one T-DNA copy, one plant with two T-DNA copies, and one plant with three T-DNA copies) was tested in the field. Their resistance levels were compared to a transgenic plant H14-xian6 (harboring the same two *R* genes and an extra *Rpi-vnt1.1* gene), cv Atlantic and to Atlantic regenerants that did not harbor either of both *R* genes. The five events with one T-DNA copy showed similar levels of resistance. The percentage of infected foliage increased gradually till 80%-90% at 21 dpi (Figure 2). However, the disease development of these five gene transfer events was clearly delayed as compared to cv Atlantic or Atlantic regenerants. In the events with two and three T-DNA copies, no infection was observed at 2 and 7 dpi. At 21 dpi, a few leaves (less than 5%) from one event with two T-DNA copies showed late blight lesions, while no infection was observed in the event with three T-DNA copies. Leaves from susceptible control clones (cv Atlantic and Atlantic regenerants) showed 15% and 100% of the leaf area infected at 2 dpi and 21 dpi, and the plants were completely blighted at 21 dpi. The event harboring *Rpi-sto1*:*Rpi-vnt1.1* (H43-KB05-4), which was tested in both 2011 and 2012, showed full resistance at 21 dpi (Fig. 2).

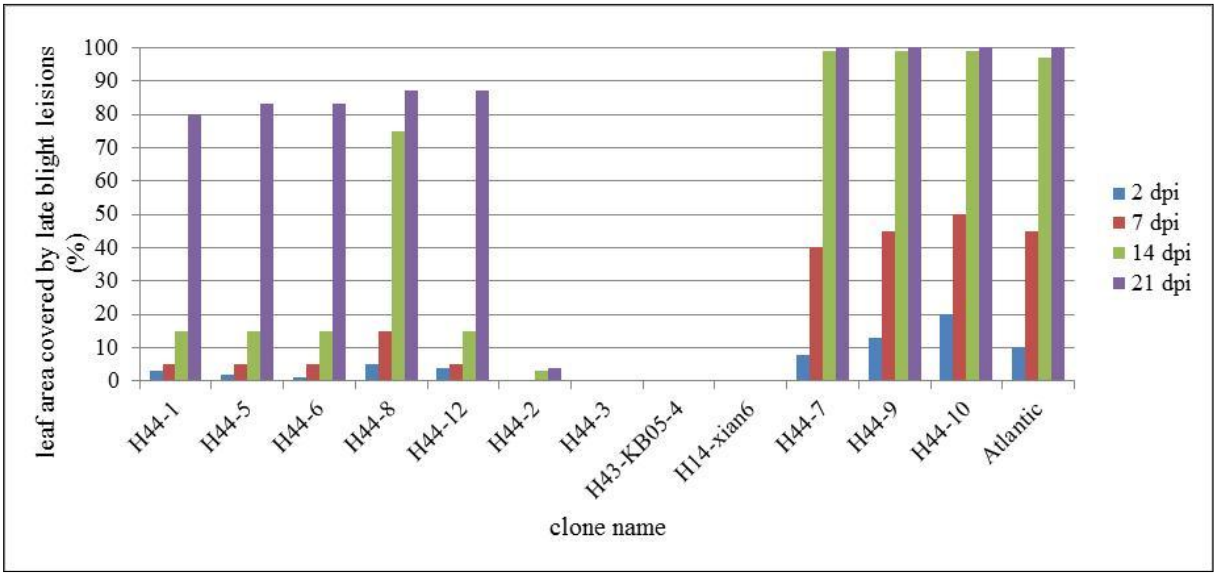

Figure 2. Disease development in the field for gene transfer events harboring two *R* genes.

Two replicates, each with six plants per plot, were tested for each transformation event.
Isolate IPO-C (*Avrsto1*, *Avrvnt1*, *avrblb3*) was used for inoculation in the field. Tested H44 Atlantic transformants in this experiment contained both *Rpi-sto1* and *Rpi-vnt1.1* genes. Plants H44-1,5,6,8,12 contained one T-DNA copy and H44-2 contained two T-DNA copies); H44-3 contained three T-DNA copies); H43-KB05-4 is a cv Atlantic transformant harboring two copies of *Rpi-sto1*:*Rpi-vnt1.1*; H14-xian6 is a cv Atlantic transformant (resistant control, containing *nptII*:*sto1*:*vnt1.1*:*blb3*); H44-7,9,10 were Atlantic regenerants, but not containing any inserted *R* genes.

In conclusion, 19 out 26 gene transfer events from construct pBINAW2:*Rpi-sto1*:*Rpi-blb3* and one out of two gene transfer events obtained from construct pBINAW2:*Rpi-sto1*:*Rpi-vnt1.1* were cisgenic. There were eleven out of 24 plants, harboring both *Rpi-sto1* and *Rpi-blb3*, that showed functionality of both genes in DLA as well as both gene transfer events (H43) harvested from construct pBINAW2:*Rpi-sto1*:*Rpi-vnt1.1*.

Resistance in tuber slices of different cisgenic R *gene transfer events*

Besides the resistance test in leaves, the gene transfer events were also tested for their late blight resistance in tubers. It is known from earlier observations that breeding clones with *Rpi-vnt1.1* provide late blight resistance to the tubers of transgenic plants (Pel, 2010). Here we would like to investigate whether the same can be observed in the marker free stacked gene transfer events. Eleven gene transfer events obtained from vector pBINAW2:*Rpi-sto1*:*Rpi-blb3* (H44) were subjected to the tuber slice assay using isolates USA618 (*Avrsto1*, *avrblb3*) and PIC99183 (*avrsto1*, *Avrvnt1*) (Table 2). A transformant of cv Atlantic H14-xian6 harboring *nptII*:*Rpi-sto1*:*Rpi-vnt1.1*:*Rpi-blb3* and a non-transformed cv Atlantic were used as positive and negative controls, respectively. All eight plants with one copy of *Rpi-sto1*, showed no clear increase of resistance in tuber slice assays against isolate USA618; whereas, plants containing two (H44-2) or three copies (H44-3) of *Rpi-sto1* showed decreased susceptibility to this isolate (Table 2, Fig. 3). The nine plants containing one copy of *Rpi-blb3* (H44-1), showed partial resistance to isolate PIC99183 in the tuber slice assay. The plant (H44-2) harboring two copies of *Rpi-blb3* showed increased resistance, and the plant (H44-3) with three copies of *Rpi-blb3* conferred full

resistance to PIC99183 (Fig. 3). The cisformant H43-KB05-4 harboring *Rpi-sto1*:*Rpi-vnt1.1* was tested for late blight resistance in tubers against isolates EC1 (*Avrsto1*, *avrvnt1*) and PIC99189 (*avrsto1*, *Avrvnt1*). Partial resistance (from *Rpi-sto1*) was observed towards EC1 and full resistance from (*Rpi-vnt1.1*) was observed with PIC99189. This showed that both inserted *R* genes were functional in tubers (Fig. 3).

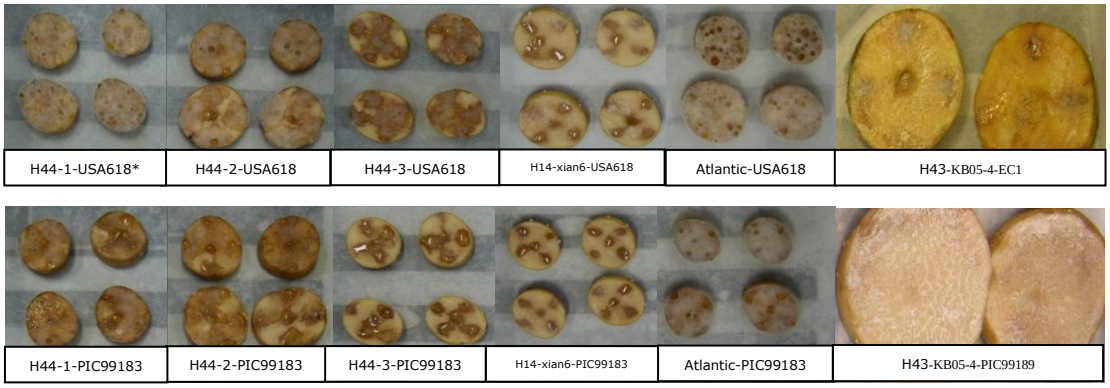

Figure 3. Late blight resistance in tubers from different gene transfer events
Tuber slices were inoculated with isolates USA618 and PIC99183 for events from pBINAW2:*Rpi-sto1*:*Rpi-blb3* (H44), and isolates EC1 and PIC99189 for events from pBINAW2:*sto1*:*vnt1.1*. (H43). Pictures were taken at 7 days after inoculation.
* Gene transfer event H44-1 was inoculated with isolate USA618.

Inheritance of the R *genes in cisformants after crossing with a susceptible cultivar*

In order to investigate the inheritance of *R* genes in cisformants, plant H43-KB05-4, which harbored two copies of *Rpi-sto1*:*Rpi-vnt1.1*, was crossed with susceptible cv Katahdin. As expected, in the resulting population (PBM12-008) it was determined by PCR that *Rpi-sto1* fully co-segregated with *Rpi-vnt1.1* in 33 offspring plants. There were 26 seedlings containing both *Rpi-sto1* and *Rpi-vnt1.1*, and seven seedlings without these two genes. The segregation of the progenies with and without both *R* genes gave a ratio of 3:1 (chi-square test, $P_{0.05}$). This indicated that the two T-DNA copies from transformant H43-KB05-4 had inserted at unlinked positions in the potato genome (Table 3).

Table 3. Inheritance of two *R* genes in the offspring of cisformants with two separate single *R* gene constructs or with one multiple *R* gene construct

		Population PBM12-007	Population PBM12-008	Foliar resistance in the lab (PBM12-007/PBM12-008)			
		sto1&*vnt1.1*	*sto1*:*vnt1.1*	PIC99189		EC1	
				R	S	R	S
R gene containing	*sto1*+*vnt1.1*	27	26	2/10	0	2/8	0/2
	sto1	5	0	0	2/0	0	2/0
	vnt1.1	7	0	0	2/0	0	2/0
	no *R* gene	6	7	0	2/2	0	2/2

Isolate EC1 (*Avrsto1*, *avrvnt1*) and PIC99189 (*avrsto1*, *Avrvnt1*) were used in the resistance tests.
ND: not done;

DLA was performed on twelve PBM12-008 offspring plants (ten plants contained both *Rpi-sto1* and *Rpi-vnt1.1*, two plants did not contain any *R* gene) from construct *Rpi-sto1*:*Rpi-vnt1.1*. All ten offspring plants with both *Rpi-sto1* and *Rpi-vnt1.1*, showed resistance to isolate PIC99189 (*avrsto1, Avrvnt1*), indicating the functional expression of *Rpi-vnt1.1*. Whereas, eight out of ten plants showed resistance to isolate EC1 (*Avrsto1, avrvnt1*), implying the conditional functionality of *Rpi-sto1* in DLA. The two seedlings, which did not contain any *R* genes, showed susceptibility to both isolates.

Co-transformation of two *R* genes in separate vectors

Selection of cisformants harboring two R genes and expressing foliar late blight resistance

Two experiments were conducted in order to investigate the feasibility of co-transformation of *R* genes deriving from two different binary vectors. In these two experiments different vectors were used that contained one or four tandem copies of the vector left border (LB). This stimulated us to address a second question: can LB copy number decrease the integration of vector backbone sequences? The first co-transformation experiment was performed with pBINAW2:*Rpi-sto1* & pBINAW2:*Rpi-vnt1.1* (H63) and the second one with pBINAW2LB4:*Rpi-sto1* & pBINAW2LB4:*Rpi-vnt1.1* (H48), both in *A. tumefaciens* strain AGL0+virG. The vector of the second experiment was constructed by adding three tandem copies of LB.

Table 4. Comparison of vector backbone integration incidence after *R* gene transfer using vectors with one (H63) or four left borders in tandem (LB; H48)

Exp. code	Explant #	Regenerants #	# of *R* gene transfer events (# of cisformants)			# of gene transfer events with full resistance in the lab			backbone incidence (%)
			sto1	*vnt1.1*	*sto1* and *vnt1.1*	*sto1*	*vnt1.1*	*sto1* and *vnt1.1*	
H63	292	334	6 (4)	7 (2)	3 (0)	3 (2$)	4 (2)	1R/R; 1RQ/R; 1S/R*	63
H48	1772	2810	28 (8)	22 (12)	16 (3)	12 (3)	18 (8)	12R/R; 3RQ/R; 1R/RQ (2R/R; 1RQ/R)	58

Isolate EC1 (*Avrsto1*, *avrvnt1*) and PIC99189 (*avrsto1*, *Avrvnt1*) were used in the resistance test.
* 1 plant was susceptible to isolate EC1 (indicating the non-functionality of *Rpi-sto1*) and resistant to isolate PIC99189 (indicating the functionality of *Rpi-vnt1.1*).
$ Two out of four *Rpi-sto1* containing cisformants showed full resistance against EC1 in the lab.

Based on PCR experiments, H63 and H48 showed a gene transfer efficiency (number of gene transfer events/number of regenerants) of 4.8% (16/334) and 2.3% (66/2810), respectively. As expected, the efficiency of cisgenic co-transfer of both genes was lower, being 0.9% (3/334) and 0.6% (16/2810) for experiment H63 and H48, respectively. The difference of gene transfer efficiency between experiments H63 and H48 might be caused by the four copies of tandem LB in H48, compared to the one copy of LB in H63.

Five specific primer pairs derived from vector backbone sequences right border (RB), *oriV3+5*, *nptIII*, *tetA*, and LB were used to screen for the presence or absence of vector backbone integration. The

incidence of vector backbone integration was high in both experiments (63% and 58%) and no obvious reduction could be observed due to the additional LB copies. As expected, in the case of co-transfer the vector backbone integration (81-100%) seemed higher than in single *R* gene transfer events (33-71%). No clear difference in the frequency of late blight resistance was found between backbone containing and backbone free events. The frequency of generating late blight resistant events by insertion of *Rpi-sto1* (44-57%) was lower than the efficiency of *Rpi-vnt1.1* (70-87%; Table 4).

In total, six (4+2) and 23 (8+12+3) cisformants were obtained out of 16 (H63) and 66 (H48) gene transfer events, respectively. Among these 29 cisformants, three had both *R* genes and two of them showed a high level of resistance for both *R* genes. One cisformant, H48-KB06-51, showed the presence of only one copy of both *R* genes. In order to further confirm the activity of the individual *R* genes in the cisformants that had both or either of both *R* genes, agro-infiltration with *Avrsto1* and *Avrvnt1* was performed. Plants which did not show an HR in all six replicates in agro-infiltration were indeed fully susceptible to the late blight isolates EC1 and PIC99189. If a plant containing *Rpi-sto1* showed HR in all replicates, at OD_{600}=0.05 in the agro-infiltration of *Avr-sto1,* this plant also showed full resistance in DLA against isolate EC1 (*Avrsto1, avrvnt1*). If clear HR was observed at OD_{600}=0.2, most *Rpi-sto1* containing plants showed full resistance, while the rest of *Rpi-sto1* containing plants showed partial resistance in DLA. For *Rpi-vnt1.1* containing plants, if a clear HR showed up in all replicates of plants in the agro-infiltration of *Avrvnt1* at OD_{600}=0.2, these plants also showed full resistance in DLA.

Resistance in tuber for cisformants harboring two R *genes*

The three cisformants harboring both *R* genes harvested from the H63 co-transformation experiment were also tested for their resistance in tuber against isolates PIC99189 and EC1 in a tuber slice assay.

Table 5. Tuber slice resistance test in three cisformants harboring both *R* genes after co-transformation

	Copy number		DLA		% of infection in tubers disease test	
	sto1	*vnt1.1*	PIC99189	EC1	PIC99189	EC1
H48-KB06-14	1	3	R	R	0	15
H48-KB06-21	1	3	R	R	0	1
H48-KB06-51	1	1	R	RQ	0	5

Rpi-vnt1.1 provided full resistance in tubers in all three cisformants and *Rpi-sto1* provided a lower level of resistance, with little mycelium growth around the inoculation sites (Table 5).

Inheritance of two R *genes in populations derived from cisformants obtained by co-transformation*

In order to investigate the inheritance of *R* genes from co-cisformants, cisformant H48-KB06-51, harboring one copy of functional *Rpi-sto1* and *Rpi-vnt1.1*, was crossed with susceptible cv Katahdin.

Among the investigated 45 seedlings from the resulting population PBM12-007 (Table 3), 27 had both genes *Rpi-sto1* and *Rpi-vnt1.1*. Five, seven, and six seedlings contained only *Rpi-sto1*, only *Rpi-vnt1.1*, or no *R* genes, respectively. This indicated that *Rpi-sto1* and *Rpi-vnt1.1* were integrated at different but linked loci so that seedlings with single *R* genes could be identified. Eight seedlings (two seedlings harbored only *Rpi-sto1*, two contained only *Rpi-vnt1.1*, two contained *Rpi-sto1* and *Rpi-vnt1.1*, two did not contain either *R* gene) were tested for their foliar late blight resistance in the lab using isolates EC1 (*Avrsto1*, *avrvnt1*) and PIC99189 (*avrsto1*, *Avrvnt1*). Surprisingly, all four single *R* gene (either *Rpi-sto1* or *Rpi-vnt1.1*) containing seedlings were susceptible to both isolates at DLA level. However, the two seedlings which harbored both *Rpi-sto1* and *Rpi-vnt1.1*, showed resistance to both isolates PIC99189 and EC1. Both seedlings, which did not contain any *R* genes, showed susceptibility to isolates PIC99189 and EC1 (Table 3).

Co-transformation with antibiotic resistance and cisgene containing vectors, followed by a sexual step for marker elimination

So far we showed that cisformants harboring stacked cisgenes can be obtained using PCR selection after marker free transformation, but the efficiency of obtaining cisformants with functional stacked genes is relatively low. We were interested to know if the high efficiency of antibiotic aided marker assisted selection could be combined with co-transfer of cisgenes to increase the efficiency. Therefore, we set up an experiment in which two constructs were used, one containing the *nptII* marker gene coding for kanamycin resistance and the other construct harboring three cisgenes (*Rpi-sto1*, *Rpi-vnt1.1* and *Rpi-blb3)* in its T-DNA. To this end cv Atlantic explants were co-cultivated with different mixture ratios of *A. tumefaciens* strain AGL-1+virG harboring pBINPLUS(*nptII*) or pBINAW2:*Rpi-sto1*:*Rpi-vnt1.1*:*Rpi-blb3* (experiment H62). As shown in Table 7, the respective *A. tumefaciens* cultures were mixed with a 1:1 volume in different ratios ranging from 1:1 to 1:3 for *nptII* and cisgenes containing vectors. After co-cultivation, regenerants were selected on kanamycin containing medium. Specific primers for *nptII* and the three *R* genes (*Rpi-sto1*, *Rpi-vnt1.1*, *Rpi-blb3*) were used to screen the regenerants by PCR. Consequently, there were 2, 30, 6, and 24 transformants harvested from mixtures 1-4, respectively (Table 7). As expected all 62 co-transformants contained the *nptII* gene. Remarkably, most of the detected transformants were only harboring *Rpi-sto1* and *nptII*, but not *Rpi-vnt1.1* and *Rpi-blb3*. Only nine transformants had all three expected *R* genes. Eight of them were from the 1:3 mixture and one was from the 0.5:1 mixture (mixture 2). The other two mixtures did not result in any events containing the three *R* genes. No plants with only *nptII*, but no *R* genes were obtained.

Table 6. Co-transformation of cv Atlantic with *nptII* & *Rpi-sto1*:*Rpi-vnt1.1*:*Rpi-blb3* with different mixture ratios of *Agrobacterium*

Mixture combination	Ratios (OD_{600}) *	Explant #	Kana resistant Regenerants #	Co-transfer events #	Only *sto1* (backbone free)	*sto1*:*vnt1.1*:*blb3* (backbone free)	Co-transformation efficiency (%)
1	0.8/0.8	200	2	2	2(0)	0	1
2	0.5/1.0	200	30	30	29(3)	1(0)	15
3	0.8/1.6	200	6	6	6(3)	0	3
4	0.5/1.6	200	24	24	16(4)	8(4)	12

* OD_{600} ratio for *Agrobacterium* containing pBINPLUS(*nptII*) : pBINAW2:*Rpi-sto1*:*Rpi-vnt1.1*:*Rpi-blb3*, respectively.

All co-transfer events were tested by PCR for backbone sequence integration using specific primers of six backbone genes (*tetR, traJ, oriV, nptIII, trfA* and *tetA*). Fourteen out of 62 events were backbone free (Table 6).

Copy number of *R* genes was determined in the plants containing all three *R* genes by qRT-PCR. Copy number of *Rpi-sto1* ranged from 6-33, copy numbers of *Rpi-vnt1.1* and *Rpi-blb3* were between 1-3. In most cases *Rpi-vnt1.1* and *Rpi-blb3* had the same copy number.

Ten events containing *nptII* and *Rpi-sto1* together with eight events containing *nptII, Rpi-sto1, Rpi-vnt1.1* and *Rpi-blb3* were tested for their resistance against late blight isolates PIC99189 (*avrsto1, Avrvnt1, avrblb3*) and EC1 (*Avrsto1, avrvnt1, Avrblb3*). As expected, the ten *nptII* and *Rpi-sto1* containing events were resistant to EC1 only, but not to PIC99189. The eight events harboring all three *R* genes conferred resistance to both isolates.

Selection of transformants from offspring plants by sexual marker gene elimination

Two *nptII* containing vector backbone free events were selected to be crossed with susceptible cv Katahdin. H62-46 harbored four copies of both *nptII* and *Rpi-sto1*, and H62-54 harbored *nptII, Rpi-sto1, Rpi-vnt1.1* and *Rpi-blb3* (copy number was not determined). This resulted in the crossing populations PBM12-006 and PBM12-005, respectively. The *nptII* and *R* genes segregated independently in both populations PBM12-005 and PBM12-006 (Table 7), indicating that cisformants with only cisgenic *R* genes could be selected.

Table 7. Selection for cisgenic seedlings by PCR after crossing H62-54 and H62-46 with cv Katahdin

Population #	*nptII* plus/minus	*Rpi-sto1* presence	*sto1+vnt1.1+blb3* presence	*Rpi* gene absence
PBM12-005 (H62-54)	*nptII* plus	7	26	1
	nptII minus	2	5	3
PBM12-006 (H62-46)	*nptII* plus	36	0	0
	nptII minus	5	0	3

In both populations PBM12-005 and PBM12-006 containing each 44 seedlings respectively, seven and five cisformants were obtained that lacked the *nptII* gene but did contain the *R* gene(s). The copy numbers of the inserted *R* genes was determined in five cisformants containing all three *R* genes from population PBM12-005 and in two cisformants containing only *Rpi-sto1* from population PBM12-006. Four out of five plants from population PBM12-005 had a higher number of *Rpi-sto1* than *Rpi-vnt1.1* and *Rpi-blb3* (present in single copy) (Table 8). The two cisformants containing only *Rpi-sto1* from population PBM12-006 harbored one and three copies of *Rpi-sto1*.

Table 8. Late blight resistance in leaves and tubers of cisgenic seedlings after crossing H62-54 and H62-46 with cv Katahdin

Plant ID	Copy number			Resistance in leaves			% of infection in tubers disease test	
	sto1	*vnt1.1*	*blb3*	IPO-C	EC1	USA618	PIC99189	EC1
PBM12-005-1	3	1	1	R	R	ND	0	0
PBM12-005-2	4	4	1	R	R	ND	0	75
PBM12-005-3	3	1	1	R	R	ND	5	0
PBM12-005-36	4	1	1	ND	ND	ND	0	0
PBM12-005-44	3	1	1	ND	ND	ND	25	5
PBM12-006-18	1	0	0	ND	ND	R	100	1
PBM12-006-19	3	0	0	ND	ND	R	100	0
H62-46	4	0	0	ND	R	ND	100	0
Desiree	0	0	0	S	S	S	100	100

ND: not done.
Isolate PIC99189 was also tested on H62-46, showed susceptibility.

Initially three and two harvested cisformants from populations PBM12-005 and PBM12-006, respectively, were inoculated with late blight isolates for their functional expression of the *R* gene(s) within these plants (Fig. 4). All tested PBM12-005 cisformants, which contained all three *R* genes, showed resistance to both isolates EC1 (*Avrsto1*, *avrvnt1*, *Avrblb3*) and IPO-C (*Avrsto1*, *Avrvnt1*, *avrblb3*). Both cisformants from PBM12-006, which contained *Rpi-sto1*, showed resistance to isolate USA618 (*Avr-sto1*).

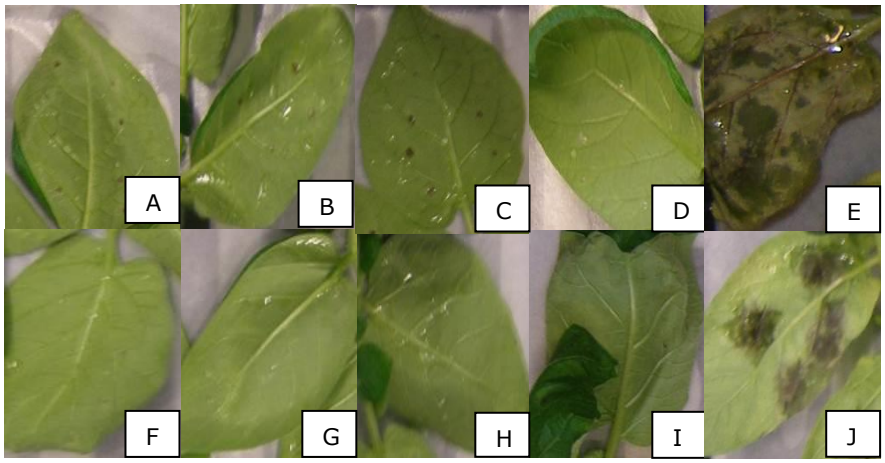

Figure 4. Foliar late blight resistance of cisgenic seedlings
A and F: PBM12-005-3; B and G: PBM12-005-2; C and H: PBM12-005-1; D: PBM12-006-19; E: Katahdin; I: PBM12-006-18; J: Atlantic. A, B, C, E and J were inoculated with isolate IPO-C; D and I were inoculated with isolate USA618; F, G and H were inoculated with isolate EC1.

The cisgenic seedlings, which were tested for late blight resistance in leaves, were also examined for resistance in tubers. The mother plant H62-46, harboring four copies of *Rpi-sto1*, showed full resistance to EC1, but susceptibility to PIC99189. Interestingly, cisformants harboring multiple copies of *Rpi-sto1* from population PBM12-006 showed high levels of resistance against isolate EC1 (*Avrsto1*, *avrvnt1*, *Avrblb3*). The five cisgenic seedlings from PBM12-005, harboring all three *R* genes, were tested for their resistance to isolates PIC99189 (*avrsto1*, *Avrvnt1*, *avrblb3*) and EC1 (*Avrsto1*, *avrvnt1*, *Avrblb3*) and these plants showed frequently high levels of resistance against both isolates (Table 8). Due to the complexity of the resistance and the isolates, it could not be indicated which *R* genes were responsible for this resistance.

As an alternative to test functional *R* gene expression, agro-infiltrations were performed in all five cisformants from population PBM12-005 (containing *Rpi-sto1*, *Rpi-vnt1.1* and *Rpi-blb3*), and two cisformants from PBM12-006 (containing *Rpi-sto1*) using the matching *Avr* genes. Three out of five seedlings from population PBM12-005 which contained *Rpi-sto1*, *Rpi-vnt1.1* and *Rpi-blb3*, showed cell death in response to all three *Avr*s (Fig. 5B), one seedling showed cell death with *Avrsto1* and *Avrvnt1* and the other one showed cell death with only *Avrsto1*. Both cisgenic seedlings from population PBM12-006 (containing *Rpi-sto1*) showed cell death with *Avrsto1*. This indicated that *R* genes do not always function at high levels in some cisgenic seedlings.

A B C

Figure 5. Agro-infiltration in seedling cisformants with *Avrsto1*, *Avrvnt1* and *Avrblb3*.
Pictures were taken at three days after infiltration. A: PBM12-006-18 (*Rpi-sto1*); B: PBM12-005-44 (*Rpi-sto1*, *Rpi-vnt1.1* and *Rpi-blb3*). C: Inoculation scheme.

Discussion

For the gene transfer experiment with two *R* genes in one vector an *in vitro* disease test was used to select gene transfer events. There were 83 (2.7% of total regenerants) plants showing resistance against isolate 90128 (*Avrsto1*, *Avrblb3*) *in vitro*, and 18% of these 83 regenerants were determined as gene transfer events by PCR. This approach provides a considerable success in comparison with only selecting by PCR reaction. There were 65% of the PCR plus plants, pre-selected from the *in vitro* disease resistance test, showing functional expression of both *R* genes; while none of the plants directly selected by PCR showed functional expression of both *R* genes at DLA level. The isolate used for the *in vitro* inoculation is 90128, which harbored both *Avrsto1* and *Avrblb3*. A mixture of two different isolates, containing *Avr* genes corresponding to each *R* gene can be used in the future to test if the rate of resistance for both *R* genes in DLA is higher than using one isolate. The transformation efficiency observed in the experiment with two *R* genes in one vector was around 0.6%. This is lower than observed in other reports on potato using similar methods (de Vetten et al., 2003). De Vetten et al. reported that cvs Karnico, Mercator, Kardal, Seresta had a transformation efficiency of 4.5%, 0.6%, 1.7% and 2.4%, respectively. The reason why our frequencies were mostly lower might be due to the different cultivars and also could be caused by the size of the T-DNA inserts. Introduction of two relatively large *R* genes (together 16 kb) in this study might be less efficient, compared to the 2.4 kb T-DNA used in the study by de Vetten et al. (2003). Alternatively, the introduction and expression of two *R* genes could cause a fitness penalty that selects against gene transfer events at cellular level.

The *in vitro* late blight resistance selection experiments are comparable with the selection for transgenic *Arabidopsis* after flower dip transformation (Harrison et al., 2006). Selection for gene transfer events is also performed later at seedling level and not directly during the transformation process. Screening for kanamycin resistance among *Arabidopsis* seedlings selects against seedlings which are exhibiting a low level of marker gene expression. From our current study in potato, the PCR selection revealed that only 42% (11/26) of the gene transfer events from the construct pBINAW2:*Rpi-sto1*:*Rpi-blb3* showed functional expression of both *R* genes in the leaves. This percentage is similar to the results of marker free transformation in potato cultivars Karnico (45%) and Seresta (44%) (de Vetten et al., 2003), and 49% in Tobacco (Jia et al., 2007). The reason of this lower frequency might be found in the variable transcriptional activity at the different T-DNA insertion sites in the genome. In *Arabidopsis*, T-DNA integration was found more frequently in the genomic regions suppressing transcription in events screened by PCR than those selected by kanamycin resistance (Francis and Spiker, 2005). These findings indicate that after PCR selection of gene transfer events a functional analysis of the inserted genes has to be a second important step during the selection of cisformants. Apart from a potential role of the T-DNA insertion site, this study revealed that transformants with one inserted T-DNA copy are less frequently providing high levels of resistance. The level of resistance is higher among plants with two or three inserts (Table 2). From the crossing population with primary gene transfer event H48-KB06-51, indications were obtained that *R* gene expression in cisformants with only a single *R* gene (*Rpi-sto1* or *Rpi-vnt1.1*) were generally too low to reach sufficiently high level of resistance, and resistance was achieved with cisformants harboring both *Rpi-sto1* or *Rpi-vnt1.1*. This might be due to the possibility that H48-KB06-51 contained an extra non-functional copy of *R* genes, which was not detected from copy number analysis. Another reason could be due to the different genetic backgrounds conferred from the crossing, which is less favorable for the expression of the *R* gene. Furthermore, this lower frequency of functional expression of *R* genes in cisformants is clearly different from transformants obtained by pre-selection for kanamycin resistance (Zhu et al., 2012). There we observed normal functionality of the introduced triple *R* genes in all transformants which were pre-selected by kanamycin resistance. This indicates that selection for functional *nptII* expression is a sufficient good selection criterion for high level *R* gene expression as well, even if they are on separate vectors and inserted separately.

The earlier described resistance in tubers conferred by *Rpi-vnt1.1* in both breeding clones (Zhu et al., unpublished) and transgenic plants (Pel, 2010) was also observed in cisformants. In this study, the frequency of resistant cisformants containing *Rpi-vnt1.1* was higher than with *Rpi-sto1* and *Rpi-blb3*. It was clear that resistance in tubers was more frequently obtained in cisformants with two or more copies of the individual *R* gene(s). Next step is to test whether the *R* genes involved can provide resistance in tubers under field and storage conditions. Also the activity of the *R* genes in both in leaves and tubers in different genetic backgrounds remains elusive. This is an important issue for both cisgenic and conventional stacking of (cisgenic) late blight resistance genes. Specific studies about *R* gene

functionality in different genetic backgrounds are not known to us and requires attention in future research.

In this paper, we only showed the resistance in tuber slices, which mainly represents the flesh component. We have also tested late blight resistance in intact tubers by inoculating *P. infestans* isolates mixtures at the tuber skin and at natural openings like the stolon and the tuber eyes. One month after inoculation, no disease symptoms were found in the susceptible control cultivars Desiree and Bintje (data not shown). Because of the lack of a late blight assay for entire tubers, we focused on resistance in tuber slices. The criteria of scoring resistance in tubers might also be related to tuber size. Tubers that were harvested in the field were bigger than the tubers from screen cage grown plants, which might influence the scoring of the susceptible tubers. However, we included resistant and susceptible controls in each experiment which reacted as predicted.

Field experiments with cisgenic plants were made with late blight isolate IPO-C, using the combinations *Rpi-sto1*:*Rpi-blb3* or *Rpi-sto1*:*Rpi-vnt1.1*. In the first combination it was observed that plants with one copy of both genes showed a lower level of resistance than plants with two or three copies of this *R* gene combination. It must be realized that *Rpi-blb3* does not contribute to resistance against IPO-C. This result is in line with our previous findings that full resistance provided by *Rpi-sto1* is found only at low frequency among cisformants in DLA. Now we have extended this notion to field conditions. This situation is different from *Rpi-sto1* in transgenic plants obtained by transformation with the selectable kanamycin resistance marker *nptII*. Among these transformants quantitative resistance in DLA was frequently found, while these plants were mostly fully resistant in the field (Ronald Hutten, Wageningen UR Plant Breeding, personal communication). From the second combination, *Rpi-sto1*:*Rpi-vnt1.1*, the cisformant with two T-DNA inserts showed full resistance till the last scoring, which is probably due to the contribution from *Rpi-vnt1.1*.

R genes introduced into cisformants using one construct appeared, as expected, to be inherited stably and clustered at the molecular level as demonstrated by PCR analysis. This is in accordance with the earlier published inheritance of introduction of three clustered *R* genes using a triple *R* gene construct in a transgenic study (Zhu et al., 2013). However, the resistance of *R* genes from cisformants (eg. H48-KB06-51) do not always provide full resistance after sexual crossing. This might be related to the fact that the altered genetic backgrounds are not always favoring the functioning of the cisgenes. This observation has consequences for using cisformants as breeding parents. Selection of cisgenes at the PCR level will not always be sufficient to ensure the functionality of the *R* gene(s) involved. Therefore, the necessity for additional tests to show functional expression (DLA, effector response, field trials) is higher in cisformants than in transformants.

The co-transfer experiments with *Rpi-sto1* and *Rpi-vnt1.1* in separate vectors resulted in the cisformant H48-KB06-51 containing one copy of both *R* genes. However, the high number of events with

only one *R* gene and the high frequency of vector backbone integration in events with both *R* genes were remarkable and drastically reduced the efficiency of getting stacked cisformants using this strategy. Plant H48-KB06-51 was also tested for the inheritance of both genes in the offspring. It turned out that 60% of offspring plants contained both *R* genes indicating linkage of both inserts.

A solution for stacking of three, four or more *R* genes for market release would be re-transformation of a cisformant that already contains one or more *R* genes. Depending on downstream applications it may be necessary to assess the cisformant, to be re-transformed, first to be "true to type" to the original variety. This means that such a host for re-transformation needs to be tested not only for functional expression of both *R* genes but also for the desired agricultural and tuber quality traits.

The transformation strategy using co-transfer followed by marker gene elimination can be performed in three ways: 1. Marker gene and gene of interest have their independent vector borders constructed on different transformation vectors in different *Agrobacterium* strains; 2. Marker gene and gene of interest are constructed in different vectors in the same *Agrobacterium* strain; 3. Marker gene and gene of interest have their independent borders (Holme et al., 2012), and are constructed in two T-DNAs in one transformation vector in one *Agrobacterium* strain (Upadhyaya et al., 2010). The second of these strategies to produce cisformants was tested in this study, using a vector with a marker gene in addition to the vector with three cisgenes. Successively, the marker gene could be eliminated after a crossing step. Using this approach, the cisformants with a stack of three different *R* genes were successfully identified. It was, however, remarkable that all of the events had higher copy numbers of *Rpi-sto1* as compared to the other two *R* genes. It might be due to the possibility that partial T-DNAs harboring only *Rpi-sto1* gene had integrated into the genome of cv Atlantic, because events were frequently found which only harbored *Rpi-sto1* and *nptII*. These observations indicated the instability of the vector harboring the *R* genes during or after different steps involved in the gene transfer. By using a 1:3 mixture for selection marker containing vector and the vector with the three *R* genes respectively, it was easy to obtain sufficient numbers (14%) of cisformant seedlings (plants did not contain *nptII* but only the *R* genes) via backcrossing (Table 8). In all mixtures used, there was no primary transformant observed that contained only *nptII* and no cisgenes, this could be partly due to the high dose of cisgenes during the co-transformation, and there were only limited numbers of transformants harvested from OD ratio 1:1. This result is different with what we obtained from the second strategy with co-transformation using two vectors each harboring one *R* gene, where many gene transfer events harbored single *R* gene. This could be due to the fact that the same OD of *Agrobacterium* was used with 1:1 volume of each culture during the co-transformation. A co-transformation on grape showed also this phenomenon that higher ratio of vector containing gene of interest and the marker gene containing vector resulted in more plants containing the gene of interest (Dutt et al., 2008). A remarkable observation was that *R* gene(s) functioned well at DLA level in all tested *Rpi-sto1* and triple *R* gene containing transformants, which were kanamycin resistant. In this case it was again observed that selection for kanamycin resistance was

sufficient to drive *R* gene functioning simultaneously even with separate insertions in the genome. After marker gene elimination in seedling cisformants, it was observed that all three *R* genes were inherited as a cluster, as earlier described by Zhu et al., (2013) for triple *R* gene transformants with the *nptII* selection marker in the same vector plasmid. It is clear from these experiments that stacking by co-transformation followed by marker gene elimination is possible, especially when the multiple *R* gene containing vector is stable during all stages of the transformation process. The obtained cisformants with stacked *R* genes in the sexual offspring could exhibit a high level of biological functioning with all three *R* genes in leaves and frequently in tubers. Similar research results were also found in barley where segregants harbored high level of expression from the gene of interest (Holme et al., 2012). It means that this method is appropriate to end up with well performing, triple *R* genes containing cisformants which can be used as a breeding parent. This way of co-transformation with two T-DNAs, of which one harboring a marker gene, was also successfully performed in maize (Ishida et al., 1996, Huang et al., 2004), rice and tobacco (Komari et al., 1996), and American Chestnut (Zhang et al., 2011). Ishida et al. (1996), observed that a very high concentration of inoculum (>5.0×10^{9} cfu/ml) could not produce any transformants; when the concentration was 1.0×10^{8} cfu/ml, transformation efficiency was 10%; if the concentration was too low (<1.0×10^{7} cfu/ml), then no transformants could be obtained. This could be the reason that we harvested less transformants in the inoculum mixture ratio 3, which had the highest OD for the transformation. Zhang et al. (2011) reported that the ratio of 4:1 for gene of interest: marker gene containing vector can produce higher co-transformation efficiency (40%) than the ratio 2:1 (20%). This might explain that we obtained higher co-transformation efficiency from inoculum mixture ratios 2 and 4.

From this study, HR produced by agro-infiltration with *Avr*s in cisformants is sometimes not matching with the resistance in the DLA. In events selected by kanamycin resistance, there is a full match between *Avr* responsiveness and resistance in DLAs. However, if cisformants showed a HR after agro-infiltration, then also resistance in DLA was found but the reverse was not always found. Therefore, agro-infiltration using *Avr* effectors can be used to test the functionality of *R* genes in cisformants. The use of DLA is, however, preferred since resistant cisformants might be overlooked due to the weak interaction between *R-Avr* in agro-infiltration. In case of *R* gene stacking, the use of DLA requires the use of isolates that can discriminate the functionality between the *R* genes. If such discriminating isolates are not available, functional expression of *R* genes can be tested using specific *Avr*s by agro-infiltration.

R genes to be used for stacking should meet several demands. Preferably, their recognition spectra are broad, and also complementary. Thus, the chance that the isolates overcome all the *R* genes stacked is decreasing. Furthermore, it was shown that *in vitro* pre-selection followed by PCR reaction can be made. It is observed that the pre-selected PCR positive events were showing full resistance in DLAs more frequently than the plants selected only by PCR. This bottle neck of functional expression of the resistance has to be considered in the process of producing cisformants. It needs the pre-selection of more PCR plus plants in order to select among them the sufficiently high expressing cisformants.

Comparison of the three investigated strategies to produce cisformants with stacked *R* genes shows that stacking is possible in all three ways but that the use of one multiple *R* gene vector is highly preferred if the resulting event will be used directly as an improved variety or as a breeding parent. Co-transfer of cisgenes through separate vectors is causing a high incidence of vector backbone integration and a low incidence of presence of both *R* genes as judged by PCR selection. Co-transfer of cisgenes, followed by marker gene elimination can produce cisformants with high levels of foliar resistance and resistance in tubers. They can be used as a breeding parent. The feasible approach to achieve high levels of functional gene stacking (i.e. four or more *R* genes) is through single vector mediated gene transfer followed by the second single vector gene transfer step (re-transformation) if the resistance from the previous transformants is broken. Re-transformed plants could be made beforehand using broad spectrum *R* genes with complementary resistance spectrum against previously collected isolates (Zhu and Jacobsen, 2012). Once the susceptibility of the plant(s) with stacked *R* genes is found in the field, another combination of gene stacking, which harbors the potential resistance could be applied immediately in order to maintain the resistance in the field. This can be achieved by testing different combinations of *R* genes in one plant with newly collected isolates from recent years.

For direct use of marker free transformants in potato varieties, the so-called "clean vector technology" could be an alternative. This transformation is made with a vector containing the desired *R* gene(s) together with kanamycin resistance gene. In addition a recombinase gene is present which can be induced site specifically in order to remove the undesired (helper) genes. The end result is the excision of these helper genes and only the *R* gene(s) of interest is left (Schaart et al., 2011). It is expected that such inserted *R* genes after "cleaning" will also have good performance. In apple this approach is practised at the moment (Vanblaere et al., 2011).

Genetically modified (GM) plants are not accepted by the European public due to questions about environmental and food safety. However, cisformants with only natural genes from the same or crossable species bypass many questions about environmental and food safety, and have a much higher acceptance rate in society consequently (Eurobarometer, 2010; ISAAA, 2012). In 2004, the US National Academy of Science suggested using natural genes from the breeders' gene pool as a non-GMO alternative (National Academy of Science, 2004). In 2011, the US Environmental Protection Agency (EPA) proposed to exempt plants with cisgenic resistance genes from the GMO regulations (Reardon, 2011). Also, the European food safety institute (EFSA) declared cisgenesis to be as safe for the environment and for public health as conventional breeding methods (EFSA, 2012). Recently, a European Working Group on new breeding techniques also suggested that transformants with less than 20 bp of foreign DNA should not be considered to be transgenic (European Working Group on new breeding techniques, unpublished data). A new argument is that transformation with wild type *Agrobacterium rhizogenes* with 4 *rol*-genes in *Kalanchoë* is treated as non-GMO because of the absence of recombinant DNA (Lutken et al., 2012). In the future, there is a chance that cisgenesis will be

exempted from GMO regulations, like mutagenesis and cell fusion are considered as non-GMO. Then it will be a new tool in plant breeding with natural genes from the breeders gene pool.

In general, cisformants with low copy number of individual *R* genes which show resistance in leaves and tubers can readily be made. In the near future, additional cisformants are expected to be produced along the strategies outlined in this report. According to different objectives, these strategies can be developed further and might help solving the late blight problem but can also be extended to many other problems in agriculture.

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Supplementary Table 1. List of primers used in the study

Label of primer pairs	Sequence (5'-3')	Annealing temperature (°C)	Length of product (bp)	Remark
nptII	TCGGCTATGACTGGGCACAACAGA AAGAAGGCGATAGAAGGCGATGCG	55.5	722	T-DNA
Rpi-sto1	ACCAAGGCCACAAGATTCTC CCTGCGGTTCGGTTAATACA	65	890	T-DNA
Rpi-vnt1.1	CCTTCCTCATCCTCACATTTAG CTCATCTAATAGATCCTCCAC	60	302	T-DNA
Rpi-blb3	AGCTTTTTGAGTGTGTAATTGG GTAACTACGGACTCGAGGG	63.5	305	T-DNA
traJ	ACGAAGAGCGATTGAGGAAA CAAGCTCGTCCTGCTTCTCT	62.5	260	backbone
oriV	ATAAGTGCCCTGCGGTATTG GCAGCCCTGGTTAAAAACAA	60.8	246	backbone
*oriV*3+5	TGCGGCGAGCGGTATCAG CTTCTTGATGGAGCGCATGGG	63	1045	backbone
nptIII	GAAAGCTGCCTGTTCCAAAG GAAAGAGCCTGATGCACTCC	60.8	162	backbone
trfA	CGTCAACAAGGACGTGAAGA CCTGGCAAAGCTCGTAGAAC	61.5	146	backbone
tetA	CTGCTAGGTAGCCCGATACG CCGAGAACATTGGTTCCTGT	61.4	296	backbone
tetR	GGGGGAGGGGATGTTGTCTA AGGGGTATGTTGGGTTTCAC	60	843	backbone
LB	AGCAACGCTCTGTCATCGTT TATCCTGCCACCAGCCAG	65	283	backbone
RB	TGACAGGATATATTGGCGGGT CTGCTGTAGTGAGTGGGTTGC	61	172	backbone
Rpi-sto1	GCTTGATCAGTTGTGGACATC TTCAATTGTGTTGCGCACTAG	60	-	qRT-PCR
Rpi-vnt1.1	ATGAATTATTGTGTTTACAAGACTTG CAGCCATCTCCTTTAATTTTTC	60	-	qRT-PCR
Rpi-blb3	TGTCGCTGAAAGAGTAGACC CACCTTTTGCCATTGGTTTAG	60	-	qRT-PCR
EF-1alfa	ATTGGAAACGGATATGCTCCA TCCTTACCTGAACGCCTGTCA	60	-	qRT-PCR

Supplementary Table 2. Transformant screening using PCR among gene transfer events obtained with pBINAW2:*Rpi-sto1*:*Rpi-blb3*

	#of *R* gene transfer events	Foliar resistance in the lab for *R* gene(s) containing plants		
		sto1	*blb3*	*sto1* and *blb3*
In vitro selection & PCR	17	12 R* 5 S	15 R 1 S 1 Q	11 RR
PCR	9	3 R 6 S	6 R 3 S	0

Isolates USA618 (*Avrsto1*, *avrblb3*) and PIC99183 (*avrsto1*, *Avrblb3*) were used for the foliar resistance test.
*12 *Rpi-sto1* containing plants showed resistance.

Supplementary Table 3. Foliar resistance and resistance in tubers for gene transfer events from construct pBINAW2:*Rpi-sto1*:*Rpi-blb3*

	In vitro disease selection/PCR	T-DNA PCR		backbone PCR					copy number		DLA		Agro-infiltration		% of infection in tubers disease test	
		sto1	*blb3*	*tetR*	*oriV*	*nptIII*	*trfA*	*tetA*	*stol*	*blb3*	USA618	PIC 99183	*Avrblb3*	*Avrsto1*	USA618	PIC99183
H44-1	R/R	+	+	-	-	+	+	+	1	1	R	R			100	62
H44-2	R/R	+	+	-	-	-	-	-	2	2	R	R	-	+	75	50
H44-3	R/R	+	+	-	-	-	-	-	3	3	R	R	+	+	50	0
H44-5	R/R	+	+	-	-	+	+	+	1	1	R	R			100	75
H44-6	R/R	+	+	-	-	-	-	-	1	1	R	R	-	+	100	75
H44-8	R/R	+	+	-	-	-	-	-	1	1	S	R				
H44-12	R/R	+	+	-	-	-	-	-	1	1	S	R				
H44-14	R/R	+	+	-	-	-	-	-	1	1	R	R	-	+		
H44-15	R/R	+	+	-	-	-	-	-	1	1	R	Q			100	100
H44-16	R/R	+	+	-	+	+	+	+	1	1	RQ	R			100	75
H44-17	R/R	+	+	-	-	-	-	-	2	2	R	R	+	+		
H44-18	R/R	+	+	-	-	-	-	-	2	2	R	R	+	+		
H44-19	R/S	+	+	-	-	-	-	-	1	1	R	R	-	+		
H44-20	R/S	-	+	-	-	-	-	-	0	1	S	RQ			100	45
H44-21	R/R	-	+	-	-	-	-	-	0	1	S	S				
H44-22	R/R	+	+	-	-	-	-	+	1	1	S	R				
H44-23	R/R	+	+	-	-	-	-	-	2	2	R	R	-	+		
H44-24	PCR	+	+	-	+	+	+	+	1	1	R	SQ			100	87
H44-25	PCR	+	+	-	+	+	+	+	1	1	RQ	SQ			87	100
H44-26	PCR	+	+	-	+	+	+	+	1	1	R	Q			100	87
H44-27	PCR	+	+	-	-	-	-	-	2	2	S	R				
H44-28	PCR	+	+	-	-	-	-	-	2	2	S	R				
H44-39	PCR	+	+	-	-	-	-	-	2	2	S	R				
H44-40	PCR	+	+	-	-	-	-	-	2	2	S	R				
H44-44	PCR	+	+	-	-	-	-	-	2	2	S	R				
H44-45	PCR	+	+	-	-	-	-	-	2	2	S	R				
H14-Xian6	PCR	+	+	+	+	+	+	+	3	3	R	R	+	+	0	0
Atlantic	PCR	-	-	-	-	-	-	-	0	0	S	S			100	100

Supplementary Table 4. PCR and resistance analysis of co-transformants

Constructs	Clone name	PCR					DLA		Agro-infiltration	
		sto1	*vnt1.1*	*oriv3+5*	*nptIII*	*tetA*	PIC99189	EC1	*Avrsto1*	*Avrvnt1*
pBINAW2-*vnt1.1* &pBINAW2-*sto1*	KB04-10	+	-		+		S	SQ		
	KB04-11	+	-	-	-	-	S	S	0	
	KB04-13	+	-		+		S	R		
	KB04-14	+	+		+		R	R		
	KB04-15	+	-	-	-	-	S	R	0,2	
	KB04-16	-	+		+		S	S		
	KB04-17	+	+		+		R	S		
	KB04-18	+	-	-	-	ND	S	Q	0,2	
	KB04-19	-	+	-	-	-	R	S		0,2
	KB04-20	-	+		+		S	S		
	KB04-21	-	+		+		RQ	S		
	KB04-22	+	+	-	+	+	R	RQ		
	KB04-3	+	-	-	-	-	S	RQ	0,2	
	KB04-5	-	+	-	-	-	S	S		0,2
	KB04-6	-	+	-	-	+	R	S		0,2
	KB04-9	-	+		+		R	S		
pBINAW2LB4-*vnt1.1*&pBINAW2LB4-*sto1*	KB06-10	+	-	ND	-	ND	S	RQ	0,2	
	KB06-11	-	+	-	-	-	S	S		0
	KB06-12	+	+		+		RQ	R		
	KB06-13	+	+		+		R	R		
	KB06-14	+	+	ND	-	-	R	R	0,2	0,2
	KB06-15	+	+		+		R	RQ		
	KB06-16	-	+	+	-	-	R	S		0,2
	KB06-17	+	-	-	-	-	S	R	0,2	
	KB06-18	-	+	-	-	ND	R	S		0,2
	KB06-19	-	+	-	-	-	S	S		0
	KB06-2	-	+	-	-	-	R	S		0,2
	KB06-20	-	+	-	-	-	S	S		0
	KB06-21	+	+	ND	-	-	R	R	0,2	0,2
	KB06-22	-	+		+		R	S		
	KB06-23	+	-	-	-	-	S	Q	0	
	KB06-24	+	+		+		R	R		
	KB06-25	-	+	-	-	-	R	S		0,2
	KB06-26	+	+		+		R	R		
	KB06-27	-	+	-	-	-	R	S		0,2
	KB06-28	-	+	-	-	ND	R	S		0,2
	KB06-29	+	-	-	-	-	S	RQ	0,2	
	KB06-3	+	+		+		R	S		
	KB06-30	-	+		+		R	S		
	KB06-31	-	+		+		R	S		
	KB06-32	+	-		+		S	R		
	KB06-33	-	+	-	-	-	R	S		0,2
	KB06-34	+	-	-	-	-	S	R	0,05	
	KB06-35	+	+		+		R	RQ		
	KB06-36	-	+		+		R	S		
	KB06-37	+	+		+		R	R		
	KB06-38	+	-	-	-	-	S	R	0,2	
	KB06-39	+	-		+		S	R		
	KB06-4	+	-		+		S	SQ		
	KB06-40	+	-		+		S	R		
	KB06-41	+	-		+		S	R		
	KB06-42	-	+	-	-	-	R	S		0,2
	KB06-43	-	+	-	-	+	R	S		0,2
	KB06-44	-	+	-	-	+	R	S		0,2
	KB06-45	+	+		+		R	S		
	KB06-46	-	+	-	-	ND	R	S		0,2

Supplementary Table 4. PCR and resistance analysis of co-transformants (continued)

pBINAW2LB4-*vnt1.1*&pBINAW2LB4-*sto1*	KB06-47	+	+		+		R	R		
	KB06-49	+	-		+		S	R		
	KB06-5	+	+		+		R	R		
	KB06-50	+	-		+		S	S		
	KB06-51	+	+	-	-	-	R	RQ	0,2	0,2
	KB06-52	+	-	-	-	+	S	RQ	0.2	
	KB06-53	-	+	-	-	-	S	S		0
	KB06-54	+	-		+		S	R		
	KB06-55	+	-	-	-	-	S	R	0,2	
	KB06-56	+	+		+		R	R		
	KB06-57	+	-	-	-	-	S	S	0	
	KB06-58	+	-		+		S	R		
	KB06-59	+	-		+		S	R		
	KB06-6	+	-	-	-	ND	S	R	0,05	
	KB06-60	+	-		+		S	R		
	KB06-61	+	-		+		S	S		
	KB06-62	+	-		+		S	R		
	KB06-63	-	+		+		R	S		
	KB06-64	+	-	-	-	-	S	Q	0,2	
	KB06-65	-	+	-	-	-	R	S		0,05
	KB06-66	+	-		+		S	R		
	KB06-67	+	-		+		S	SQ		
	KB06-68	+	-	-	-	-	S	R	0	
	KB06-7	+	+		+		R	R		
	KB06-8	-	+	-	-	-	R	S		0,2
	KB06-9	+	-		+		S	SQ		
	Atlantic	-	-	-	-	-	S	S	0	0

ND: not done; shaded data indicated that the results were not repeatable.

Supplementary Table 5. Foliar resistance under field conditions of gene transfer events from construct pBINAW2:*Rpi-sto1*:*Rpi-blb3* in cv Atlantic harboring two *R* genes

days post inoculation	2dpi		7dpi		14dpi		21dpi		average			
replication	1st*	2nd**	1st	2nd	1st	2nd	1st	2nd	2dpi	7dpi	14dpi	21dpi
H44-1***	3&	3	5	5	10	20	70	90	3	5	15	80
H44-2	0	0	0	0	0	3	3	5	0	0	1.5	4
H44-3	0	0	0	0	0	0	0	0	0	0	0	0
H44-5	1	3	5	5	20	10	85	80	2	5	15	82.5
H44-6	1	1	5	5	10	10	85	80	1	5	10	82.5
H44-8	5	5	10	20	70	80	85	90	5	15	75	87.5
H44-12	3	5	5	5	10	20	85	90	4	5	15	87.5
H44-7	10	5	40	40	99	99	100	100	7.5	40	99	100
H44-9	20	5	40	50	99	99	100	100	12.5	45	99	100
H44-10	20	20	70	30	100	95	100	100	20	50	97.5	100
H43-KB05-4	0	0	0	0	0	0	0	0	0	0	0	0
H14 xian6-1	0	0	0	0	0	0	0	0	0	0	0	0
Atlantic	10	10	40	50	95	99	100	100	10	45	97	100

* first replication; ** second replication; *** clone name; & 3% leaves infected.
Isolate IPO-C (*Avrsto1*, *Avrvnt1*, *avrblb3*) was used for the inoculation in the field. Tested H44 plants in this experiment contained both *Rpi-sto1* and *Rpi-vnt1.1* genes. Plants H44-1,5,6,8,12 contained one T-DNA copy and H44-2 (two T-DNA copies) were infected; H44-3 (three T-DNA copies); H43-KB05-4 is a cv Atlantic transformant harboring two copies of *Rpi-sto1*:*Rpi-vnt1.1*; H14-xian6 is a cv Atlantic transformant (resistant control, containing *nptII*:*sto1*:*vnt1.1*:*blb3*); H44-7,9,10 were susceptible, Atlantic regenerants, not containing *R* genes.

CHAPTER 5

An updated conventional- and a novel GM potato differential set for virulence monitoring of late blight resistance genes

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Abstract

Late blight is an important disease in potato that is caused by the oomycete *Phytophthora infestans*. Genetically encoded disease resistance is amply available from related wild species and many resistance (*R*) genes have been identified. Already in the previous century eleven *R* genes, deriving from *Solanum demissum*, were introgressed into cultivated potato and plants for a differential set were selected by Mastenbroek and Black on the premise that each individual differential plant contained one *R* gene. These sets were used to characterize the virulence spectrum of *P. infestans* isolates and to monitor the occurrence and dynamics of virulence in *P. infestans* populations. In this and other studies, the Mastenbroek differentials Ma*R3*, Ma*R5* - MaR*11* are shown to harbor extra *R* gene(s). We extended the differential set with plants with reduced *R* gene complexity SW8540-025 (*R3a*), SW8540-325 (*R3b*), 3020-330 (*R8*) and 3151-04 (*R9a*). The differential set was also extended with parental plants containing the recently cloned *R* genes derived from related *Solanum* spp. However, the newly provided plants have highly divergent genetic backgrounds and to cope with this problem, a differential set based on GM Desiree plants containing single late blight *R* genes was set up.

Successively, the virulence spectra of a panel of current late blight isolates from different geographic locations was tested in DLA using the novel differential sets. The experiments suggest the presence of additional late blight *R* genes in the existing and extended conventional differential set and implicate that the GM differential set is more accurate for late blight isolate virulence typing. Furthermore, a distinction could be made between broad spectrum (*Rpi-blb3, Rpi-blb1, Rpi-chc1, R8, R9, Rpi-vnt1.1* and *Rpi-blb2*) and narrow spectrum (*R1, R3a, R3b, R4*) *R* genes. Remarkably, none of the *R* genes investigated showed full spectrum resistance, which has serious consequences for the deployment of single *R* genes in cultivars. Besides the use in detached leaf assays, the novel differential sets were shown to be useful as trap plants to isolate novel late blight strains and to monitor virulence in late blight populations towards particular *R* genes `on site´. The results largely confirmed the distinction that was made between broad and narrow spectrum resistance genes using our panel of late blight isolates in DLA. Also for these `on site´ monitoring experiments the GM differentials were superior to the updated conventional differential set. Legislative restrictions are however limiting the use of the GM differential set. An alternative cisgenic version might alleviate these problems in the future.

Introduction

Worldwide, breeding efforts for crop improvement are made. Important traits to be introduced into novel cultivars include disease resistance (*R*) genes against a variety of different pests and diseases. The interaction between pathogen races and *R* gene containing plants often follow the gene-for-gene concept for host-pathogen interaction (Flor, 1942). This concept implies that the loss of an a-virulence gene from the pathogen results in its virulence and consequent *R* gene breakdown. A differential set (also named differential varieties, differential hosts, or plant differentials) is a set of genotypes (also named

differential plants), ideally each containing a unique single resistance (*R*) gene, used to e.g. define the pathogen virulence races based on the resulting susceptible and resistant reactions (International Seed Federation, 2013). Differential sets play an important role in mechanistic studies on the host-pathogen interaction and in monitoring pathogen adaptation and pathogen population genetics. A lack of consistency between various "local" differential sets limits their application and thus calls for the creation of a well-defined standardized *R* gene differential set for international use (Goyeau et al., 2012). Plant differential sets so far consist of cultivars or accessions harboring a (set of) specified *R* gene(s) such as e.g. for downy mildew in spinach (Irish et al., 2003), Fusarium in melon (Sandlin and Webb, 2012) and black spot in rose (Whitaker et al., 2010). For potato late blight, caused by *P. infestans*, a differential set of eleven *Solanum* genotypes (Ma*R1*-Ma*R11*), expected to contain eleven different *R* genes, were derived from the hexaploid species *Solanum demissum* (Black and Mastenbroek, 1953; Malcolmson and Black, 1966).

Late blight, an important disease in potato and tomato (Haverkort et al., 2008), is caused by the oomycete *Phytophthora infestans*. Recent *P. infestans* population shifts are resulted from migration, mutation and recombination between both mating types A1 and A2 (McDonald and Linde, 2002, Cooke et al., 2003, Haas et al., 2009, Raffaele et al., 2010, Kim et al., 2012) and resulted in e.g. the dominance of genotypes US-1 and US8 in the United States and Canada (Goodwin et al., 1998) and Blue-13 in Europe (Fry et al., 2009; Li et al., 2012). Host resistance is considered to be an important component in an integrated potato late blight control strategy. Recent history, however, shows that host resistance with major *R* genes(s) can be overcome by the pathogen quickly after the introduction of resistant varieties (Lowe et al., 2011, Drenth et al., 1994). Fast-evolving effector genes, localized at highly dynamic regions of the *P. infestans* genome, in combination with the capability to produce huge numbers of spores are keys to the rapid adaptability of the pathogen (Haas et al., 2009). Therefore, characterization of *P. infestans* populations for virulence towards specific *R* genes, is of additional practical and scientific value to current monitoring efforts mostly using neutral markers (Li et al., 2012). Major *R* genes *R1*, *R2*, *R3*, *R4* and *R10* were introgressed into potato cultivars and released to the consumer market (Wastie, 1991). The resistance conferred by these genes was, however, overcome by the fast evolving *P. infestans* isolates in a relatively short time period (Colon et al., 1995). Virulence towards the Mastenbroek (Ma) differential plants Ma*R5*, and especially Ma*R8* and Ma*R9*, is still rare and the *R* genes involved can still be employed for a durable resistance strategy (Swiezynski et al., 2000, Kim et al., 2012).

From the Mastenbroek differential set, four *R* genes have been cloned: *R1* (Ballvora et al., 2002), *R2* (Lokossou et al., 2009), *R3a* (Huang et al., 2005), *R3b* (Li et al., 2011). Genes *R6*, *R7*, *R10* and *R11* were mapped on chromosome XI (ElKharbotly et al., 1996, Bradshaw et al., 2006). *R5*, *R8* and *R9* were suggested to reside on chromosome XI as well (Huang et al., 2005), but recently this suggestion was rejected since *R8* and *R9* were shown to reside on chromosome IX (Jo et al., 2011, Jo, 2013). Besides these late blight *R* genes from *S. demissum*, *R* genes like *Rpi-sto1* from *S. stoloniferum*, *Rpi-blb1* from *S.*

bulbocastanum, *Rpi-pta1* from *S. papita* (van der Vossen et al., 2003, Vleeshouwers et al., 2008), *Rpi-blb2* from *S. bulbocastanum* (van der Vossen et al., 2005), *Rpi-blb3* from *S. bulbocastanum* (Lokossou et al., 2009), *Rpi-vnt1.1* from *S. venturii* (Pel et al., 2009) and *Rpi-chc1* from *S. chacoense* (J. Vossen, unpublished data) have also been cloned. In the near future, new varieties containing these *R* genes are likely to be exposed to the current, highly adaptive *P. infestans* populations. Therefore, virulence monitoring toward these *R* genes will require an updated differential set.

Virulence towards a major *R* gene is acquired by the loss of the cognate *P. infestans* component, encoded by a-virulence genes. So far, *Avr1* (Guo, 2008), *Avr2* (Saunders et al., 2012), *Avr3a* (Armstrong et al., 2005), *Avr3b* (Li et al., 2011), *Avr4* (van Poppel et al., 2008), *Avr8* (Jo KR, unpublished data), *Avr10* (Rietman, 2011), *Avrsto1* (Vleeshouwers et al., 2008), *Avrblb2* (Oh et al., 2009), *Avrvnt1* (Pel, 2010), and *Avrchc1* (J. Vossen unpublished data) were cloned. The availability of *Avr* genes provides an additional methodology for virulence typing of late blight isolates, besides differential set studies. Furthermore, *Avr*s can be used to detect specific functional *R* genes and characterize breeding materials (Rietman et al., 2012). When the current late blight differential plants were tested using marker technology and *Avr* responsiveness, doubts were raised about the monogenic nature. Ma*R5*, Ma*R6* and Ma*R9* were found to also contain *R1* (Trognitz and Trognitz, 2007); Ma*R3* was shown to harbor two *R* genes: *R3a* and *R3b* (Huang et al., 2005, Li et al., 2011) and Ma*R8* and Ma*R9* were shown to harbor four (*R3a*, *R3b*, *R4* and *R8*) and seven (*R1*, *Rpi-abpt1*, *R3a*, *R3b*, *R4*, *R8* and *R9*) *R* genes, respectively (Kim et al., 2012).

It is clear that both breeders and growers can benefit from further purification and extension of the potato late blight differential set. The purified, extended differential set can play an important role in monitoring efforts of the fast evolving *P. infestans* populations in different parts of the world. In this study, we further investigated the presence of additional *R* genes in the Mastenbroek late blight differential set using agro-infiltration with cloned *Avr* effectors. Meiotic segregation was then used to remove surplus *R* genes from Ma*R3*, Ma*R8* and Ma*R9*. Finally, the differential set is supplemented with *Solanum* spp. known to carry at least one recently cloned *R* gene to result in an updated conventional differential set. In a parallel approach, a genetically isogenic differential set in potato cultivar Desiree is presented using transformation of cloned *R* genes. Since *R* genes are introduced in the same genetic background (cv Desiree), this approach excludes the disadvantages caused by the heterogeneity of the genetic background as found in the current Dutch differential set Ma*R1* – Ma*R11*. This GM differential set is therefore expected to provide more accurate characterization of *P. infestans* isolates.

Materials and Methods

Plant Materials

The Mastenbroek (Ma) differential set Ma*R1* – Ma*R11* (Ma*R1*: CEBECO 43154-5; Ma*R2*: CEBECO 44158-4; Ma*R3*: CEBECO 4642-1; Ma*R4*: CEBECO 4431-5; Ma*R5*: Black 3053-18; Ma*R6*: Black XD2-21; Ma*R7*: Black 2182 ef (7); Ma*R8*: Black 2424 a (5); Ma*R9*: Black 2573 (2); Ma*R10*: Black 3681 ad (1); Ma*R11*: Black 5008 ab (6)) and genotypes blb99-256-3, SW8540-025, SW8540-325, sto389-4, blb8005, Pta767-1, blb2002, vnt367-1 and chc543-5, containing the recently cloned *R* genes *Rpi-blb3, R3a, R3b, Rpi-sto1, Rpi-blb1, Rpi-pta1, Rpi-blb2, Rpi-vnt1.1* and *Rpi-chc1*, respectively were maintained *in vitro* at Wageningen UR Plant Breeding. F1 and BC1 populations from Ma*R8* and Ma*R9* were previously described (Kim et al., 2012). F1 population '3020' and '3025' resulted from crosses between cv Concurrent (harboring *R3b* and *R10*) and Ma*R8* or Ma*R9*, respectively . BC1 populations were made by crossing the individual F1 plants 3025-43, 3025-48, 3025-53, 3025-71 and 3025-89 with cv Katahdin. The resulting BC1 populations were coded 3150, 3151, 3153, 3154 and 3155, respectively.

In total, 40 F1 plants from population 3020 (MaR8 x Concurrent), and nine BC1 plants from the "Ma*R9*" populations above were selected in pursuit of plant material only containing resistance genes *R8* or *R9*. The selected F1 plants from the 3020 population displayed a hypersensitive response (HR) after agro-infiltration with *Avr8* and harbored *R8* specific marker CDP3 (Jo et al., 2011). BC1 plants from populations 3150, 3151, 3153, 3154 and 3155 ((Ma*R9* x Concurrent) x Katahdin), which did not harbor the *R8* specific marker CDP3, but showed resistance against isolate IPO-C in the field were selected.

Desiree transformants containing *R1* were kindly provided by Dr. Christiane Gebhardt (Max-Planck Institute, Germany). Binary plasmids pBINPLUS:*Rpi-blb3*, pBINPLUS:*R3a*, pBINPLUS:*R3b*, pBINPLUS:*Rpi-sto1*, pBINPLUS:*Rpi-blb1*, pBINPLUS:*pta1*, pBINPLUS:*Rpi-blb2*, pBINPLUS:*Rpi-vnt1.1*, pBINPLUS:*Rpi-chc1* were constructed in house and transformed into cv Desiree using *Agrobacterium* mediated transformation (Zhu et al., 2012).

Phytophthora infestans

Sixteen in house, well defined *Phytophthora* isolates: IPO-0, PIC99171, PIC99189, PIC99183, EC1, 88069, H30P04, Dinteloord, 90128, VK98014, F95573, 428-2, 89148-09, Katshaar, IPO-complex and USA618 (Vleeshouwers et al., 2008), were used to characterize the differential plants. PCR assays using *R* gene specific primers for *R1*, *R2*, *R3a* and *R3b* were used to check for the presence or absence of these *R* genes in the Ma*R8* and Ma*R9* differential plants and their offspring plants (Supplementary Table 1).

Quantitative Real Time PCR (qRT-PCR)

nptII specific primers (Supplementary Table 1) were employed to determine the copy number of inserted *R* genes in cv Desiree transformants using the Bio-Rad qRT-PCR kit and Bio-Rad iCycler iQ machine. Reaction mixtures contained 5 ng of genomic DNA, 5 µl of Sybr green qRT-PCR mix, 3 mM forward primer and 3 mM reverse primer in 10 µl. *EF-1alfa*, an endogenous single copy gene, was used for delta Ct calculations. Three transgenic events with one T-DNA insertion (as determined by Southern blot) of

nptII were used as standards for the Ct values of single copy plants. Negative controls included the non-transformed potato cv Desiree and Milli-Q water. Southern blot was used to detect the copy number for *R1* Desiree transformants using the *nptII* PCR product as probe.

Detached Leaf Assays (DLA)

Leaves of greenhouse grown plants were inoculated using two different methods to assess the foliar resistance of specific host genotypes: droplet inoculation with zoospore/sporangia suspension of $5*10^4$ spores per ml (Vleeshouwers et al., 1999) or spray inoculation with a suspension of $2*10^4$ sporangia per ml (Förch et al., 2010).

Infections resulting from droplet inoculations were scored as follows:
R, all of the inoculation spots on a detached leaf showed resistance (no visual necrosis or showed hypersensitive response);
RQ, 6–7 out of eight spots on a leaf showed resistance;
Q, 3–5 out of eight spots on a leaf showed resistance;
SQ, 1–2 out of eight spots on a leaf showed resistance;
S, all spots on a leaf showed susceptibility.

The scoring criteria for DLA using spray inoculation are:
R, no sporulation observed on the inoculated leaf;
RQ, sporulation occurred on around 25% of leaf surface;
Q, around 50% of the leaf surface covered with sporulation;
SQ, around 75% of inoculated leaf surface covered with sporulation;
S, 100% of leaf surface covered with sporulation.

Agro-infiltration was performed according to Rietman et al., (2012) using *Agrobacterium* strain AGL-1 containing the seven different *Avr* genes (*Avr* effectors *Avr1*, *Avr2*, *Avr3a*, *Avr3b*, *Avr4*, *Avr8* and *Avr10* in binary vector pK7WG2) at OD_{600}: 0.2. For each clone, two replicate plants, each plant with two leaves, were subjected to agro-infiltration.

Late blight field trials

Late blight resistance assays under field conditions were performed essentially as described previously (Kim et al., 2012). Potato tubers were planted in the field in the beginning of May. Two replications, each with six plants per genotype, were planted. Inoculation with isolate IPO-C (1,2,3,4,5,6,7,10,11) was performed in the beginning of July. The percentage of the leaf area covered by late blight lesions was estimated by visual inspection.

Results

Resistance in the Mastenbroek differential set when exposed to modern* P. infestans *isolates

P. infestans isolates IPO-0, PIC99171, PIC99189, PIC99183, EC1, 88069, H30P04, Dinteloord, 90128, VK98014, F95573, 428-2, 89148-09, Katshaar, IPO-C and USA618 were droplet inoculated on Ma*R1* – Ma*R11*, Desiree and Bintje leaflets in a Detached Leaf Assay (DLA).

Table 1. Disease test of the Mastenbroek differential set against sixteen in house isolates of *P. infestans*

Isolate / Plant	IPO-0	PIC99177	PIC99189	PIC99183	EC1	88069	H30P04	Dinteloord	90128	VK98014	F95573	428-2	89148-09	Katshaar	IPO C	USA618
Desiree	S	S	S	S	S	S	S	S	S	S	SQ	S	S	S	S	S
Bintje	S	S	S	S	S	S	S	S	S	S	S	S	S	S	S	S
Ma*R1*	R	SQ	S	S	S	S	R	S	S	S	S	S	R	RQ	S	S
Ma*R2*	R	S	S	Q	R	R	R	Q	R	Q	R	Q	RQ	R	S	S
Ma*R3*	SQ	S	R	S	S	S	S	R	S	R	S	S	R	S	S	S
Ma*R4*	RQ	S	R	S	SQ	S	R	S	S	S	Q	S	R	S	S	R
Ma*R5*	R	R	RQ	R	R	R	R	R	R	R	R	R	R	R	SQ	R
Ma*R6*	R	R	R	R	R	R	R	R	R	R	R	R	R	R	S	S
Ma*R7*	Q	S	RQ	S	Q	S	S	Q	S	R	S	S	R	S	S	S
Ma*R8*	R	R	R	S	R	R	R	R	RQ	R	R	RQ	R	RQ	R	R
Ma*R9*	R	Q	R	Q	R	R	R	R	R	R	R	R	R	R	RQ	R
Ma*R10*	S	Q	S	S	S	SQ	SQ	R	S	R	S	Q	R	S	S	S
Ma*R11*	S	S	S	S	S	S	S	R	S	R	S	S	R	S	S	S

Susceptible controls Desiree and Bintje were found to be susceptible to all *P. infestans* isolates included in the experiment. The scores “R” and “RQ” are considered to result from incompatible interactions. Differential plants Ma*R5*, Ma*R6*, Ma*R8* and Ma*R9* were resistant to 14 or 15 out of 16 isolates; whereas Ma*R1*, Ma*R3*, Ma*R4*, Ma*R7*, Ma*R10* and Ma*R11* were susceptible to the majority of these 16 isolates. Ma*R2* conferred resistance against eight out of the 16 isolates (Table 1). The wide susceptibility found for the latter set of differential plants indicates a restricted usefulness of the *R* gene(s) involved.

The Mastenbroek differential set MaR1 – MaR11 contains extra* R *genes

Recently, questions had arisen about the monogenic nature of the Mastenbroek differential set. These questions were addressed by agro-infiltration of *Avr1*, *Avr2*, *Avr3a*, *Avr3b*, *Avr4*, *Avr8* and *Avr10* on all the members of the Mastenbroek differential set.

Table 2. Agro-infiltration assay with seven *Avr* effectors on the eleven plants of the Mastenbroek differential set

	Avr1	*Avr2*	*Avr3a*	*Avr3b*	*Avr4*	*Avr8*	*Avr10*	Predicted *R* gene content
Ma*R1*	+	-	-	-	-	-	-	*R1*
Ma*R2*	-	+	-	-	-	-	-	*R2*
Ma*R3*	-	-	+	+	-	-	-	*R3a, R3b*
Ma*R4*	-	-	-	-	+	-	-	*R4*
Ma*R5*	+	+	-	+	-	-	-	*R1, R2, R3b**
Ma*R6*	+	+	+	-	-	-	-	*R1, R2, R3a**
Ma*R7*	-	-	+	-	+	-	-	*R3a, R4**
Ma*R8*	-	-	+	-	+	+	-	*R3a, R4, R8*
Ma*R9*	+	+	+	+	+	+	-	*R1, Rpi-abpt1, R3a, R3b, R4, R8**
Ma*R10*	-	-	-	+	-	-	+	*R3b, R10*
Ma*R11*	-	-	-	+	-	-	+	*R3b, R10**

'+' indicates HR recognition, '-' indicates absence of HR recognition.
* Additional *R* genes may exist (unpublished data).
(all agro-infiltrations applied an OD_{600} of 0.2)

Ma*R1*, Ma*R2* and Ma*R4* only responded to the *Avr1*, *Avr2*, and *Avr4* treatments respectively and not to additional *Avr* genes. All other differential plants responded to their designated *Avr* gene but also to various additional *Avr* treatments. As previously reported by Huang et al. (2005) and Li et al. (2011), Ma*R3* contained *R3a* and *R3b* and indeed this plant responded to both *Avr3a* and *Avr3b*. Ma*R5* and Ma*R6* were shown to respond to *Avr1*, *Avr2* and *Avr3b* or *Avr1*, *Avr2* and *Avr3a*, respectively. Ma*R7* responded to *Avr3a* and *Avr4*. Ma*R8* responded to *Avr8* but also to *Avr3a* and *Avr4* as previously described but not to *Avr3b* as was anticipated by the *R3b* marker (Kim et al., 2012). Ma*R9* harbored at least six additional *R* genes (*R1, Rpi-abpt1*, *R3a*, *R3b*, *R4* and *R8*) (Kim et al., 2012) which were all confirmed using our co-infiltration experiments. Ma*R10* also contained *R3b*, whereas Ma*R11* contained *R3b* and *R10*.

Selection of new* R8 *and* R9 *differential plants from Ma*R8 *and Ma*R9 *crossing populations

The presence of multiple *R* genes in most of the Mastenbroek plants limits the usefulness of these differentials. It is therefore worthwhile to produce new differential plants only harboring *R5, R6, R8* or *R9*. Because the *Avr*s and molecular markers matching *R5* and *R6* were not available, we set out to select plants only containing *R8* or *R9* from characterized populations (Kim et al., 2012). In a step-wise process, plants containing only the target *R* gene were selected: 40 F1 plants from the Ma*R8* population 3020 were found to harbor *R8* through agro-infiltration with *Avr8*. Because *Avr9* was not available, *R9* containing plants were selected because they were found to be resistant against isolate IPO-C (*Avr8*, *Avr9*) in the field and did not harbor *R8* as shown through agro-infiltration with *Avr8*. Next, PCR assays using

R gene specific markers for *R1*, *R2*, *R3a* and *R3b* were used to exclude plants with undesired additional *R* genes. The resulting eight F1 plants from Ma*R8* population 3020 and four BC1 plants deriving from the Ma*R9* were subsequently infiltrated with *Avr4*. Five F1 plants from the Ma*R8* population 3020 and three BC1 plants from the Ma*R9* populations responded to *Avr4* and these plants were discarded. Finally, the remaining plants were tested for their performance in DLA using droplet method with isolate IPO-C (*Avr8*, *Avr9*).

Plants 3020-330 from the Ma*R8* population and 3151-04 from a Ma*R9* BC1 population were selected as *R8* and *R9* containing plants with reduced *R* gene complexity, because these two plants showed negative results in PCRs using *R1*, *R2*, *R3a* and *R3b* specific markers, did not respond to *Avr4* infiltration, and showed resistance against isolate IPO-C (*Avr8*, *Avr9*) in DLA with the droplet method. However, these two plants 3020-330 and 3151-04, together with Ma*R8* and Ma*R9* were tested with all the 55 isolates collected in 2011 with the spraying assay. More severe virulence symptoms were observed on 3020-330 and 3151-04, compared to Ma*R8* and Ma*R9* from all 55 isolates. Isolates showing complete susceptibility on Ma*R8* and Ma*R9* were also found (G. Kessel, unpublished data). Therefore, it is still useful to keep the old Ma*R8* and Ma*R9* in the extended list to monitor the pathogen population.

An improved differential set

Besides adding new differential plants containing only *R3a*, *R3b*, *R8* and *R9*, the differential set was extended by including *R* gene containing plants (blb99-256-3, sto389-4, blb8005, pta767-1, blb2002, vnt367-1, chc543-5) that served as the new resistance source from recently cloned *R* genes (Table 3). The individual members of the improved differential set were named DS-R1, DS-R2, etc., coding for "Differential Set" plus the relevant *R* gene. If more than one known resistance gene was present, just a sequential number is provided.

Table 3. Improved Mastenbroek differential set with plants containing recently cloned *R* genes and less complex seedlings selected from crosses with Ma*R3*, Ma*R8* and Ma*R9*, respectively

New name	Genotype	plant species	*R*-gene content*
DS-R1	MaR1: CEBECO 43154-5	*S. demissum*	*R1*
DS-R2	Ma*R2*: CEBECO 44158-4	*S. demissum*	*R2*
DS-R3a	SW8540-025	*S. demissum*	*R3a*
DS-R3b	SW8540-325	*S. demissum*	*R3b*
DS-R4	Ma*R4*: CEBECO 4431-5	*S. demissum*	*R4*
DS-5	Ma*R5*: Black 3053-18	*S. demissum*	*R1, R2, R3b***
DS-6	Ma*R6*: Black XD2-21	*S. demissum*	*R1, R2, R3a***
DS-7	Ma*R7*: Black 2182 ef (7)	*S. demissum*	*R3a, R4***
DS-8	Ma*R8*: Black 2424 a (5)	*S. demissum*	*R3a, R4, R8*
DS-R8	3020-330	*S. demissum*	*R8*
DS-9	Ma*R9*: Black 2573 (2)	*S. demissum*	*R1, Rpi-abpt1, R3a, R3b, R4, R8, R9***
DS-R9	3151-04	*S. demissum*	*R9***
DS-10	Ma*R10*: Black 3681 ad (1)	*S. demissum*	*R3b, R10*
DS-11	Ma*R11*: Black 5008 ab (6)	*S. demissum*	*R3b, R10***
DS-blb3	blb99-256-3	*S. bulbocastanum*	*Rpi-blb3*
DS-sto1	sto389-4	*S. stoloniferum*	*Rpi-sto1***
DS-blb1	blb8005	*S. bulbocastanum*	*Rpi-blb1***
DS-pta1	pta767-1	*S. papita*	*Rpi-pta1***
DS-blb2	blb2002	*S. bulbocastanum*	*Rpi-blb2***
DS-vnt1.1	vnt367-1	*S. venturii*	*Rpi-vnt1.1*
DS-chc1	chc543-5	*S. chacoense*	*Rpi-chc1***

* *R* gene content was determined using a combination of molecular marker analysis and *Avr* responsiveness.
** Indications are available for additional *R* genes in these plants, besides the *R* genes listed in this table (unpublished data).

This plant collection now contains ten plants that are presumed to contain only one *R* gene (DS-R1, DS-R2, DS-R3a, DS-R3b, DS-R4, DS-R8, DS-blb3, DS-sto1, DS-pta1, DS-vnt1.1). Also five plants are included (DS-5, DS-R9, DS-blb1, DS-blb2, DS-chc1) that contain additional unknown *R* genes besides the known *R* genes, because the resistance spectrum of these plants is broader as the sum of the known *R* genes (data not shown). Finally for three plants, DS-6, DS-7, and DS-11 it is not clear if they contain additional *R* genes besides the genes that are indicated in Table 3.

A GM differential set in the genetic background of cv Desiree

As already noted, additional *R* genes are present in some of the differential plants presented in Table 3. Also additional unknown *R* genes might be present and accessory genes affecting the plants' physiology

vary between the different plants. This will blur the outcome of disease assays that will be performed with this or any other differential set obtained using conventional breeding. To exclude the possibility that different genetic backgrounds may influence the results obtained, a second path was explored. Cloned *R* genes, listed in Table 4, were transformed to cv Desiree. Transformants were tested by PCR using *nptII* specific primers, followed by the copy number analysis tested by qRT-PCR with *nptII* primers. Then resistance against avirulent isolates was tested in DLA using droplet assay, followed by the foliar resistance test in the field sprayed with isolate IPO-C. For the *R* genes (*R1, Rpi-blb3, R3a, R3b*), from which their resistance was broken by isolate IPO-C, their resistance were only performed at DLA level. Finally, individual *R* gene transformants in the genetic background of cv Desiree containing a single copy of functional *R* genes, showing resistance in DLA/field, were selected as GM differential plants (Table 4).

Table 4. A monogenic GM differential set of ten *Rpi* genes in cv Desiree with their matching effectors

Name	Genotype	*R* gene	*R* gene donor	Donor species	matching effector
DSD	Desiree	*R0*	-	-	-
DSD-R1	A(10-2-4)	*R1*	Ma*R1*: CEBECO 43154-5	*S. demissum*	*Avr1*
DSD-blb3	A03-142	*Rpi-blb3*	blb99-256-3	*S. bulbocastanum*	*Avr2*
DSD-R3a	A04-33	*R3a*	SW8540-025	*S. demissum*	*Avr3a*
DSD-R3b	A25-11	*R3b*	SW8540-325	*S. demissum*	*Avr3b*
DSD-sto1	A09-277	*Rpi-sto1*	sto389-4	*S. stoloniferum*	*Avrblb1*
DSD-blb1	A01-20	*Rpi-blb1*	blb8005	*S. bulbocastanum*	*Avrblb1*
DSD-pta1	A23-43	*Rpi-pta1*	pta767-1	*S. papita*	*Avrblb1*
DSD-blb2	A02-33	*Rpi-blb2*	blb2002	*S. bulbocastanum*	*Avrblb2*
DSD-vnt1.1	A13-13	*Rpi-vnt1.1*	vnt367-1	*S. venturii*	*Avrvnt1*
DSD-chc1	A17-27	*Rpi-chc1*	chc543-5	*S. chacoense*	*Avrchc1*

DSD: differential set Desiree.

This differential set contains three cloned *R* genes from *S. demissum* (*R1*, *R3a*, *R3b*), three *R* genes from *S. bulbocastanum* (*Rpi-blb1*, *Rpi-blb2* and *Rpi-blb3* as a homolog for *R2* (Lokossou et al., 2009), and one *R* gene from *S. stoloniferum* (*Rpi-sto1*), *S. papita* (*Rpi-pta1*), *S. venturii* (*Rpi-vnt1.1*) and *S. chacoense* (*Rpi-chc1*). *Rpi-sto1*, *Rpi-blb1* and *Rpi-pta1* are highly homologous genes, only differing by a few nucleotides (Vleeshouwers et al., 2008). *Rpi-blb2*, *Rpi-vnt1.1* and *Rpi-chc1* are recently cloned *R* genes, displaying a broad resistance spectrum (unpublished data) in experiments with the *P. infestans* isolates listed in Table 1. Furthermore, the availability of *Avr*s can help to recognize the functionality of *R* genes in the differential plants. This set of GM differential plants harboring single *R* genes in an identical genetic background is expected to provide accurate characterization of the virulence spectrum of *P. infestans* isolates.

Comparison of the GM and the expanded conventional differential set

In order to investigate whether extra *R* genes exist in the plants blb8005, sto389-4, pta767-1, blb2002, blb99-256-3, SW8540-025, SW8540-325, vnt367-1 and chc543-5 containing *Rpi-blb1*, *Rpi-sto1*, *Rpi-pta1*, *Rpi-blb2*, *Rpi-blb3*, *R3a*, *R3b*, *Rpi-vnt1.1* and *Rpi-chc1*, respectively, they were inoculated with eight *P. infestans* isolates showing different virulence spectra on the old differential set (Table 1).

Table 5. Virulence spectrum of eight isolates on *R* gene containing differential plants and on GM plants of cv Desiree containing the same inserted *R* genes

Plant genotype	*GM/ Conventional*	*R* gene	*P. infestans* isolate							
			EC1	PIC99189	IPO-0	H30P04	NL07434	NL08797	NL09129	NL11389
A01-20	*GM*	*blb1*	R	S	R	R	R	S	R	R
blb8005	*Conv.*	*blb1*	R	R	R	R	R	R	R	R
A09-268	*GM*	*sto1*	R	S	R	R	R	S	R	R
sto389-4	*Conv.*	*sto1*	R	SQ	R	R	R	RQ	R	R
A23-29	*GM*	*pta1*	R	S	RQ	R	R	S	R	R
pta767-1	*Conv.*	*pta1*	R	SQ	R	R	R	R	R	R
A02-33	*GM*	*blb2*	R	R	R	R	SQ	RQ	R	R
blb2002	*Conv.*	*blb2*	R	R	R	R	R	R	R	R
A03-142	*GM*	*blb3*	R	S	R	R	RQ	RQ	S	RQ
blb99-256-3	*Conv.*	*blb3*	R	SQ	R	R	R	R	S	R
A04-33	*GM*	*R3a*	S	R	R	S	S	S	S	S
SW8540-025	*Conv.*	*R3a*	S	R	R	S	S	S	S	S
A25-11	*GM*	*R3b*	S	S	S	S	S	S	S	S
SW8540-325	*Conv.*	*R3b*	S	S	S	S	S	S	S	S
A13-13	*GM*	*vnt1.1*	S	R	R	R	R	R	R	R
vnt367-1	*Conv.*	*vnt1.1*	Q	R	R	R	R	R	R	R
A17-27	*GM*	*chc1*	R	R	R	RQ	R	S	R	R
chc543-5	*Conv.*	*chc1*	R	R	R	R	R	SQ	R	R
MaR9	Conv.	*	R	R	R	R	R	R	RQ	R
Desiree	Conv.	-	S	S	S	S	S	S	S	S
Resulting *P. infestans* race (*R* gene containing transformants)			*R3a, R3b, vnt1.1*	*Rpi-sto1, Rpi-blb1, Rpi-pta1, Rpi-blb3, R3b*	*R3b*	*R3a, R3b*	*Rpi-blb2, R3a, R3b*	*Rpi-sto1, Rpi-blb1, Rpi-pta1, R3a, R3b, Rpi-chc1*	*Rpi-blb3, R3a, R3b*	*R3a, R3b*
Race spectrum from Table 1			1,3,4,7,10,11	1,2,10,11	7,10,11	3,7,10,11	ND	ND	ND	ND

*Ma*R9* contains *R1*, *Rpi-abpt1*, *R3a*, *R3b*, *R4*, *R8*, *R9* (Kim et al., 2012).
R, all of the inoculation spots on a detached leaf showed resistance (no visual necrosis or hypersensitive response); RQ, 6–7 out of eight spots on a leaf showed resistance; Q, 3–5 out of eight spots on a leaf showed resistance; SQ, 1–2 out of eight spots on a leaf showed resistance; S, all spots on a leaf showed susceptibility.
ND: not done; RQ was considered to be resistance; SQ and Q were considered to be susceptibility.

Table 5 shows that most plants-isolate combinations display an identical compatible or incompatible interaction pattern on GM and conventional differential plants containing the same *R* gene. However, also several discrepancies were found between the conventional and GM differential material containing the same *R* gene. These discrepancies might indicate the presence of additional *R* genes in the conventional differential plant. Alternatively, it could show a race specific background dependence of the *R* genes involved.

With respect to *Rpi-blb1*, *P. infestans* isolates PIC99189 and NL08797 were incompatible with blb8005 and compatible with GM differential A01-20. This indicates *Rpi-blb1* has been overcome by PIC99189 and NL08797 and it indicates the presence of additional *R* genes in blb8005 effective against isolates PIC99189 and NL08797. Interestingly, isolate NL08797 also showed different results between the GM and conventional differentials for *Rpi-pta1* and *Rpi-sto1*. This is another indication for the presence of additional *R* genes effective against NL08797 in conventional differential plants sto389-4 and pta767-1 except *Rpi-pta1* and *Rpi-sto1*, respectively. Differential results shown between blb2002 (containing *Rpi-blb2*) and *Rpi-blb2* containing transformant A02-33 also indicate that *Rpi-blb2* itself has been overcome by isolate NL07434 and that differential plant blb2002 might contain additional *R* genes.

Furthermore, *R3b* containing conventional plant SW8540-325 and transformant A25-11 were susceptible to all eight isolates tested.

Based on these experiments, virulence spectra (races) for the eight *P. infestans* isolates can be provided. According to the results obtained with the GM differential plants, five and six *R* genes were overcome by PIC99189 and NL08797, respectively. *R3b* is the only *R* gene overcome by isolate IPO-0. The other five isolates overcame two or three of the *R* genes tested (Table 5). From the race spectrum of four isolates (EC1, PIC99189, IPO-0 and H30P04), the *R* genes *R1*, *R7*, *R10* and *R11* were widely overcome.

Application of GM differential set in the field

Apart from their role in characterizing *P. infestans* isolates, (GM) differential plants can also be used as trap plants in the field. In this role, trap plants serve to detect the presence of specific virulence in the local *P. infestans* population. For this purpose, differential plants, wild *Solanum* spp. and susceptible and resistant commercially available cultivars were planted in “trap fields” in the Netherlands without fungicide spraying from 2008 - 2011. Simple observations of these plants were made and already provided an indication about the frequency of virulence towards a particular *R* gene (Fig. 1).

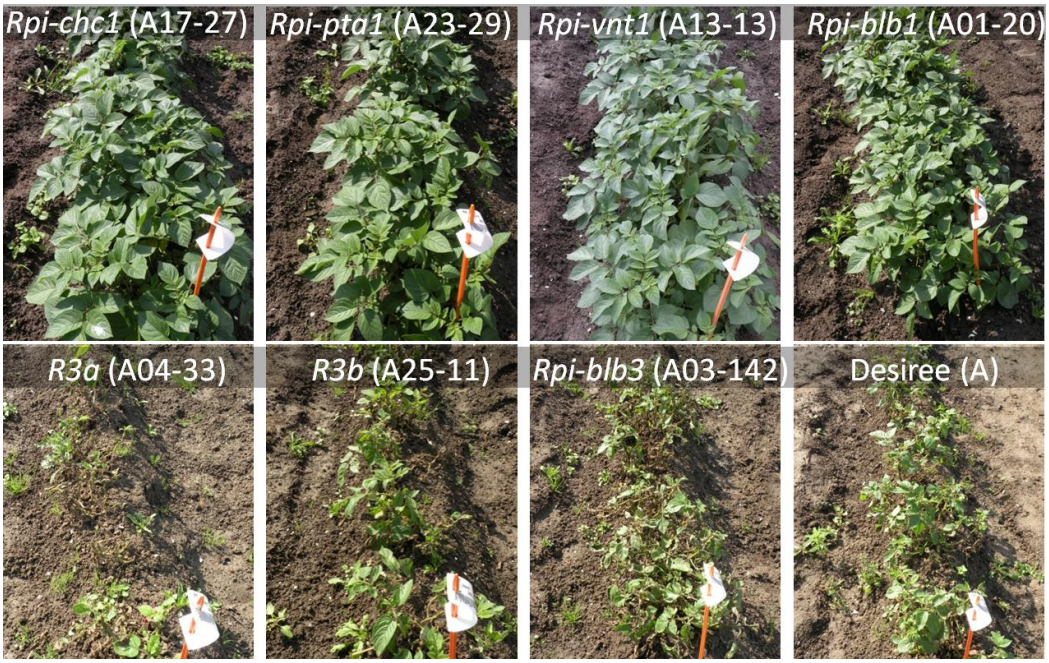

Figure 1. Members of the GM differential set as trap plants with natural infection in a Dutch potato bait field in Lelystad in 2011. (pictures were taken 49 days after planting the *in vitro* plants)

When natural *P. infestans* infections occurred on these trap plants, pure cultures (isolates) were produced in the laboratory. The resulting isolates were re-tested using spray inoculated DLA to confirm or reject virulence towards the *R* gene harbored in the plant and to establish the virulence spectra of individual isolates towards the *R* genes tested. During 2008-2011, one or more *P. infestans* isolates were found capable of overcoming each of the characterized *R* genes in the field and in the subsequent DLA (Table 6).

Table 6. Origin, genetic characteristics and virulence spectrum of *P. infestans* strains isolated in the trap fields with *R* gene containing (GM) plants

P. infestans isolate	Year of isolation	Trap plant	*R* gene in trap plant	Isolate Mating type	Isolate Haplo type	No.*	Detached Leaf Assay										
							A(10-23-2) *R1*	A01-20 *blb1*	A09-276$$$ *sto1*	A23-29 *pta1*	A02-33 *blb2*	Bionica *blb2*	A03-142 *blb3*	A04-33 *R3a*	A25-04 *R3b*	A13-13$$ *vnt1.1*	A17-27$ *chc1*
NL11452	2011	A01-20	*blb1*	A2	ND	2/2	ND	S	S	S	R	R	R	ND	ND	R	R
NL09067	2009	A01-84	*blb1*	A1	Ia		S	S	S	ND	R	ND	R	S	S	R	S**
NL11592	2011	A09-277	*sto1*	A2	ND	1/1	ND	S	S	S	R	R	R	ND	ND	R	R
NL11593	2011	A23-29	*pta1*	A1	ND	1/1	ND	S	S	S	R	R	R	ND	ND	R	R
NL09068	2009	A02-33	*blb2*	A2	Ia	1/1	SQ	R	R	ND	SQ	ND	R	S	S	R	R**
NL09030	2009	Bionica	*blb2*	A2	Ib	6/1	S	R	S	ND	S	ND	S	S	S	R	S**
NL10216	2010	Toluca	*blb2*	A2	Ia	3/2	ND	S	ND	S	ND	S	S	ND	ND	R	S
NL09300	2009	Toluca	*blb2*	A2	Ia		S	R	Q	ND	ND	S	R	S	S	R	R
NL11027	2011	A03-142	*blb3*	A2	ND	1/1	ND	R	R	R	R	R	S	ND	ND	R	S
NL11052	2011	A04-33	*R3a*	A1	ND	1/1	ND	S	S	S	R	R	R	ND	ND	R	S
NL11054	2011	A25-11	*R3b*	A2	ND	1/1	ND	S	S	S	R	R	S	ND	ND	R	R
NL11479	2011	A13-13	*vnt1.1*	A1	ND	1/1	ND	S	S	S	R	R	R	ND	ND	S	S
NL11480	2011	A17-27	*chc1*	A1	ND	1/1	ND	S	S	S	R	R	R	ND	ND	R	S
NL08645	2008	chc543-5	*chc1*	A2	IIa	2/1	ND	S	S	R***	R	ND	ND	ND	ND	R	S**

*: Total number of isolates picked up from the trap plants/number of isolates that were confirmed to break this resistance.
** *Rpi-chc1* containing plant chc543-5 was used instead of A17-27.
*** *Rpi-pta1* containing plant pta767-1 was used instead of A23-29.
$ A17-28, an independent Desisree transgenic event harboring *Rpi-chc1*, instead of A17-27 was used for DLA with isolates collected in 2009.
$$ A13-1 instead of A13-13 was used for the characterization of the isolate (NL08645) collected in 2008.
$$$ plant A09-1 was used for 2008 isolates, and A09-7 was used for 2011 isolates.
R, no sporulation observed on the inoculated leaf; RQ, sporulation occurred on around 25% of leaf surface; Q, around 50% of the leaf surface covered with mycelium; SQ, around 75% of inoculated leaf surface covered with mycelium; S, 100% of leaf surface covered with mycelium. ND: Not Done

Two *P. infestans* isolates (NL09067 and NL11452) were collected from *Rpi-blb1* containing, two Desiree transgenic events A01-84 and A01-20 in 2009 and 2011, respectively. Virulence of these *P. infestans* isolates towards *Rpi-blb1* and its homologs *Rpi-sto1* and *Rpi-pta1* was confirmed in subsequent DLA (Table 6). Also isolates NL11592 and NL11593 collected from the *Rpi-sto1* and *Rpi-pta1* containing GM differential plants, respectively, were compatible with the *Rpi-sto1*, *Rpi-pta1* and *Rpi-blb1* plants after re-testing in DLA. In addition to this, *Rpi-blb1*, *Rpi-sto1* and *Rpi-pta1* containing GM differentials also displayed a susceptible interaction following inoculation with *P. infestans* isolates NL11052, NL11054, NL11479 and NL11480 collected in the field from *R3a*, *R3b*, *Rpi-vnt1.1* and *Rpi-chc1* containing GM differential plants, respectively. Isolate NL08645, collected in the field from chc543-5, is interesting as it displayed an incompatible interaction with conventional *Rpi-pta1* containing plant pta767-1, while a compatible interaction with *Rpi-blb1* and *Rpi-sto1* containing GM differential plants was observed in the DLA. This observation might indicate the presence of additional *R* genes in pta767-1 or it is an indication that *P. infestans* isolate NL08645 is capable of differentiating between *Rpi-pta1* on the one hand and *Rpi-blb1* and *Rpi-sto1* on the other hand.

Additionally, isolate NL09030, collected from *Rpi-blb2* containing conventional potato cultivar Bionica, displayed an incompatible interaction with *Rpi-blb1* containing GM differential plant A01-20, but a compatible interaction with *Rpi-sto1* containing GM differential plant A09-276. Four out of ten isolates collected in the field on *Rpi-blb2* containing plants (A02-33, Bionica and Toluca) were confirmed to be compatible with *Rpi-blb2* GM plants in DLA (Table 6). Virulence towards *Rpi-vnt1.1* and *Rpi-blb2* is currently rare. Plants containing only *R1*, *R3a* or *R3b* were completely overcome by all members of the current Dutch *P. infestans* isolates (G. Kessel, unpublished data).

Discussion

Most differential sets currently available are for pathogens in seed propagated crops, such as bacterial spot on pepper and *Fusarium* wilt on watermelon (International Seed Federation, 2013). These differential sets mostly consist of varieties or near-isogenic lines which potentially only differ by the presence of individual resistance genes. This is, however, difficult to achieve for vegetatively propagated crops, like apple, rose, and potato due to their high levels of heterozygosity, polyploidy or long generation time. Previous attempts to produce differential sets in these crops include black spot in Rose (Whitaker et al., 2010), bacterial wilt in potato (DFID, 2013) and apple scab in apple (Bus et al., 2011). For potato late blight, a Mastenbroek differential set was built up, however, it was indicated that it needed further improvement (Kim et al., 2012).

For this purpose, the investigation of current Mastenbroek differential set Ma*R1* – Ma*R11* for resistance against late blight in potato, was investigated using 16 in house isolates. Ma*R5*, Ma*R6*, Ma*R8* and Ma*R9* harbored broader spectrum resistance compared to other Mastenbroek differential plants. This indicates the potential usefulness of the *R* genes in these plants for breeding programs, which might be

related to the limited exposure to the pathogen population. Most other Mastenbroek differential plants harbor relative narrow resistance spectrum, which calls for new genetic resources. For this purpose, four genotypes were added with a reduced *R* gene complexity for *R3a*, *R3b*, *R8* and *R9*. Also, the differential set is supplemented with *Solanum* spp. known to carry at least one recently cloned *R* gene. The result is a new, conventional differential set.

Previously, individual members of the Mastenbroek differential set Ma*R1* – Ma*R11* were demonstrated to harbor additional *R* genes (Huang et al., 2005, Li et al., 2011, Kim et al., 2012). Agro-infiltration with *Avr1*, *Avr2*, *Avr3a*, *Avr3b*, *Avr4*, *Avr8* and *Avr10* on Ma*R1* – Ma*R11* in this study demonstrated the presence of extra *R* genes in Ma*R5*, *6*, *7*, *10* and *11* (Table 2) and confirmed the presence of *R3a* and *R3b* in Ma*R3* (Huang et al., 2005, Li et al., 2011); *R3a*, *R4*, *R8* in Ma*R8*; *R1*, *Rpi-abpt1*, *R3a*, *R3b*, *R4*, *R8*, *R9* in Ma*R9* (Kim et al., 2012); and *R1* in Ma*R5*, *6* and *9* (Trognitz and Trognitz, 2007).

For Ma*R8*, Kim et al., (2012), using PCR, reported the presence of *R3a*, *R3b*, *R4* and *R8*. In the present study, using *Avr3b*, *R3b* activity was not found in Ma*R8*. Possibly, a *R3b* homolog, which does not respond to agro-infiltration with *Avr3b*, exists in Ma*R8*. *R3a* and *R3b* are closely linked in coupling phase in SH83-92-488, which was derived from Ma*R3*, and used for the cloning of both *R3a* and *R3b*. However, *R3a* or *R3b* were separately confirmed in Ma*R5*, *6*, *7*, *8*, *10* and *11* from the agro-infiltration experiments (Table 2). Additionally, the resistance caused by Ma*R6* and Ma*R7* may be only due to the stacking effects from *R1*, *R2*, *R3a* and *R3a*, *R4*, respectively. However, the results from Table 1 showed that isolate PIC99177 gave susceptibility on Ma*R1*-Ma*R4*, but resistance on Ma*R6* (containing *R1*, *R2*, *R3a*). If Ma*R6* does not contain additional *R* genes than *R1*, *R2* and *R3a*, then it should show susceptibility instead of resistance, with the assumption that the residual effect of resistance from *R* genes which were overcome is not big enough to induce the resistance. A similar case occurred also on Ma*R7* against isolate USA618. Ma*R7* (containing *R3a*, *R4*) should have performed resistance instead of susceptibility against isolate USA618, due to the resistance shown on Ma*R4* plants against isolate USA618, and Ma*R4* was shown to harbor only *R4*. No indications have shown the existence of *R11* in Ma*R11* so far. However, the existence of another *R* gene in Ma*R5* is expected, due to its resistance shown in the field against isolate IPO-C. The resistance gene in Ma*R5*, might be *R8* or *R8-like*, due to the weak response on Ma*R5* once detected during agro-infiltration using *Avr8*. However, further proof needs to be collected.

In a parallel approach, an isogenic GM differential set in potato cultivar Desiree was produced. It started with ten Desiree transformants, each harboring a single *R* gene: *R1*, *Rpi-blb3*, *R3a*, *R3b*, *Rpi-sto1*, *Rpi-blb1*, *Rpi-pta1*, *Rpi-blb2*, *Rpi-vnt1.1* and *Rpi-chc1* (Table 4). The availability of all the *Avr*s corresponding to these cloned *R* genes provides the possibility to quickly identify the *R* genes present in *Solanum* spp. with an unknown *R* gene content. This GM differential set is expected to give more

accurate results of the virulence characterization from *P. infestans* isolates due to the absence of interfering *R* genes in the genetic background. For the same reason, the GM differential set is expected to give more detailed results as trap plants in *P. infestans* population monitoring programs, to directly predict the functionality of specific *R* gene (stacks) in the field/lab. Furthermore, additional GM differential plants containing a range of small and larger *R* gene stacks would provide the possibility to investigate and predict the durability of resistance. This GM differential set is a continuously developing plant set. It is unique in the sense that it is the first isogenic differential set for a vegetatively propagated crop.

In Table 5, isolate H30P04 interacted in a susceptible way with *R3b* containing (GM) plants. This result deviates from the results reported by Huang et al., (2005). Rietman (2011) also showed that isolate H30P04 conferred susceptibility on *R3b* containing plant. The reason for this difference is unresolved so far.

In Table 5 and 6, *R1*, *R2*, *R3a* and *R3b* were shown for their relatively narrow spectrum of resistance against modern *P. infestans* isolates collected in the Netherlands. Unsurprisingly, *R1*, *Rpi-blb3* (*R2* homolog), *R3a* and *R3b* GM Desiree transformants were found to be susceptible to all the Dutch field isolates collected from trap plants in recent years. This is another confirmation of wide spread virulence to *R1*, *R2* homologs, *R3a* and *R3b* in the Dutch *P. infestans* population as reported earlier (Vleeshouwers et al., 2011).

In Table 6, *Rpi-pta1* containing plant pta767-1 was resistant to isolate NL08645 while *Rpi-blb1* and *Rpi-sto1* containing GM plants were susceptible in DLA. This is a strong indication for the presence of an extra *R* gene or *R* genes in the genetic background of pta767-1. Similar results indicated that the additional *R* gene(s) in pta767-1 also provide resistance against isolate NL08797 (Table 5). Similar observations were made between the *Rpi-blb1*, *Rpi-sto1* and *Rpi-blb2* containing plants blb8005, sto389-4, blb2002 and their GM counter parts. Additional *R* genes in the conventional wild *Solanum* spp. provided resistance against *P. infestans* isolates NL08797, PIC99189 and NL07434 respectively. This finding is in line with findings by Zhu et al. (2010) who reported indications in DLA results for the presence of extra *R* genes in blb8005 and sto389-4 using isolate PIC99192. Another reason that isolate NL08797 conferred resistance on the *Rpi-sto1*, *Rpi-blb1*, *Rpi-pta1* containing plants sto389-4, blb8005 and pta767-1, and susceptibility on their GM plants in cv Desiree, could be due to the genetic background of cv Desiree, that the resistance level of these three *R* genes expressed in cv Desiree background is not high enough to confer resistance against isolate NL08797. Another possibility is that *Rpi-blb1*, *Rpi-sto1* and *Rpi-pta1* co-evolved from the same ancestor, together with another gene which is closely linked with *Rpi-blb1*, *Rpi-sto1* or *Rpi-pta1* into different species. Additional studies are required to test the above hypotheses. The conventional way to test the presence of additional *R* genes is by

crossing the plant with a susceptible parent, and determining the (co-) segregation between resistance and molecular markers.

Rpi-blb1, *Rpi-sto1* and *Rpi-pta1* are highly homologous genes with only a few nucleotides difference and reacting to the same *Avr* gene (Vleeshouwers et al., 2008). Interestingly, isolate NL09030, collected from a *Rpi-blb2* containing plant, showed resistance on the *Rpi-blb1* containing GM Desiree plant, but susceptibility on the *Rpi-sto1* containing GM Desiree plant (Table 6). Therefore, isolate NL09030 could potentially be used as a differential isolate to distinguish *Rpi-blb1* from *Rpi-sto1* and *Rpi-pta1*.

An interesting observation came from the evaluation of *P. infestans* strains that were collected from resistant trap plants (Table 6). Not all isolates collected from *R* gene containing plants in the field (such as cv Toluca, cv Bionica and chc543-5) displayed virulence to the GM plant material containing the same *R* gene when they were re-tested in DLA. Only one out of six and two out of three isolates collected from cvs Bionica and Toluca, respectively, confirmed their virulence to the *Rpi-blb2* containing (GM) plant in DLA. One out of these two isolates also displayed virulence to *Rpi-chc1* in DLA (Table 6). The phenomenon, that not all collected isolates were confirmed their virulence in DLA towards the resistance involved, might also be caused by physiological aging of the trap plants. On the other hand, all the isolates directly collected from the GM differential plants in the field displayed virulence to the GM differentials after re-testing in DLA. This further indicates the improved accuracy of the GM differential set as trap plants under field conditions.

These findings predict the less accurate results from the conventional differential set as compared to the GM differential set due to the presence of additional *R* genes in the genetic background of at least some of its members, especially the wild *Solanum* spp. As a tool, the GM differential set is thus preferred.

Both (conventional and GM) differential sets have an important role in creating a better understanding of *P. infestans* dynamics with respect to virulence, monitoring and understanding *P. infestans* population dynamics at local, regional, national and possibly larger scales and in gaining understanding of the contribution of stacking *R* genes towards achieving durable resistance. A disadvantage of the GM differential set is the need for permits when used in field situations. This requirement restricts the use of the GM differential set in this application. In the future, a cisgenic version of the same differential set might alleviate these problems if the competent authorities decide to lift or reduce the current requirements for the introduction of cisgenic breeding products.

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Supplementary Table 1. List of primers

Label of primer pairs	Sequence (5'-3')	Reference
R1	CACTCGTGACATATCCTCACTA	Ballvora *et al.*, 2002
	CAACCCTGGCATGCCACG	
R2	GCTCCTGATACGATCCATG	Kim et al., 2012
	ACGGCTTCTTGAATGAA	
R3a	ATCGTTGTCATGCTATGAGATTGTT	Huang et al., 2005
	CTTCAAGGTAGTGGGCAGTATGCTT	
R3b	TAAAAGAGTTTGAAGTTGCTGATGCTCA	Rietman et al., unpublished
	TCTATTTCTGGACAAGCTTGCACAAC	
nptII	TCGGCTATGACTGGGCACAACAGA	Southern blot
	AAGAAGGCGATAGAAGGCGATGCG	
EF-1alfa	ATTGGAAACGGATATGCTCCA	qRT-PCR
	TCCTTACCTGAACGCCTGTCA	
nptII	CGTTGGCTACCCGTGATATTGC	qRT-PCR
	GTCCCGCTCAGAAGAACTCGTC	

CHAPTER 6

General discussion

Potato (*Solanum tuberosum*) is the third most important food crop in the world after wheat and rice. It originates from South America, where varieties, landraces and wild *Solanum* species are grown side by side (Delgado et al 2013). Potato late blight is one of the most serious diseases in potato, which causes 15% yearly losses (Haverkort et al., 2008). Late blight is caused by the pathogen *Phytophthora infestans*, which is an oomycete (Lee et al., 2012). Agriculture of potato in the Netherlands requires around 15 sprays of fungicides per growing season (Haverkort et al., 2008). However, this chemical prevention and protection strategy is not environmental friendly, and can cause residuals of fungicide(s) in the plants which might be harmful for human health (Latin, 2006). Therefore, the use of potato varieties with natural endogenous resistance is preferred. Single major *R* genes from *Solanum demissum*, also referred to as vertical resistance, were introgressed into cultivars by interspecific crossing and backcrossing. Remarkably, none of the single *R* genes used so far gave durable resistance to *P. infestans* populations. This moved the focus of breeding to horizontal resistance which is often partial and selects against major *R* genes. Also partial resistance did not always be proved to be durable in potato (Leonard-Schippers et al., 1994) but also not in other plant-pathogen interactions like barley against *Puccinia hordei* (Marcel et al., 2007). Apart from the durability problem, introgression breeding programs often introduced linkage drag with negative effects on different traits.

Several years before this thesis work started at Plant Breeding, research programs were initiated to revisit vertical resistance from *Solanum* species that are crossable with potato (Van der Vossen et al., 2003, Van der Vossen et al., 2005, Huang et al., 2005, Lokossou et al., 2009; Pel et al., 2009). A long term project in this field with genetic modification of major *R* genes is the DURPh project which is for 10 years funded by the government from the Netherlands (**DU**rable **R**esistance to ***Ph****ytophthora infestans*; Haverkort et al, 2008).

The main objective of this thesis was to investigate the possibility of gene stacking by transgenesis and cisgenesis to achieve durable resistance against potato late blight. Transgenesis is the genetic modification of an organism with transgenes, (partly) originating from non-crossable species or synthetic genes. Cisgenesis is the transfer of cisgenes into cultivated plants via genetic modification. A cisgene is a natural gene from the crop species itself or from a sexually compatible plant that can be used in conventional breeding (Cisgenesis, 2012). Durable resistance to a disease is the phenomenon that the resistance in a crop remains effective for a long time during wide cultivation. Besides the development and evaluation of tools, such an approach also requires a resistance management strategy to support durability. The availability of a reliable differential set for the characterization of *Phytophthora infestans* isolates and populations is an essential tool for an effective resistance management strategy. Therefore, the existing differential set has been updated and a novel GM differential set has been introduced. This thesis reports that plants with stacked *R* genes show a broadened resistance spectrum resulting from the sum of the spectra of the individual *R* genes in leaves and in tubers. During the investigations, cognate *Avr* genes became available, which are essential for the selection of GM plants with functionally stacked,

broad spectrum *R* genes (**Chapters 2, 3, 4**). The *R* genes, that were stacked, can be inherited stably to the next generation. Transgenic and cisgenic plants containing the *R* gene stacks showed resistance in the field trails in Wageningen, the Netherlands (**Chapters 3, 4**). Since it is known that *P. infestans* populations can differ tremendously between geographic locations, the transgenic plants containing *R* gene stacks were not only tested in the multiple locations in the Netherlands but also in Belgium and at two sites in North Korea. Interestingly, also in these unrelated locations, the transgenic plants containing *R* gene stacks showed full late blight resistance. Another way to study the employability of *R* genes and *R* gene stacks in different geographic locations is by analysing pathogen populations using *R* gene differential sets. Two late blight *R* gene differential sets, the Mastenbroek and Black's differential set, are available (Black et al., 1953; Malcolmson and Black, 1966). However, it turned out that too many members of these sets contained multiple *R* genes instead of a single one. In addition severalnovel *R* genes, are not represented in this set. **Chapter 5** suggests to extend the conventional differential sets with genotypes containing novel *R* genes and with new genotypes harboring reduced *R* gene complexity. To avoid the problems of unforeseen *R* gene complexities and different genetic backgrounds in the individual differential set members, we started a GM set with single *R* genes in the same genetic background. The use of these differential sets in the field as trap plants to catch new virulent isolates for individual *R* genes, and the use as a tool to compare novel isolates in a detached leaf assay is illustrated. The GM differential set is expected to better characterize *Phytophthora infestans* isolates and monitor the pathogen population, which could bring a breakthrough in global late blight *R* gene management.

This thesis is trying to solve the potato late blight problem from two sides: the plant side and the pathogen side. **Chapters 2, 3 and 4** are focused on the plant side to try to breed plants to be resistant to *Phytophthora infestans* isolates with various virulence spectra. **Chapter 5** is trying to build up a system which can monitor the adaption of the pathogen population by investigating the resistance spectrum of the existing breeding materials against the current *P. infestans* isolates. By investigating this host-pathogen interaction, it is expected to bring better understanding towards achieving durable resistance against potato late blight.

Strategies to achieve durable resistance

Gene stacking

So far, no single *R* genes were shown to harbor resistance to all tested *P. infestans* isolates. However, recent studies showed that plants with *R* genes stacks, introduced by introgression breeding, showed broad spectrum resistance, which is expected to delay and even prevent late blight disease (Verzaux, 2010, Kim et al 2012, Rietman et al 2012). This indicates that stacking of *R* genes is a potential strategy to tackle plant diseases in a durable way. On the other hand, cv Escort which is containing at least 4 *R* genes (*R1*, *R2*, *R3*, *R10*) (Bormann et al., 2004) is not considered a durable resistant variety. This indicates that *R* gene stacking alone is not enough and that the choice of *R* genes to be stacked is equally

important. The strategy to stack transgenes via genetic transformation has been successfully applied for protection against insects (Que et al., 2010). This thesis and another recent thesis from Plant Breeding (Jo, 2013) provide the first reports about stacking of cisgenic *R* genes.

Availability of cisgenes for stacking

Among the *R* genes against *P. infestans* isolates, those with broad and complementary resistance spectra can be expected to provide durable resistance. The *R* genes, *R1, R3a, R3b, R4, R10* have a narrow resistance spectrum because the majority of the isolates from different parts of the world showed virulence towards these genes (**Chapter 5**, Rietman, 2011). *R2* homologs (eg. *R2, R2*-like, *Rpi-blb3* have a broader resistance spectrum than *R1, R3a, R3b, R4, R10*, because the frequency of avirulence among tested isolates was higher (**Chapters 2**, **5**). The spectra of *Rpi-vnt1.1* and *Rpi-sto1* are referred to as broad (**Chapters 2**, **5**) because isolates that can break the resistance are very rare. From the functional point of view, the resistance from *R1, R2, R3a, R3b, R4* is widely defeated by isolates that were collected recently at different geographic locations. *Rpi-blb1* homologs (*Rpi-blb1*, *Rpi-sto1*, *Rpi-pta1*) and *Rpi-chc1* have relatively narrower resistance spectrum compared to *Rpi-blb2* and *Rpi-vnt1.1* (**Chapters 2**, **5**). The employment of narrow spectrum resistance genes should, however, not be excluded since defeated *R* genes were reported to show residual effects (Rauscher et al 2010) and contribute to the resistance spectrum *R* genes (**Chapter 2**, Kim et al., 2012).

The best way to test the resistance spectrum of *R* genes is by transforming them separately into an identical susceptible genetic background, and by successively testing these transformants with collected isolates from destinations according to the breeding objectives. The *R* genes (*Rpi-sto1, Rpi-vnt1.1* and *Rpi-blb3*) used for stacking in this thesis were selected in this way (**Chapters 2, 3 4**). Single *R* gene containing transformants harboring *Rpi-sto1, Rpi-vnt1.1* or *Rpi-blb3* showed broad resistance spectrum in DLA. Another way of identifying genes with broad resistance spectrum was suggested by Birch et al. (2008). They proposed to employ *R* genes cognate to invariant and functionally non-redundant effectors from the *P. infestans* population. One way to recognize these invariant and functionally non-redundant effectors could be via sequencing. With the current low prices for sequencing, more field isolates can be collected and sequenced. The sequence of the conserved regions between isolates can contribute to the recognition of these effectors. After cloning of these conserved *Avr* effectors into *Agrobacterium*, agro-infiltration can be used to efficiently screen potato accessions or wild species for resistance genes recognising these invariant effectors. This strategy is called effector genomics (Vleeshouwers et al., 2006). A similar sequencing strategy could be applied to identify genes with NBS-LRR structure in *Solanum* accessions. With the availability of a map position, the *R* gene candidates can be selected and cloned for functional validation. However, this approach is not very straight forward because *R* genes are located in clusters of highly repetitive nature, which is a tremendous challenge for sequence assembly. Therefore, more advanced sequencing techniques are needed.

Foliar resistance versus resistance in tubers

Most researchers have focused on foliar resistance against late blight, due to the idea that resistance in foliage can decrease the amount of inoculum exposed to tubers. However, if foliage blight is only partly controlled, sporangia produced from leaf and stem can be washed into the soil, which may result in tuber infection (Naerstad et al., 2007). Infected tubers can spread the disease further during storage, especially in tubers with insect induced wounds, or mechanical damage (Nyankanga et al., 2007). Especially in seed potatoes, late blight infection is a problem because it is a potential source for an epidemic in the next season. Therefore, late blight resistance in tubers is very important, especially in countries without the availability of sufficient amounts of fungicides and without good postharvest facilities.

Late blight spreads quickly in cool and high moisture environment, as Lapwood (1977) found out that tuber blight incidence was largely increased after 25mm rain than after 8mm rain. Pathak and Clarke (1987) hypothesized that resistance in tubers could be contributed by three major components: the periderm, the cortex cell layers and the medulla. Among which, the periderm can form a barrier at the surface to prevent pathogen infection (Lulai, 2002).

Previously, researchers studied the resistance in tubers on cultivars and seedling clones (Bhatia and Young, 1985, Pathak and Clarke, 1987, Inglis et al., 1996, Dorrance and Inglis, 1998). There are different methods to assess resistance in tubers against late blight, such as whole tuber assay (Swiezynski et al., 1991), tuber slice assay (Dorrance and Inglis, 1998) and tuber wounded assay (Nyankanga et al., 2008). Whole tuber assay can evaluate the pathogen penetration and colonization into the periderm and the cortical tissues, whereas tuber slice assay can assess the resistance based on *R* gene recognition. In this thesis, we used the tuber slice assay to assess the resistance caused by *R* genes.

One question which was raised while investigating the resistance in leaf and tuber, is whether resistance in leaf and tuber has any overlap or not (Wastie et al., 1987). To answer this question, they investigated 250 clones for their resistance in foliage and in tubers. These clones were from five different crossing populations, from which one parent was resistant in both foliage and tubers, and the other one was susceptible. By assessing the foliage resistance in the field and resistance in tubers in the laboratory from glasshouse-grown tubers, a correlation of resistance between foliage and tuber was determined. This research indicated that both resistance in foliage and in tuber are determined by the same gene or closely linked genes (Stewart et al., 1994). Furthermore, a major quantitative trait locus (QTL) was found with effects on resistance in leaf and in tuber against late blight which was on potato linkage group V (Oberhagemann et al., 1999). However, later on it turned out to be the major *R* gene *R1* (Ballvora et al., 2002). Besides these observations, Park et al., (2005) also showed that differences in resistance observed between cultivars in leaf and in tuber may be caused by single major *R* genes which are active in one or both tissues.

From the major *R* genes from *Solanum demissum*, *R1* (Ballvora et al., 2002, Park et al., 2005) and *R3b* (Lapwood and McKee, 1961, Park et al., 2005) can confer resistance both in leaf and in tuber (Pel, 2010). *R2*, *R3a* and *R4* could only confer foliar resistance (**Chapters 3 and 4**, Lapwood and McKee, 1961, Roer and Toxopeus, 1961, Park et al., 2005). For the *R* genes from other wild species, *Rpi-phu1* from *S. phureja*, $R_{pi\text{-}ber}$ from *S. berthaultii*, and *Rpi-rzc1* from *S. ruiz-ceballosii*, it was reported that they could confer resistance both in leaf and in tuber (Sliwka et al., 2006, Mayton et al., 2011, Sliwka et al., 2012). However, *Rpi-abpt1* (homologous to *R2*, *Rpi-blb3*) and the *RB* gene (Song et al., 2003), which is also known as *Rpi-blb1* (van der Vossen et al., 2003) can only confer foliar resistance against late blight (**Chapters 3 and 4**, Park et al., 2005, Halterman et al., 2008). Recently, via a transgenic approach, Pel (2010) confirmed that *R3a* can only show foliar resistance, but *Rpi-vnt1.1* showed resistance in conventional breeding material and after transformation both in leaf and in tuber. Furthermore, Pel (2010) also showed that transformants harboring *Rpi-blb3* (homologous to *R2* and *Rpi-abpt1*), can confer resistance both in leaf and in tuber. This is also shown in this thesis.

In our experiments, we confirmed that *Rpi-vnt1.1* and *Rpi-blb3* could confer resistance in both leaves and tubers (**Chapters 3 and 4**). However, transformants containing *Rpi-sto1*, homologous to *Rpi-blb1* and *Rpi-pta1*, showed a lower frequency and lower levels of resistance in tubers than in leaves, which appeared to be related to the copy number integration (**Chapters 2, 3, 4**).

Furthermore, the resistance in tubers was also tested on transformants containing *Rpi-blb2*. No clear infection symptoms were observed visually. However, around 100 sporangia per ml were found from each sample after washing each tuber slice with 20 ml water, compared to around 40,000 sporangia per ml from the susceptible control cv Desiree. Therefore we concluded that *Rpi-blb2* can confer resistance in the tuber (unpublished data). This is in accordance with earlier observations that moderate resistance in tubers was found in some tested breeding clones containing *Rpi-blb2* (Richard Mooijweer, Agrico, pers. communication). It can be concluded that *R* genes with known resistance in leaf and tuber such as *Rpi-vnt1.1* are also showing the similar phenomenon, like *Rpi-blb2*, after transformation. Other *R* genes with only known resistance in leaves can show after transformation in tubers: 1. only susceptibility, like *R3a*, 2. low levels of increased resistance, such as *Rpi-sto1*, or 3. relatively high levels of resistance, like *Rpi-blb3*.

Resistance in tuber can only be investigated in an efficient way if tubers with sufficient size are available. This is a problem in many plants from wild species, which can produce only a few tubers, with very small size. Therefore, an experiment was performed to investigate the induction of tuberization by grafting such plants with a potato variety as a scion. This could assist early screening of wild species based germplasm for resistance in tubers. Accessions from wild species plants blb8005 (containing *Rpi-blb1*), pta767-1 (*Rpi-pta1*), sto389-4 (*Rpi-sto1*), blb2002 (*Rpi-blb2*), blb99-256-3 (*Rpi-blb3*), SW8540-025 (*R3a*), vnt367-1 (*Rpi-vnt1.1*) were grafted with cv Desiree as scion, and cv Desiree was also grafted

reciprocally with blb2002 and SW8540-025 as scion to observe whether grafting can influence tuber size and the number of tubers produced or not. Non-grafted wild species plants and cv Desiree were used as controls. Tuber slices assay showed that all accessions which were used as stocks, together with the non-grafted wild species showed resistance to avirulent isolate 89148-09 (containing *Avr* genes for *Rpi-blb1*, *Rpi-blb2*, *Rpi-blb3*, *R3a*, *Rpi-vnt1.1*), except SW8540-025 (containing *R3a*). Cv Desiree, together with cv Desiree grafted with wild species plants as scions, showed as expected complete susceptibility. Furthermore, the number of tubers from wild species vnt367-1 was increased after grafting. Tubers harvested after grafting blb2002 with cv Desiree as scion, had bigger size but a lower number of tubers, compared to non-grafted blb2002. By using the reciprocal grafting between cv Desiree and the wild species plants, it is indicated that grafting itself, as expected, does not influence the resistance (unpublished data). This pilot experiment showed a positive effect of grafting on tuberization of wild material but it was performed only one time so solid conclusions can only be made after further investigation.

Transgenesis versus cisgenesis

In potato, commercial varieties harboring important characteristics such as consumption quality, yield, and earliness, do not often possess sufficient resistance against late blight at the same time (Cooke et al., 2011, Runno-Paurson et al., 2013). Therefore, a genetic transformation strategy with *R* genes can be applied on important existing cultivars, while at the same time preserving their desired characteristics. In this thesis, cultivars Desiree and Atlantic were chosen, since cv Desiree is widely grown, and cv Atlantic is famous for making potato chips (Maier et al., 1997). Furthermore, the genetic background of these two cultivars known so far is good for being the recipient for *R* gene stacking by transformation. Cv Desiree does not contain any known *R* gene against potato late blight, and cv Atlantic is known to harbor only *R1* (Grunwald et al., 2001). More importantly, these two cultivars were shown to have efficient regeneration and transformation ability from previous in house experience (unpublished data). In **Chapter 2**, three broad spectrum *R* genes; *Rpi-sto1*, *Rpi-vnt1.1* and *Rpi-blb3* were selected using a set of in house isolates and 28 isolates collected from China. These three selected *R* genes were later on used (described in **Chapters 2 and 3)** for the transgenic *R* gene stacking and (in **Chapter 4**) for the cisgenic *R* gene stacking.

For the transgenic gene stacking, transformants containing *nptII*:*Rpi-sto1*:*Rpi-vnt1.1*:*Rpi-blb3*, were shown to functionally express all three *R* genes. The resistance spectrum of the plants with stacked *R* genes is the sum of the individual *R* genes in leaves and in tubers (**Chapters 2 and 3**). These functional *R* genes were also stably inherited (**Chapter 3**). The plants with stacked *R* genes also showed field resistance during two years in Wageningen, the Netherlands. It was evident that plants with more than one copy of the integrated *R* gene(s) more often showed a good expression of the resistance in tubers. The triple *R* gene transformants also showed full resistance in two regions in North Korea in 2012 (Kim,

2013), and strong resistance for two years in Belgium (DuRPh project, unpublished data). The next step is to test the transformants containing *Rpi-sto1*, *Rpi-blb3* and *Rpi-vnt1.1* and the transformants with the single *R* genes *Rpi-sto1*, *Rpi-blb3* and *Rpi-vnt1.1* in the four regions in China, where the 28 isolates were collected. This is to observe their performance against the local *P. infestans* population.

If indications of resistance breakage are found on transgenic plants for one or two *R* genes used in the triple *R* gene containing plants, re-transformation with a new *R* gene, which can be potentially functional in the field, can be considered to prolong duration of the resistance. Such (re-) transformed plants can be made earlier by using *R* genes harboring broad and complementary resistance spectra, which can confer resistance to recently collected field isolates (Zhu and Jacobsen, 2012). By following these procedures of testing local *P. infestans* isolates, collected from the *R* gene containing trap plants, a scheme of *R* gene breeding can be made to contribute to local breeding programs. By testing locally collected isolates it is possible to start resistance breeding for a broader set of countries.

In our research, the transformation efficiency, with three *R* genes *Rpi-sto1*, *Rpi-blb3* and *Rpi-vnt1.1* in binary vector pBINPLUS harboring a T-DNA size of 22 Kb in cv Desiree, was clearly delayed in time (14% and 59% at five and nine months after the transformation) (**Chapter 2**); while the transformation efficiency for a single *R* gene *Rpi-pta1* (highly homologous to *Rpi-sto1*; Vleeshouwers et al., 2008) harboring a T-DNA size of 6.7 Kb with the same transformation vector was 33% and 43% (Zhu et al., 2010). A size effect on transformation efficiency of T-DNA in maize was also earlier found (personal communication Dr. Qiudeng Que, Syngenta Biotechnology, Inc.). Furthermore, 45% of *R* gene containing plants harbored one or two copies of T-DNA without the integration of vector borders and backbone (**Chapter 3**). It means that in our case, stacking of three *R* genes can be made in one step with sufficient numbers of useful plants to come to a transgenic variety with three *R* genes. In the single *R* gene transformants with *Rpi-sto1*, *Rpi-vnt1.1* and *Rpi-blb3*, it was observed that all three *R* genes were functional in cv Desiree. Therefore, it was expected that also the triple *R* gene construct was able to deliver transformants with three well-functioning *R* genes. However, this observation is not always made. Unpublished data from our group showed genotype dependent performance of *R* genes inserted by transformation but also by sexual crossing (Jack Vossen, personal communication). This observation has consequences for gene stacking research. In a transgenic approach, every *R* gene can first be tested as a single gene in the variety of interest, before it can be used in a construct with multiple *R* genes. For the three *R* genes that were selected in this thesis, no obstacles like genotype dependency or epistatic effects were found (**Chapters 2 and 3**).

Due to the public acceptance issue of transgenic plants which will be discussed in more detail later in this chapter, cisformants, which contain only cisgenes, without a transgenic selection marker, are expected to be preferred. Therefore, in our study cisformants containing stacked cisgenes were prepared in three ways: 1. by single vector transformation containing two *R* genes; 2. by co-transformation with

two vectors, each harboring one *R* gene; 3. by co-transformation with a *nptII* containing and a cisgenes containing vector, followed by removing *nptII* by recombination after sexual crossing. Cisformants were successfully obtained from all three approaches with proper functionality, and the resistance spectrum from plants with the stacked *R* genes was the sum of the stacked individual *R* genes. However, the frequency of cisgenic plants with functional *R* genes was different compared to what was obtained with transgenesis (**Chapter 4**).

Only 4% of the PCR plus plants produced from the second approach (co-transformation with two vectors each containing one *R* gene) were cisformants harboring stacked *R* genes, due to the high vector backbone incidence (81-100%) in the transformed plants containing both *R* genes. This is another disadvantage of co-transformation. Therefore, the application of single vectors with two or more *R* genes is the most useful strategy for the improvement of existing susceptible varieties. The third way of using co-transformation with the *nptII* containing vector could be useful if resistant breeding parents have to be created. After crossing and removing of *nptII*, a high percentage of the selected cisgenic plants will harbor well performing *R* genes. The only drawback of this procedure is that it will not be considered as full cisgenesis because by regulators it is interpreted to be a transgenic plant even when the *nptII* gene is removed afterwards (Evert Jacobsen, personal communication).

One important difference between transgenesis and cisgenesis was the frequency of plants with a high level of resistance. The percentage of plants with functional *R* genes produced via cisgenesis was lower than that from transgenesis. This high frequency of functional inserts in transgenic plants might be due to the presence of the *nptII* gene and kanamycin in the medium which is actively stimulating the functional selection of kanamycin resistant cells in the initial stage of transformation (**Chapter 2**). A remarkable observation was that cisformants produced from the third way (co-transformation with *nptII* and the three *R* genes on different vectors) showed 100% foliar resistance (**Chapter 4**). This indicates that the insertion place of *R* genes in the recipient genome, in this situation, is more frequently favoring *R* gene functioning together with the presence of functional *nptII*. This is to a certain degree remarkable because we observed in sexual offspring that the *R* genes were also good functioning if they were inserted at another position of the genome than the *nptII* gene (**Chapter 4**). Another observation was that the frequency of plants with only *Rpi-sto1* was much higher than after transformation with all four genes (*nptII*:*Rpi-sto1*:*Rpi-vnt1.1*:*Rpi-blb3*) on one vector used during transgenesis with the triple *R* gene transformation (**Chapters 2, 3 & 4**). It indicates that the high percentage of transformants with all three *R* genes (*Rpi-sto1*:*Rpi-vnt1.1*:*Rpi-blb3*) was different from the situation after co-transformation with *nptII* in a separate vector. Selection of transformants with kanamycin in the medium using vectors with *nptII* near the left border is discarding almost all instabilities which can occur during the early gene transfer process. This is not the case during (co-)transformation, providing opportunities to incorporate all kind of instabilities during the transformation process which is leading to a lower frequency of plants with all three *R* genes. In our case this was accompanied with insertion of additional copies of *Rpi-sto1*. This

observation stimulates the idea to come to the development of cisgenic selection markers, such as recently described marker for apple with the *Myb-10* gene leading to a visual selection marker by anthocyanin production (Kortstee et al., 2011). In this way, like observed with kanamycin resistance, a higher frequency of cisgenic plants can be directly selected with sufficient *R* gene functioning. However, a cisgenic selection marker near the left border, with selection at cellular level like kanamycin, helps also to avoid incorporation of T-DNA products resulting from instabilities during the transformation process.

The comparison between trans- and cisgenesis brought a few important observations: 1. Selection for transformation by PCR is really different and is accompanied with different types of T-DNA insertion compared to the *nptII* pre-selection from transgenesis, on the one hand leading more frequently to introgression loci with no or weak expression of the introduced *R* gene(s) (strategy 1, **Chapter 4**), and on the other hand to introgression loci with sufficient expression of the introduced *R* genes (strategy 3, **Chapter 4**) as observed after *nptII* selection in transgenic plants (**Chapters 2 and 3**). It means that more transformation events are needed for the selection of sufficient numbers of cisformants with well performing *R* genes; 2. The stability of the vector during the marker free transformation process seems to be low. This is especially observed when multiple *R* genes are present and no *nptII* selection marker is present and used near the left border (strategy 3, **Chapter 4**). Until now it was possible to introduce two *R* genes present on one vector by marker free transformation without using *nptII*. By using the co-transformation with *nptII* and cisgenes containing vectors, it was possible to produce cisformants with three *R* genes (**Chapter 4**). With respect to stability during the transformation process, a vector with three or more *R* genes appeared in our case to be problematic. Cisgenic stacking of three or four *R* genes can best be made by marker free transformation of two *R* genes followed by re-transformation with one or two additional *R* genes.

It can be concluded that cisgenic transformation brings a number of additional problems as discussed above. These problems need to be solved if cisgenesis in future would be allowed to be treated as a technology leading to a non-GMO or as an exempted GMO.

R-Avr *system for functional detection of* R *genes in plants with stacked* R *genes*

After discussing the kind of *R* genes used during stacking and the methods to achieve gene stacking, the question has to be answered how to detect the functionality of each inserted individual *R* gene. It is not always possible to find the differential isolates which could distinguish the individual functionality of each *R* gene in the *R* gene stacked plants, especially when broad spectrum *R* genes are combined.

Already in 1942, Flor proposed a gene for gene theory, which is still valid nowadays. That is: a plant contains a resistance (*R*) gene, versus the cognate a-virulence (*Avr*) gene possessed by the pathogen. A resistance symptom, such as hypersensitive response (HR), can only be achieved when the plant

harbors the *R* gene, and the invading pathogen contains its cognate *Avr* gene at the same time. Many scientists wonder why the pathogen has *Avr* genes to prevent themselves from further invading into plants. It was later determined that a-virulence genes have the primary function of being virulence effectors to suppress the pathogen associated molecular pattern (PAMP)-triggered immunity (Jones and Dangl, 2006, Kamoun, 2007). Plant *R* genes evolved later to recognize some of these a-virulence effectors (*Avr* genes) to trigger a hypersensitive response (HR).

The technique of agro-infiltration containing *Avr* genes was used to discover cognate *R* genes in accessions by observing whether a HR was achieved (Vleeshouwers et al., 2008). In this thesis, agro-infiltration was used to detect the functionality of inserted *R* genes in the transformants. This is especially necessary to determine the individual function of all inserted *R* genes in a single transformant with stacked *R* genes, since differential isolates, which could detect the functionality of each stacked *R* gene separately, are often not available when genes with broad and complementary resistance spectra are used. The combined system of DLA and ATTA appeared to be a strong tool for the detection of plants with multiple *R* genes. This is especially needed after cisgenesis because of the lower frequency of well-functioning *R* genes after transformation.

This technique is not only helpful to be used in leaves. Pathak and Clarke (1987) observed spots in tuber tissue after infection with *P. infestans* isolates, which were similar to the hypersensitive response (HR) in leaves. Recently, Pel showed that co-expression of the matching *R* and *Avr* genes in tuber slices can trigger a HR. The expression level of a *R* gene in combination with the expression level of the matching *Avr* gene can determine late blight resistance in tubers (Pel, 2010). Therefore, agro-infiltration with cognate *Avr* genes is expected to be widely used in the future for detecting the functionality of individual *R* genes in tubers of plants with stacked *R* genes, like it is used in leaves.

Susceptibility (S) genes

Susceptibility genes (S-genes) are plant genes required for reprogramming plant cells causing susceptibility to pathogens (Pavan et al., 2010). If such genes are mutated (loss of function), the pathogen is not able to reprogram host cells for a successful infection with the pathogen. The first susceptibility gene cloned was *PMR6*, it was reported to be required in the process to make *Arabidopsis* susceptible to powdery mildew (Vogel et al., 2002). Thus, the recessive non-functional allele of this gene *pmr6* caused resistance against this pathogen. This type of resistance was considered to be different from race-specific resistance caused by major *R* genes. In another research of screening 26,000 M2 *Arabidopsis* plants, after EMS treatment, for mutants with resistance to powdery mildew, four recessive loci, named *pmr1*, *pmr2*, *pmr3* and *pmr4*, were observed. Except the powdery mildew resistance from all four mutants, three of them *pmr1*, *pmr3* and *pmr4* showed also severe phenotypic defects. The *pmr1* mutant showed a defect in pollen tube growth, *pmr3* mutants performed a dwarf phenotype, and *pmr4* mutants showed abnormal growth of leaves (Vogel and Somerville, 2000). These results showed that the susceptibility genes harbor

not only the function of supporting pathogen invasion, but they also have their own tasks in the plant, like the a-virulence effectors/genes do have virulence functions in the pathogen.

Already in 1942, a mutant in barley conferring resistance against all powdery mildew isolates was described (Jorgensen, 1992). This resistance is caused by *mlo*, a recessive allele of the *Mlo* gene, which confers susceptibility to powdery mildew in barley. Later on, loss of function of a homolog of the barley *Mlo* gene was found in cherry tomato to confer resistance to tomato powdery mildew (Bai et al., 2008). Until now, most S genes were identified in *Arabidopsis* (Pavan et al., 2010), no S genes were observed in potato yet; however, a new project is going on in this field at Wageningen UR Plant Breeding.

It is expected that S genes against *P. infestans* isolates will also be discovered in potato which could be a new source of durable resistance genes. Such S genes could be used for the gene stacking strategy, together with earlier described major *R* genes harboring broad and complementary spectrum, to achieve durable resistance. However, potato is autotetraploid and not easy in breeding for recessive loss of function mutations. Therefore the RNAi approach is needed with dominantly expressed silencing of gene function, which can directly be applied in tetraploid varieties. The silencing of plant own genes is included nowadays in the so-called intragenesis approach (Rommens et al., 2007). Intragenes are a combination of functional gene parts from different cisgenes and are not known to exist in this combination in nature. Despite of having the same origin as cisgenes, intragenes are still considered to be treated as transgenes (EFSA, 2012). At the moment, the intragenic approach is in development, in combination with marker free transformation, and we have to see what type of GM regulation has to be applied for the release of intragenic varieties.

PAMP effector Inf1 receptor

Inf1 was recognized as a pathogen associated molecular pattern (PAMP), secreted by many *P. infestans* isolates. Inf1 is a protein produced by *Phytophthora.* It can work as an a-virulence factor to induce a HR reaction in *N. benthamiana* (Kamoun et al., 1998). In tomato, it can induce resistance to bacterial wilt disease by activating jasmonic acid and ethylene-mediated signaling pathways, but this does not produce a HR reaction (Kawamura et al., 2009). In *Solanum*, seven *S. huancabambense* and four *S. microdontum* accessions showed HR reaction to Inf1, but correlation between Inf1 response and resistance to Inf1 (non-)containing isolates was not observed (Vleeshouwers et al., 2006). By making a crossing population between a responding accession and a non-responding accession in *S. microdontum*, a receptor gene *Rinf1-mcd*, which was called elicitin response (ELR) was identified to respond to Inf1 (Verzaux, 2010). Stable transformants of cv Desiree for this ELR were later obtained and they showed an enhanced resistance against *P. infestans* isolates. In addition, ELR could also recognize elicitins from other *Phytophthora* pathogens (Du, 2013).

This kind of Inf1 receptors can be used as PAMP effectors in breeding programs to achieve enhanced resistance against potato late blight which is expected to be broad spectrum and durable.

Differential sets

Due to the narrow resistance spectrum in some members of the Mastenbroek differential set, and the existence of additional *R* genes in other members of the differential set, additional research on the Mastenbroek differential set was necessary. An updated conventional differential set was made, together with a new GM differential set, harboring ten transformants in cv Desiree, each with a single *R* gene (**Chapter 5**). The updated differential set was extended from the Mastenbroek set, with plants containing recently cloned *R* genes, and with selected plants harboring less complex *R* gene compositions. The GM differential plants showed resistance in the lab against avirulent isolates, and also in the field (except for the transformants containing *R* genes, from which the resistance was already overcome by the isolate IPO-C). An issue related to GM regulations is that some of these GM differential plants still contained backbone sequences, which might have constraints for field application in some regions or countries. Regulations for field trials can be strict. If in the near future plants produced via cisgenesis would be allowed to be treated as non GMO, a cisgenic version of the GM differential set could solve this problem. For the GM differential set, except the ten transformants in Desiree, another GM set containing *nptII* was also made available in *Nicotiana benthamiana* (unpublished data). The seeds from the selfing of the primary transformants, containing single integrations of each *R* gene, were sown on kanamycin containing medium. Populations with a 3:1 segregation for kanamycin resistance/susceptibility were selected. Kanamycin resistant seedlings were further selfed in the greenhouse, and the T2 seeds (after two selfings) were sown on kanamycin containing medium again in order to select for homozygosity of the inserted *R* gene. These homozygous plants are expected to be more convenient for conservation and multiplication than the GM plants of cv Desiree. One important point that needs attention is the observation that the resistance of *N. benthamiana* against potato late blight is age-related (Shibata et al., 2010). Young plants showed susceptibility, while mature plants show resistance to *P. infestans* isolates. Therefore, the GM differential set of potato *R* genes in *N. benthamiana* can only be used for this aim at the young plant stage.

Furthermore, cv Desiree with two stacked genes *Rpi-sto1*:*Rpi-blb3*, or *Rpi-sto1*:*Rpi-vnt1.1* (**Chapter 4**), three stacked genes *Rpi-sto1*:*Rpi-vnt1.1*:*Rpi-blb3* (**Chapter 2**), and even four stacked genes *Rpi-sto1*:*Rpi-vnt1.1*:*Rpi-blb3*:*Rpi-chc1* are available (unpublished). These plants with multiple *R* genes can be used in the field together with the GM differential set containing single *R* genes, to test the functionality of the plants with stacked *R* genes, and to monitor *P. infestans* population for new isolates which have broken one or more resistance genes.

The extended differential set of Mastenbroek is a starting point. This set can be extended with plants with less complex *R* genes selected from other members than Ma*R8* and Ma*R9*, and also from

plants with other new *R* gene resources, which are being investigated and will become available in the near future. The GM differential set can also be extended with new cloned *R* gene transformants like the recently cloned *R9a* gene (Jo, 2013). The GM differential set against potato late blight mentioned above is the first isogenic differential set in a vegetatively propagated crop (potato). This disease can also partly be investigated in a seed propagated isogenic differential set of *N. benthamiana*. Similar GM differential sets can also be set up in other crops for different pathogens to accurately characterize pathogen populations and isolates.

Future perspectives on public acceptance of cisformants

Genetic transformation has many unique functions, such as in our case: 1. the efficient improvement of existing varieties by the introduction of economic important traits in potato cultivars like cv Bintje and cv Russet Burbank without any linkage drag while maintaining their (quality) characteristics; 2. setting up a GM differential set harboring an isogenic genetic background to better characterize *P. infestans* isolates. These improvements are very difficult to achieve by conventional breeding in vegetatively propagated crops, such as potato, apple and banana, which are highly heterozygous. However, genetically transformed plants are generally not appreciated by the public, especially in Europe. Therefore, cisgenesis has been developed at the beginning of this century. The product of cisgenesis, called cisformants, only harbors cisgenes, which are natural copies of genes from the same species or crossable species, with its own promoter and terminator. Cisformants should not harbor any vector backbone sequences.

Although resistance can be obtained both from transgenesis and cisgenesis, cisgenesis was shown to be relatively more favorable than transgenesis by the consumers from a survey on consumers from 32 countries using transgenic and cisgenic apple with scab resistance as example. Cisgenic products perceived to be more natural, and safer for the environment (European commision, 2010). In 2012, a report on the practical consequences of the application of novel techniques in plant breeding showed that the risk for unintended effects of varieties produced by cisgenesis and conventional breeding is similar (Herzog, 2012). Another report from the Joint Research Centre (Institute for Prospective Technological studies) also mentioned that cisgenic products are similar to the products obtained via conventional plant breeding (Lusser et al., 2011). The European Food Safety Authority (EFSA) published a report about the safety assessment of plants developed by cisgenesis. It was concluded that cisgenic plants and conventionally bred plants harbor similar hazards, but intragenic and transgenic plants can produce new hazards (EFSA, 2012). A similar conclusion was drawn in a report by the Netherlands Commission on Genetic Modification (Commission on Genetic Modification, 2006). The US National Academy of Science suggested using the nowadays called cisgenes as a non-GMO alternative (The National Academy of Science, 2004). In 2011, the US Environmental Protection Agency (EPA) also proposed to exempt cisgenic plants from the GMO regulations (Reardon, 2011). Very recently, the European Working Group on new breeding techniques suggested that plants harboring less than 20 bp foreign DNA should not be

considered transgenic, due to the chance that such sequences with high homology can also be randomly found in the DNA of conventionally bred plants (European Working Group on new breeding techniques, unpublished data). Corresponding to the regulations on GMO, countries like Canada developed regulations based on the product, but EU regulations are mainly based on the technique itself as described in Directive 2001/18/EC, which is complicating the release of GM products (Food and Feed Safety, 2013). Regulations based on the process are inconsistent, since conventionally bred plants can also harbor similar safety concerns (Advisory Committee on Releases to the Environment, 2007). Thus regulations based on end products are preferred, in which case, cisgenic plants should be considered as non-GMO. Therefore, a separate non-GMO class between GMO and conventionally bred plants should be created for those cisgenic plants. Furthermore, regulations should also be clarified if these are based on the technique used or on the end product. International agreement on how to manage GMO and other plants like cisformants should be achieved to bring more convenience for international trading (Podevin et al., 2012).

In the future, under the described conditions, cisgenesis can be seen as a non-GMO and will be a new tool in conventional plant breeding.

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Summary

Among the many diseases of potato (*Solanum tuberosum* L.), which is the third food crop in the world after wheat and rice, late blight caused by the oomycete pathogen *Phytophthora infestans*, is one of the most serious diseases. In the last century, major resistance (*R*) genes were introgressed mainly from the wild species *Solanum demissum* into the cultivated potato *Solanum tuberosum*. However, introgression of late blight resistance genes by interspecific crosses followed by backcrosses, proved to be associated with linkage drag problems. The desired *R* gene is then closely linked with one or more unfavorable genes. Moreover, the obtained resistance in the varieties could be easily overcome by fast evolving virulence among *P. infestans* isolates. The introduction of the A2 mating type from Mexico to Europe resulted in genetically more diverse and complex *P. infestans* offspring, since initially only the A1 mating type existed. Therefore, new strategies for breeding varieties with durable and broad spectrum resistance needed to be developed.

Previous research indicated that varieties containing single major *R* genes did not show durable resistance. Therefore, the potato breeding and research community abandoned the introgression of major *R* genes and started breeding for horizontal resistance by combining multiple partial resistance genes. This quantitative resistance breeding approach was also not successful because the levels of resistance were too low, breeding was too complicated and the spectrum was not as broad as anticipated. Nowadays, the introgression of major *R* genes regained interest and two ways of resistance breeding can be distinguished: 1. molecular marker assisted resistance breeding or 2. genetic modification (GM) of existing varieties with cloned major *R* genes.

In this thesis, the time-saving GM approach has been investigated to achieve durable resistance against potato late blight in existing varieties by stacking of major *R* genes via transgenesis and cisgenesis (**Chapters 2, 3, 4**). These *R* genes are so called cisgenes and are unmodified copies of genes from the same or crossable species, harboring their own promoter and terminator sequences.

The main difference between cisgenesis and transgenesis is the resulting (end) product. The end products for the latter case are transformants, which contain transgenes, that can come from a very different species, such as the selection marker gene *nptII* coding for antibiotic resistance from bacteria. However, the end products of cisgenesis, called cisformants, only harbor cisgenes (which are natural genes from the same or crossable species). These cisformants are selected by PCR for the presence of *R* gene(s) and for the absence of vector backbone sequences. In our study, functionality of the individual *R* genes, in trans- and cisformants containing stacked *R* genes, was determined by detached leaf assays (DLA) using avirulent isolates and by agro-infiltration with *Avr* genes matching every single *R* gene. Their foliar resistance was also tested in the field, and their resistance in tubers was tested in the lab.

In order to ensure durability, an accurate and robust system must be available to monitor virulence in *P. infestans* populations. Differential sets with plants containing single *R* genes are important and developed

in many crops in order to facilitate both resistance breeding and genetic research on pathogen populations in different locations worldwide. The existing conventional differential potato set of Mastenbroek was updated and a start was made to develop a GM differential set with cloned *R* genes in individual transformants of cv Desiree (**Chapter 5**).

In **Chapter 2**, *R* genes with broad and complementary resistance spectrum were selected as a first step for *R* gene stacking. Selection for these *R* genes was performed using DLA with 44 selected late blight isolates. Out of four *R* genes (*Rpi-sto1, Rpi-vnt1.1*, *Rpi-blb3*, and *R3a*), three were selected for stacking experiments, *Rpi-sto1* from *S. stoloniferum*, *Rpi-vnt1.1* from *S. venturii* and *Rpi-blb3* from *S. bulbocastanum*. Cv Desiree transformants containing these three single *R* genes conferred resistance to 40, 43 and 37 out of 44 isolates, respectively. The *R3a* containing transformant conferred resistance to only five out of 44 isolates. These three broad spectrum *R* genes were then combined in one binary vector pBINPLUS containing *nptII* as kanamycin resistance marker. Transformants containing *nptII* and the three *R* genes showed foliar resistance in DLA against two isolates PIC99189 (*avrsto1*, *Avrvnt1*, *avrblb3*) and EC1 (*Avrsto1*, *avrvnt1*, *Avrblb3*). Furthermore, the functions of these three individual *R* genes were confirmed using the cross reacting *Avr* genes from the pathogen, since no isolates were available to distinguish the function of each *R* gene individually due to the broad resistance spectrum. The resistance spectrum of transformants containing the three *R* genes *Rpi-sto1, Rpi-vnt1.1* and *Rpi-blb3* showed after DLA the expected sum of resistance spectrum from all three individual *R* genes and no indications for epistatic effects were observed (**Chapter 2**). These triple *R* genes containing transformants showed also full resistance in the field after inoculation with IPO-C (*Avrsto1, Avrvnt1*, *avrblb3*) both in 2011 and 2012. Furthermore, these three *R* genes were inherited to the next generation as a cluster and retained their functionality after crossing. Generally, resistance in tubers of these plants showed also the summed spectrum of all individual *R* genes in both generations, as was the case in the foliar resistance test. It was remarkable that transgenic Desiree plants, harboring *Rpi-sto1* or *Rpi-blb3*, showed increased resistance in tubers, while their functional homologs *Rpi-blb1* and *R2,* did not show resistance in tubers of conventionally bred materials. The integration of T-DNA borders and vector backbone sequences was also investigated. Around 45% of the triple *R* gene containing transformants harbored one or two T-DNA copies, without the integration of T-DNA borders and vector backbone (**Chapter 3**).

The introduction of multiple *R* genes was also applied to produce cisformants, plants containing only cisgenes. Three approaches were taken: 1) two cisgenes were introduced through one marker free transformation vector, 2) two cisgenes were introduced through two separate marker free vectors by co-transformation, 3) co-transformation of two vectors, one only containing *nptII*, and the other one is a marker free transformation vector harboring three cisgenes. This co-transformation was followed by sexual crossing to remove selection marker *nptII*. All three approaches were successful in the production of cisformants. The first approach produced a high percentage (73%) of cisformants but, in contrast to transgenic plants, the percentage of plants showing full resistance in DLA was relatively low (42%). The

second approach produced only 4% of cisformants with stacked *R* genes, due to the high incidence of vector backbone sequence integration from two vectors used for co-transformation. All transformants obtained by the third approach showed full late blight resistance, which was very efficient compared to the first two approaches. This must be due to the use of the *nptII* selection marker. After crossing, the integration of both T-DNAs appeared to be unlinked in all tested transformants. Therefore, cisformants with active *R* genes could be obtained. The resistance level in tubers of cisformants was more frequently sufficient in plants with integration of two or more T-DNA copies, as it was also observed in the triple *R* gene transformants (**Chapter 3**). Not only the *R* genes from cisformants obtained using the third approach but also the cisformants from the first approach showed clustered inheritance in a crossing population, while the *R* genes segregated independently in the crossing population from a cisformant obtained using the second approach (**Chapter 4**).

The potato late blight differential set is used to characterize the virulence of *P. infestans* isolates, consisting of eleven plants which are expected to represent eleven different late blight *R* genes. Most differential plants were found to be susceptible to current late blight isolates, with the exception of the Ma*R8* and Ma*R9* plants. It had already been described that additional *R* genes were present in some members of this differential set. In **Chapter 5,** all eleven differential plants were tested for a hypersensitive reaction towards seven *Avr* genes. Only in three differential plants (Ma*R1*, Ma*R2* and Ma*R4*) no additional *R* genes were found, while for example Ma*R3*, Ma*R8* and Ma*R9* contained multiple *R* genes. The conventional differential set was extended with F1 and BC1 segregants harboring a reduced number of these *R* genes and potentially containing only one *R* gene (*R3a, R3b, R8* or *R9*, respectively) and with plants containing recently cloned *R* genes (*Rpi-blb3, Rpi-sto1, Rpi-blb1, Rpi-pta1, Rpi-blb2, Rpi-vnt1.1* and *Rpi-chc1*). A disadvantage of the (extended) conventional differential set is that their genetic background is different which is complicating the use of this set. Moreover, for none of the extended differential plants it can be ruled out that different additional *R* genes are present. Therefore, a GM differential set consisting of ten transformants of cv Desiree, each harboring a single *R* gene was compiled. This GM differential set is more reliable for characterization of *P. infestans* isolates and for the functional test of individual *R* genes, due to the isogenic background. As a proof of concept, the conventional and the GM differential sets were compared using recently collected isolates from Dutch fields in detached leaf assays. It was found that plants containing *Rpi-blb3, Rpi-blb1, Rpi-chc1, R8, R9, Rpi-vnt1.1* and *Rpi-blb2* showed a broader resistance spectrum as compared to *R1, R3a, R3b* and *R4*. Furthermore, the application of the GM differential set to monitor virulence towards the different *R* genes in local late blight populations using trap fields was investigated. The extended conventional and the GM differential sets are on continuously growing lists, which can be in the future updated with better performing, genetically more isogenic plants harboring novel *R* genes, or when new *R* genes are transformed into cv Desiree.

In the general discussion (**chapter 6**), related topics from different experimental chapters are discussed simultaneously, some additional experimental data are provided and a broader view on the research area is given.

In summary, five main conclusions can be drawn from this work: 1. broad spectrum resistance in leaf and tuber with stable inheritance can be achieved by gene stacking via transgenesis and cisgenesis; 2. The frequency of cisformants with sufficient resistance at foliage and tuber level is lower than in transformants; 3. *Avr* genes are highly needed to test for functionality of all stacked *R* genes in trans- or cisformants; 4. the GM differential set can be used to accurately characterize *P. infestans* isolates and to assess the employability of certain *R* genes in particular geographic locations; and 5. genetic transformation is a unique way to improve existing susceptible potato varieties such as the cvs Bintje and Russet Burbank which are grown at relatively large areas worldwide.

Samenvatting

Van de vele ziekten bij aardappel (*Solanum tuberosum* L.), het 3e wereldvoedingsgewas na rijst en tarwe, is de aardappelziekte die door de oömyceet *Phytophthora infestans* veroorzaakt wordt één van de meest hardnekkige ziekten. In de vorige eeuw werden dominante resistentie genen (*R*), voornamelijk van de wilde soort *Solanum demissum*, door introgressie in de cultuuraardappel *Solanum tuberosum* geïntroduceerd. Echter, introgressie van *Phytophthora R* genen via soortskruisingen gevolgd door herhaalde terugkruising met de cultuuraardappel bleek in de praktijk met negatieve zij-effecten gepaard te gaan.

Het gewenste *R* gen blijkt dan nauw gekoppeld te zijn met één of meer negatief werkende genen. Bovendien, kon de verkregen resistentie gemakkelijk doorbroken worden door zich snel ontwikkelende virulentie onder *P. infestans* isolaten. De introductie vanuit Mexico van het A2 paringstype in de Europese *P. infestans* populatie, die tot dan toe alleen uit het A1 paringstype bestond, resulteerde in meer diverse en complexe *P. infestans* nakomelingen. Daardoor werd het noodzakelijk om nieuwe veredelingsstrategiën te ontwikkelen, voor een meer duurzame en breedspectrum resistentie.

Omdat rassen met (enkelvoudige) dominante *R* genen geen duurzame resistentie gaven werd de introgressie van *R* genen door de veredelings- en onderzoeksgemeenschap opgegeven en werd een begin met de zogenaamde horizontale resistentieveredeling gemaakt waarbij meerdere partiële resistentiegenen gecombineerd worden. Ook deze kwantitatieve resistentieveredeling bleek bij aardappel niet succesvol te zijn omdat het niveau van de resistentie te laag was, de veredeling te complex, en ook het resistentiespectrum niet zo breed bleek te zijn als verwacht. Tegenwoordig heeft de introgressie van *R* genen weer nieuwe aandacht gekregen en er kunnen nu twee benaderingswijzen onderscheiden worden: 1. resistentieveredeling met *R* genen met gebruik van moleculaire merkers, en 2. genetisch modificatie (GM) van bestaande rassen met gekloneerde *R* genen.

In dit proefschrift is de tijdsbesparende GM benadering onderzocht om tot duurzame resistentie tegen *P. infestans* in bestaande rassen te komen door, met behulp van transgenese en cisgenese, meerdere dominante *R* genen te stapelen (**Hoofdstukken 2,3,4**). Deze *R* genen bevatten hun eigen promotor en terminator sequenties en komen uit dezelfde soort of uit kruisbare soorten, zogenaamde cisgenen.

Het belangrijkste verschil tussen cisgenese en transgenese is het resulterende eindproduct. De eindproducten van het laatst genoemde geval zijn transformanten die transgenen bevatten die van een duidelijk verschillende bron afkomstig zijn, zoals de bacteriële selectiemerker *nptII* die voor een antibioticumresistentie codeert. Echter, de eindproducten van cisgenese, cisformanten genoemd, bevatten alleen cisgenen, die natuurlijke genen van dezelfde of kruisbare soorten zijn. Deze cisformanten zijn met behulp van PCR geselecteerd op de aanwezigheid van ingebrachte *R* genen en op de afwezigheid van vector "backbone" sequenties. De functionaliteit van de ingebrachte individuele *R* genen in trans- en cisformanten met gestapelde *R* genen werd vastgesteld met behulp van een bladtoets (DLA) met

(a)virulente isolaten maar ook door middel van agro-infiltratie van avirulentie (*Avr*) genen, die een kruisreactie met individuele *R* genen veroorzaken. De resistentie in het loof werd ook in het veld getoetst en de resistentie in de knol werd in het laboratorium onderzocht.

Om duurzaamheid te bewerkstelligen, is een accuraat en robuust system nodig om de virulentie van *P. infestans* populaties te controleren. Planten die enkelvoudige *R* genen bevatten zijn hiervoor belangrijk. Deze zogenaamde "differential sets" zijn in verschillende gewassen ontwikkeld om zowel de resistentieveredeling als genetisch onderzoek aan pathogeenpopulaties op verschillende locaties wereldwijd te faciliteren. De bestaande aardappelziekte "differential set" van wijlen Ir. Mastenbroek is bestudeerd en geactualiseerd en er is een begin gemaakt om een GM "differential set" van cv Desiree te ontwikkelen door transformatie van gekloneerde *R* genen (**Hoofdstuk 5**).

Als eerste stap om tot *R* genstapeling te komen, werden *R* genen geselecteerd die een breed- en complementair spectrum bezaten (**Hoofdstuk 2**). Selectie van deze *R* genen werd uitgevoerd middels DLA met 44 geselecteerde *P. infestans* isolaten. Van de 4 geteste *R* genen (*Rpi-sto1*, *Rpi-vnt1.1*, *Rpi-blb3*, en *R3a*) werden er 3 geselecteerd om tot stapelingsexperimenten te komen, Dit waren *Rpi-sto1* van *S. stoloniferum*, *Rpi-vnt1.1* van *Solanum venturii* en *Rpi-blb3* van *Solanum bulbocastanum*, respectievelijk. Transformanten van cv Desiree met deze 3 individuele *R* genen waren respectievelijk resistent tegen 40, 43 en 37 van de 44 geteste isolaten. De *R3a* bevattende transformant was resistent tegen slechts 5 van de 44 isolaten. De 3 *R* genen met een breedspectrum resistentie werden toen in de binaire vector pBINPLUS gecombineerd die ook het *nptII* gen als kanamycine resistentiemerker bevatte. Transformanten met het *nptII* gen en de 3 *R* genen lieten in DLA toetsen de verwachte bladresistentie tegen de isolaten PIC99189 (*avrsto1*, *Avrvnt1*, *avrblb3*) en EC1 (*Avrsto1*, *avrvnt1*, *Avrblb3*) zien. Verder werd het functioneren van deze 3 individuele *R* genen bevestigd aan de hand van de reactie op de kruisreagerende *Avr* genen omdat er geen isolaten beschikbaar waren die in staat waren om de functionaliteit van elk geintroduceerd *R* gen te onderscheiden. Het resistentiespectrum van transformanten met de 3 *R* genen *Rpi-sto1, Rpi-vnt1.1* en *Rpi-blb3* liet op basis van DLA de verwachte som van de resistentiespectra van alle 3 individuele *R* genen zien (**Hoofdstuk 2**). Voor deze combinatie van 3 genen werden geen epistatische effecten waargenomen. Deze transformanten met 3 *R* genen vertoonden ook volledige resistentie in het veld na inoculatie met het isolaat IPO-C (*Avrsto1*, *Avrvnt1*, *avrblb3*) zowel in 2011 als in 2012. Verder erfden deze 3 *R* genen na kruising als cluster naar de volgende generatie over en behielden zij hun activiteit. In het algemeen werd waargenomen dat de resistentie in de knollen van deze planten ook de som van de spectra van alle individuele *R* genen liet zien zoals dat ook voor de bladresistentie test was waargenomen. Het was opmerkelijk dat transgene Desiree planten met *Rpi-sto1* of *Rpi-blb3* een verhoogde resistentie in de knollen lieten zien, terwijl hun functionele homologen *Rpi-blb1* en *R2*, respectievelijk in klassiek veredelingsmateriaal geen knolresistentie gaven. De integratie van "T-DNA borders" en vector "backbone" sequenties werd ook onderzocht. Ongeveer 45% van de transformanten met 3 *R* genen

bezaten 1 of 2 T-DNA kopieën zonder de ongewenste integratie van "T-DNA borders" en vector "backbone" (**Hoofdstuk 3)**.

De transgene introductie van meerdere *R* genen werd ook toegepast om cisformanten te produceren die alleen cisgenen bevatten. Drie benaderingen werden bestudeerd: 1) twee cisgenen werden mbv één merker vrije transformatie vector geïntroduceerd, 2) twee cisgenen werden mbv co-transformatie geïntroduceerd dmv twee merker vrije vectoren, 3) co-transformatie met twee vectoren, één bevatte alleen *nptII* en de andere was een merker vrije vector met 3 *R* genen. Deze co-transformatie werd opgevolgd door seksuele kruisingen om de selectie merker *nptII* te verwijderen in de nakomelingen. Alle 3 de benaderingen waren succesvol bij de productie van cisformanten. Benadering 1) produceerde een hoog percentage (73%) cisformanten, maar het percentage planten dat volledige resistentie in de DLA liet zien was relatief laag (42%), dit in tegenstelling tot transgene planten verkregen mbv kanamycine resistentie. Benadering 2) produceerde slechts 4% cisformanten met gestapelde *R* genen, als gevolg van het hoge percentage vector "backbone" integratie dat veroorzaakt werd door het gebruik van twee vectoren tijdens de co-transformatie. Alle transformanten die via de benadering 3) verkregen werden waren volledig resistent tegen *Phytophthora*, wat veel efficiënter was dan bij de benaderingen 1) en 2). Dit moet duidelijk samenhangen met het gebruik van de *nptII* selectie merker. Na kruising bleken beide T-DNA's in nakomelingen van de twee geteste transformanten niet gekoppeld te zijn. Daarom konden cisformante zaailingen met actieve *R* genen eenvoudig geselecteerd worden. Het resistentieniveau in de knollen van cisformanten met meerdere geïntegreerde T-DNA's was vaak hoger dan in planten met 1 T-DNA zoals dat ook al in de transformanten met 3 *R* genen in **Hoofdstuk 3** was waargenomen. Niet alleen de cisformanten verkregen uit benadering 3) maar ook die van benadering 1) lieten een geclusterde overerving in kruisingspopulaties zien, terwijl de genen onafhankelijk splitsten in de kruisingspopulatie van een cisformant uit benadering 2) (**Hoofdstuk 4**).

De aardappelziekte "differential set" wordt onder andere gebruikt om de virulentie van *P. infestans* isolaten tegen de betreffende *R* genen te testen. De set bestaat uit 11 planten (Ma*R1*-Ma*R11*) die naar verwachting 11 verschillende resistentiegenen tegen *P. infestans* vertegenwoordigen. Veel individuele Ma*R* planten bleken vatbaar te zijn voor recent verkregen isolaten met uitzondering van de Ma*R8* en Ma*R9* planten. Het was al eerder in de literatuur beschreven dat er extra *R* genen in sommige Ma*R* planten aanwezig kunnen zijn. In **Hoofdstuk 5** werden alle 11 Ma*R* planten op een hypersensitiviteitsreactie (HR) met 7 *Avr* genen getest. Slechts in 3 planten (Ma*R1*, Ma*R2* en Ma*R4*) werden geen extra *R* genen gevonden terwijl bijvoorbeeld Ma*R3,* Ma*R8* en Ma*R9* meerdere *R* genen bezaten. De klassieke "differential set" werd aangevuld met F1 en BC1 recombinanten die een verminderd aantal *R* genen en potentieel nog maar 1 *R* gen bezitten (*R3a, R3b, R8* en *R9*) en met planten die recent gekloneerde *R* genen bezitten (*Rpi-blb3, Rpi-sto1, Rpi-blb1, Rpi-pta1, Rpi-blb2, Rpi-vnt1* en *Rpi-chc1*).

Een nadeel van de (uitgebreide) klassieke" differential set" is dat de genetische achtergrond van de Ma*R* planten verschillend is, wat het gebruik van deze set complexer maakt. Bovendien, kan voor geen van deze toegevoegde "differentials" uitgesloten worden dat andere, extra *R* genen aanwezig zijn. Het is zelfs aangegeven dat daar aanwijzingen voor zijn. Daarom werd er ook een GM "differential set" met 10 transformanten van cv Desiree samengesteld, waaraan slechts één *R* gen is toegevoegd. Deze GM "differential set" is, vanwege de isogene achtergrond, betrouwbaarder voor het karakteriseren van *P. infestans* isolaten en voor het functioneel testen van individuele *R* genen. Om dit concept te bewijzen werden de klassieke en de GM "differential sets" in bladtoetsen (DLA) geïnoculeerd met recent van Nederlandse aardappelvelden verkregen isolaten. Er werd duidelijk waargenomen dat de planten met *Rpi-blb3, Rpi-blb1, Rpi-chc1, R8, R9, Rpi-vnt1.1* en *Rpi-blb2* een breder resistentiespectrum vertoonden dan die met *R1, R3a, R3b* en *R4*. Verder werd de toepassing van de GM "differential set" onderzocht om de virulentie van *R* genen van de verschillende isolaten van lokale *P. infestans* populaties in vangvelden te monitoren. De uitgebreide klassieke en de GM "differential sets" vormen een voortdurend groeiende lijst die in de toekomst met beter presterende, genetisch meer isogene planten met nieuwe *R* genen geactualiseerd kunnen worden.

In de algemene discussie (**Hoofdstuk 6**), worden gerelateerde onderwerpen van de verschillende hoofdstukken bediscussieerd en sommige aanvullende experimentele resultaten en een bredere visie op het onderzoeksgebied besproken.

Op grote lijnen kunnen uit dit onderzoek vijf conclusies getrokken worden: 1. via trans- en cisgenese kan door *R* genstapeling breed spectrum blad- en knolresistentie tegen *Phytophthora infestans* verkregen worden die stabiel is en geclusterd overerft; 2 De frequentie van cisformanten met voldoende resistentie op blad en knolniveau is lager dan bij transformanten; 3. *Avr* genen zijn noodzakelijk om de functionaliteit van alle gestapelde *R* genen in trans- en cisformanten te kunnen testen; 4. de GM "differential set" kan gebruikt worden om op accurate wijze *P. infestans* isolaten te kunnen karakteriseren en om de bruikbaarheid van bepaalde *R* genen in bepaalde geografische gebieden vast te stellen; 5. genetische transformatie is een unieke manier om bestaande vatbare aardappelrassen, zoals Bintje en Russet Burbank die nog steeds op relatief grote oppervlakten verbouwd worden, snel te verbeteren.

中文摘要

马铃薯是世界第三大粮食作物，位居小麦和水稻之后。晚疫病是马铃薯最严重的病害之一，它是由卵菌纲疫霉属致病疫霉 *Phytophthora infestans* 所致。上世纪，很多抗马铃薯晚疫病的主效基因被从野生种马铃薯 *Solanum demissum* 引入栽培种马铃薯 *Solanum tuberosum* 中。然而通过种间杂交然后再回交的途径引入抗病基因存在遗传连锁累赘的问题（目标基因与一个或多个不利基因连锁，从而被同时引入栽培种中）。另一问题是引入栽培种中的抗性会很快被快速进化的晚疫病菌株克服。晚疫病菌株从前只有 A1 交配型，但后来墨西哥的 A2 交配型入侵欧洲，致使两种交配型的后代菌株的基因型更加多样化和复杂化。因此，预培育出具有持久和广谱抗病性的品种，需要制定新的策略。

前人研究表明，只含有一个主效抗病基因的品种不具有抗病持久性。因此，马铃薯育种研究从引进垂直抗性（主效抗病基因或质量抗病基因）转向引进水平抗性（微效多基因或数量抗病基因）。由于数量抗病性育种的复杂性，其抗性水平低以及抗病广谱性比较差，这个策略未取得成效。目前，引进主效抗病基因重新成为研究热点。两种育种方式分为：1. 分子标记辅助育种。2. 通过遗传转化将主效抗病基因引入到现有品种中。

本文通过遗传转化（同源转基因和异源转基因）的方法将多个主效基因叠加到栽培种中，预期达到对马铃薯晚疫病的持久抗性。本文中所应用的抗病基因是同源基因。同源基因是来自于同种或可杂交种未经任何修饰的基因拷贝，这些基因含有本身的启动子和终止子。

同源基因和异源基因的主要不同点在于最终产物。后者是异源转化体，含有来自不同种间的异源基因，例如对抗生素有抗性的选择标记基因 *nptII*，来自于细菌。然而同源转化体只含有来自同种或可杂交种的同源基因。这些同源转化体利用 PCR 技术来检测抗病基因的存在，然后剔除带有载体骨架序列的植株。在本文中，我们采用离体叶片菌株接种和单个无毒基因的根瘤农杆菌浸润法来检测抗病基因的功能性。我们也进行了叶片田间抗性和在实验室对马铃薯块茎抗性的检测。

为了达到持久抗病型，我们需要一个准确并且高效的体系来检测晚疫病群体的致病力。鉴别寄主是由含不同基因型的植株组成。鉴别寄主通过鉴别菌株的生理小种来辅助抗病育种。本文中，对现存的 Mastenbroek 马铃薯晚疫病鉴别寄主进行了更新。本文还建立了一套转基因鉴别寄主，这些鉴别寄主是由同一栽培种的转基因植株所组成，每个植株含有不同的抗病基因。

在第二章，我们通过利用 44 个晚疫病菌株对含有单个抗病基因的转化体进行离体叶片接种，选择出了具有广谱且互补抗性的基因来进行基因叠加。从四个基因中（*Rpi-sto1, Rpi-vnt1.1, Rpi-blb3,* 和 *R3a*），三个被鉴定为具有广谱且互补抗性，他们分别是来自 *S. stoloniferum* 属的

Rpi-sto1，来自 *S. venturii* 属的 *Rpi-vnt1.1*，和来自 *S. bulbocastanum* 属的 *Rpi-blb3*。三个广谱抗性基因（*Rpi-sto1, Rpi-vnt1.1* 和 *Rpi-blb3*）的栽培种 Desiree 的转化体分别对 40，43，和 37 个菌种表现出抗性。而含有 *R3a* 的栽培种 Desiree 转化体只对 5 个菌种表现出抗性。这三个选出的基因被构建到含有卡那霉素抗性标记 *nptII* 的双元载体 pBINPLUS 中。从而产生的含有 *nptII* 和三个抗病基因的转化体在离体叶片接种中对两个菌种 PIC99189 (*avrsto1, Avrvnt1, avrblb3*) and EC1 (*Avrsto1, avrvnt1, Avrblb3*) 均产生抗性。由于所选用基因的广谱性，无法找出 3 个菌株来分别验证这三个基因的功能，三个基因的功能进一步通过相应无毒基因的农杆菌接种法得到了验证。三基因转化体的抗性谱是三单个基因的总和（**第二章**）。在 2011 和 2012 年的田间试验中，含三个抗病基因的转化体对 Wageningen 当地菌株及接种菌株 IPO-C (*Avrsto1, Avrvnt1, avrblb3*)表现出完全抗性。此外，经过杂交实验这三个抗病基因作为一个整体被稳定遗传到下一代，并均表现其抗性功能。总体来讲，含三个抗病基因的转化体的薯块抗性谱和叶片抗性谱均是三个单个基因抗性谱的总和，而且这个抗性谱均被稳定遗传到下一代。从薯块抗性来看，含单个基因 *Rpi-sto1* 或 *Rpi-blb3* 的 Desiree 品种转化体相对未转化的 Desiree 薯块表现出提高的抗性；而含单个基因 *Rpi-blb1* 或 *R2* 的传统育种植株并未表现出相对 Desiree 薯块的薯块抗性。我们也对 T-DNA 边界和载体骨架序列的整合进行了研究，大约 45%的三基因转化体含有 1-2 个 T-DNA 拷贝，并且不含有 T-DNA 边界和载体骨架序列（**第三章**）。

我们也将多个抗病基因引入只含有同源基因的同源转化体。我们采用了三种方法：1）通过不含标记基因的载体转入两个同源基因；2）共转化两个同源基因，它们分别来自两个不含标记基因的载体；3）共转化两个载体，其中一个只含有 *nptII*，另一个是只含有三个同源基因的载体。我们对经在这个共转化得到的转化体进行杂交，从而移除 *nptII*。这三种方法均成功地获得了同源转化体。第一种方法产生了高达 73％的同源转化体，但是和英语标记基因的异源遗传转化相比，在离体叶片接种中表现完全抗病的植株比例相对较低（42％）。第二种方法只产生了 4％的含有叠加基因的同源转化体，由于应用两个载体进行共转化，载体骨架的插入率也相应提高。所有通过第三种方法得到的转化体均表现在离体叶片接种中表现出完全抗性。这可能是由于应用标记基因 *nptII* 的缘故。我们选取了三个同时含有 *nptII* 和三个同源基因的植株进行回交，结果表示在这三株中，*nptII* 和同源基因未发生遗传连锁。因此，我们可以获得具有抗病功能基因插入的同源转化体。在薯块抗病检测中，足够的抗病表型多出现在具有两个或多个 T-DNA 插入的同源转化体中；而在异源转化体中，不存在这种抗性和拷贝数相关联的现象（**第三章**）。从第一种和第三种方法获得的同源转化体中的多个抗病基因经杂交后表现出聚集的遗传；从第二种方法获得的同源转化体中的两个抗病基因经杂交后表现出遗传分离（**第四章**）。

马铃薯晚疫病鉴别寄主晚疫病菌菌株的毒力谱。现有的鉴别寄主是由十一棵植株组成，分别命名为 Ma*R1*－Ma*R11*，这十一棵植株过去被认为含有十一个不同的抗病基因。目前大多数鉴

别寄主植株，除了 Ma*R8* 和 Ma*R9*，均对当前的晚疫病菌株表现出感病。而且已有文献表明有些鉴别寄主植株不只含有一个抗病基因。在本文的第五章，我们对所有十一棵鉴别寄主植株分别进行了含有七个无毒基因的农杆菌接种试验。通过检测接种结果是否表现过敏性反应，我们得出结论只有三棵鉴别寄主植株不含油额外的抗病基因。我们对传统鉴别寄主用含有较少抗病基因的杂交和回交植株，和含有近期被发现的抗病基因的植株进行了延伸。传统鉴别寄主的弊端是各个植株均具有不同的遗传背景。而且单个植株可能含有一个以上的抗病基因。于是，我们建立了一套转化体鉴别寄主。这套鉴别寄主是由十棵栽培种 Desiree 的转化体所组成，每棵植株含有一个抗病基因。由于这套鉴别寄主的近等基因背景的特性，它能更可靠地用于鉴别菌株的毒力谱。通过对这些鉴别寄主接种近年来采集的菌株，我们发现和 *R1*，*R3a*，*R3b*， *R4* 相比，*Rpi-blb3*， *Rpi-blb1*， *Rpi-chc1*， *R8*， *R9*， *Rpi-vnt1.1* ， *Rpi-blb2* 具有较广谱的抗性。本文列出的扩展的传统鉴别寄主和新的转化体鉴别寄主都只是个开端，在未来的研究中可以继续延伸。

总体讨论（**第六章**）对不同试验章节相关的主题进行了讨论。在本章中，也添加了一些额外的试验数据。

总体来看，我们可以做五个总结：1. 通过同源和异源的遗传转化进行的基因叠加，我们可以达到叶片和薯块的广谱抗性，并且这种抗性会稳定遗传到下一代；2. 具有叶片和薯块抗病功能性的同源转化体频率低于异源转化体频率；3. 对于检测同源转化体和异源转化体中的叠加基因的功能性，无毒基因是必要的；4. 转化体鉴别寄主可以准确地鉴别菌株的毒力谱，并且可以用于判定特定抗病基因是否对指定地区具有应用性；5. 遗传转化是用于提高现有感病品种的独特的方式，例如我们可以针对大面积栽培的优良品种 Bintje 和 Russet Burbank 进行抗病性提高，而保留其现有的其他优良特性。

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Wageningen University

18.10.2013

ABOUT THE AUTHOR

Suxian Zhu was born on 25th May 1981 in Fushun, Liaoning Province, the Peoples' Republic of China. In Fushun, she did her study till high school. In 2006, she obtained her Bachelor degree in Shenyang Agricultural University and continued her Master study majoring in Horticulture in Qingdao Agricultural University, Shandong Province. In 2007, her supervisor Prof. Jun Zhu introduced her to Prof. Evert Jacobsen from Wageningen UR Plant Breeding. Prof. Jacobsen brought her the opportunity to obtain a Master degree in Wageningen UR with the funding from Wageningen UR Plant Breeding. After obtaining the double Master degrees from Wageningen UR and Qingdao Agricultural University in 2009, she continued her PhD study in Wageningen UR Plant Breeding under the supervisor of Prof. Evert Jacobsen, Prof. Richard Visser and Dr. Jack Vossen.

Her email address for further contact is: zhusuxianaaa@hotmail.com.

Publications

ZHU, S., ZHU, J., LI, Y., NIJENHUIS, M., BERGERVOET, M., RIETMAN, H. & JACOBSEN, E. 2010. Broad spectrum resistance from *Rpi-blb1* homologous *R*-genes has been broken by *Phytophthora infestans* isolates collected from *Solanum stoloniferum*. *Acta Horticulturae Sinica,* 37(2)**,** 241-246.

ZHU, S., LI, Y., VOSSEN, J. H., VISSER, R. G. & JACOBSEN, E. 2012. Functional stacking of three resistance genes against *Phytophthora infestans* in potato. *Transgenic Res,* 21**,** 89-99.

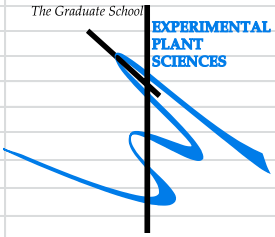
ZHU, S. & JACOBSEN, E. 2012. Towards durable resistance by stacking broad spectrum cisgenic resistance genes. *Information Systems for Biotechnology,* July**,** 4-6.

ZHU, S., DUWAL, A., SU, Q., VOSSEN, J. H., VISSER, R. G. F. & JACOBSEN, E. 2013. Vector integration in triple *R* gene transformants and the clustered inheritance of resistance against potato late blight. *Transgenic Research,* 22(2)**,** 315-325.

ZHU, S., KIM, T. Y., LIU, T., PAEK, Y. G., VOSSEN, J. H., VISSER, R. G. F. & JACOBSEN, E. Strategies of producing potato cisformants with functional resistance genes against late blight. In preparation.

ZHU, S., BERGERVOET, M., NIJENHUIS, M., VOSSEN, J. H., KESSEL, G. J. T., VLEESHOUWERS, V., VISSER, R. G. F. & JACOBSEN, E. An updated conventional- and a novel GM potato differential set for virulence monitoring of late blight resistance genes. In preparation.

Education Statement of the Graduate School
Experimental Plant Sciences



Issued to: Suxian Zhu
Date: 6 January 2014
Group: Plant Breeding, Wageningen University & Research Centre

1) Start-up phase		date
▶	**First presentation of your project**	
	Durable resistance to Phytophthora infestance by stacking of broad spectrum resistance (Rpi) genes into potato	Dec 03, 2009
▶	**Writing or rewriting a project proposal**	
	Durable resistance to Phytophthora infestans by stacking broad spectrum resistance genes into potato	Sep 30, 2009
▶	**Writing a review or book chapter**	
▶	**MSc courses**	
	Plant-Microbe Interactions, code PHP - 30306	Mar 01-Apr 30, 2010
▶	**Laboratory use of isotopes**	
	Course 'Safe handling with radioactive materials and sources'	Jan 2010
	Subtotal Start-up Phase	*12.0 credits**

2) Scientific Exposure		date
▶	**EPS PhD Student Days**	
	EPS PhD Student day, Utrecht University	Jun 01, 2010
	ExPectations 2010 Career Day Event, Wageningen University	Nov 19, 2010
	EPS PhD Student day, University of Amsterdam	Nov 30, 2012
▶	**EPS Theme Symposia**	
	EPS Theme 2 Symposium 'Interactions between Plants and Biotic Agents', Utrecht University	Jan 23, 2009
	EPS Theme 2 Symposium 'Interactions between Plants and Biotic Agents', Utrecht University	Jan 15, 2010
	EPS Theme 2 Symposium 'Interactions between Plants and Biotic Agents', University of Amsterdam	Feb 03, 2011
▶	**NWO Lunteren days and other National Platforms**	
	ALW- meeting "Experimental Plant Science", Lunteren	Apr 19-20, 2010
	ALW- meeting "Experimental Plant Science", Lunteren	Apr 04-05, 2011
▶	**Seminars (series), workshops and symposia**	
	Invited Seminar Valerie M. Williamson	Oct 23, 2009
	Plant Breeding Research Day 2010	Feb 08, 2010
	Invited Seminar Paul Birch	May 20, 2010
	Seminar 'How to write a world-class paper'	Oct 26, 2010
	Bioexploit Workshop on Marker Assisted Selection: from discovery to application	Feb 09-10, 2011
	Plant Breeding Research Day 2011	Mar 08, 2011
	Invited Seminar Regine Delourme	Oct 05, 2011
	Plant Breeding in the Genomics Era	Nov 25, 2011
	Plant Breeding Research Day 2012	Feb 29, 2012
	Invited Seminars (Howard S. Judelson, Rays H.Y. Jiang, Brian Staskawicz)	May 2013
▶	**Seminar plus**	
▶	**International symposia and congresses**	
	Potato Breeding after completion of the DNA Sequence of the Potato	Jun 27-30, 2010
	The International Conference Plant Transformation Technologies II	Feb 19-22, 2011
	The International Conference Plant Gene Discovery Technologies	Feb 23-26, 2011
	The 18th Triennial Conference of the European Association for Potato Research	Jul 24-29, 2011
	Next Generation Plant Breeding	Nov 12-14-2012
▶	**Presentations**	
	Potato Breeding after completion of the DNA Sequence of the Potato (poster)	Jun 29, 2010
	NVPW Plant Tissue Culture Symposium (oral)	Nov 26, 2010
	The International Conference Plant Transformation Technologies II (poster)	Feb 21, 2011
	ALW meeting "Experimental Plant Science", Lunteren (oral)	Apr 05, 2011
	The 18th Triennial Conference of the European Association for Potato Research (Oral and Poster)	Jul 26, 2011
▶	**IAB interview**	Nov 14, 2012
▶	**Excursions**	
	Green Life Sciences company visit-Enza Zaden	Jun 23, 2011
	Subtotal Scientific Exposure	*18.9 credits**

3) In-Depth Studies		date
▶	**EPS courses or other PhD courses**	
	Spring school "RNAi & the World of Small RNA Molecules"	Apr 14-16, 2010
	Basic Statistics	Jun 19, 20, 25-27, 2012
	Summer School "Natural Variation of Plants"	Aug 21-24, 2012
	Introduction to R for Statistical Analysis	Oct 22-23, 2012
▶	**Journal club**	
	Literature discussion "plant breeding"	2009-2013
▶	**Individual research training**	
	Subtotal In-Depth Studies	*7.2 credits**

4) Personal development		*date*
►	**Skill training courses**	
	Academic writting II	Sep 2010-Jan 2011
	Teaching and supervision thesis student	Nov 18-19, 2009
	PhD Competence Assessment	Jun 22, 2010
	Information Literacy, including Introduction Endnote	Dec 07-08, 2009
	Techniques for Writing and Presenting Scientific Papers	Apr 18-21, 2011
	Interpersonal Communication for PhD Students	Dec 12-13, 2012
►	**Organisation of PhD students day, course or conference**	
►	**Membership of Board, Committee or PhD council**	
	Subtotal Personal Development	*5.3 credits**
	TOTAL NUMBER OF CREDIT POINTS*	**43.4**
Herewith the Graduate School declares that the PhD candidate has complied with the educational requirements set by the Educational Committee of EPS which comprises of a minimum total of 30 ECTS credits		
** A credit represents a normative study load of 28 hours of study.*		

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Cover & layout: Suxian Zhu

The cover depicts that a person is running, in the shape of an *R* (resistance). This person is consisted of pictures of traditional and modern breeding techniques. The head of the person is filled with diverse potato genotypes, which indicates gene stacking. This design hints that gene stacking using traditional and modern techniques is a current trend in resistance breeding.

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