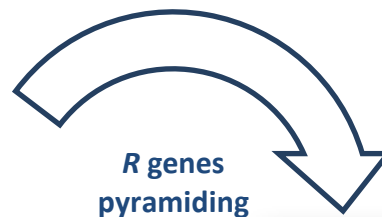WAGENINGEN UNIVERSITY
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Cisgenic Late Blight Resistance Gene Pyramiding in Potato (*Solanum tuberosum*)

A study on transformation of combinations of late blight resistance genes, T-DNA integration, vector backbone gene analysis and selection of desirable plants

R genes pyramiding

MSc Thesis Plant Breeding

Course code: PBR-80436

Student: Yeshiwas Alemnew Abate

Registration no. 840227003070

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March, 2016

The pictures on the cover page are from the DLA disease symptom scoring where the first is non-transformed variety Desiree and the second one is transformant A115-36 with two *R* genes (*R*8:*Rpi-edn*2).

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Abstract

Potato, Solanum tuberosum L., is an extremely valuable food crop cultivated in every continent but late blight, a devastating disease caused by the Oomycete Phytophthora infestans, remained confronting its production. The problem of late blight disease can better be tackled by breeding resistant varieties via introgression of R genes from the gene pool of crossable species. Due to heterozygosity problem of the crop and rapid evolution of Pi population, breeding through genetic transformation plays significant role over conventional breeding for pyramiding multiple broad spectrum R genes from wild resistant species into commercial varieties. This multiple cisgenic R genes pyramiding provide durable resistance and can be performed in a single step with genetic transformation techniques. However, in genetic transformation, bacterial backbone genes are also known to integrate into host genomes together with the genes of interest. In this research, six transformation series with two-R genes stacked together (R8:Rpi-sto1, R8:Rpi-edn2, Rpi-blb2:R8, Rpi-blb3:Rpi-edn2, Rpi-vnt1.1:R8, and Rpi-blb2:Rpi-edn2) were studied for T-DNA gene integration by PCR and resistance expression with DLA and whole plant inoculation assays. Possible causes and alternative solutions for problematic constructs, the combinability and resistance expression of two stacked R genes and the frequency to which bacterial backbone gene free transformant events could be obtained, were investigated from the six transformation series. Partial and complete deletion of genes was identified from problematic construct and transformation with inverted gene constructs with two A. tumefaciens strains was initiated (unfinished research) for better combinability. From the five transformation series, 32 transformant events (7, 6, 6, 7 and 6 respectively from R8:Rpi-edn2, Rpi-blb2:R8, Rpi-blb3:Rpi-edn2, Rpi-vnt1.1:R8, and Rpi-blb2:Rpi-edn2) were free from vector backbone genes and showed better resistance for the corresponding Pi isolates than single R gene transformants as well as from non-transformed variety Desiree. These events are also true to type for variety Desiree and recommended for T-DNA copy number analysis and further field trial to test field condition resistance.

Key words: Cisgenesis, Potato, Late blight, *R* gene pyramiding, T-DNA integration, Vector integration, Functional expression, Durable resistance, potato transformation

Abbreviations and acronyms

A.tum   *Agrobacterium tumefaciens*

Avr   Avirulence

BAP   6-Benzylaminopurine

bp   Base pairs

DLA   Detached Leaf Assay

EH   Extreme resistance

ETI   Effector triggered immunity

ETS   Effector triggered susceptibility

GM   Genetic Modified

HR   Hypersensitive Response

I   Intermediate

LB   Left Border

LB   Liquid Broth

NAA   1-Naphthaleneacetic acid

NPTII   Neomycin phosphotransferase

OD   Optical Density

PAMP   Pathogen Associated Molecular Patterns

PCR   polymerase chain reaction

Pi   *Phytopthora infestans*

PTI   PAMP triggered immunity

qPCR   Quantitative PCR

R   Resistance

RB   Right border

RSA   Rye-Sucrose-Agar medium

S   Susceptible

T-DNA   Transfer DNA

ZCVK   Zeatine, Cefotaxime, Vancomycin, Kanamycin

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1. Introduction

Potato is an ancient crop domesticated in South America and later distributed to extensive areas of the world reaching 19 million hectares of land coverage and 325 million tonnes production across the globe (Harris 2012). Potato cultivation across the world encompasses more countries than any other crops next to maize and is therefore an extremely valuable food crop for mankind (Horton and Sawyer 1985). Potato's, *Solanum tuberosum* L., worldwide importance as a food crop ranks 3rd next to rice and wheat. The overall worldwide production within the periods of 1991 to 2007 is increased by 21% (Birch et al. 2012) and contributes vital role in food security for the growing world population. In addition, potato has a reputable history in non-food applications. For instance, the starch from potato has long been used in adhesive and textile industries (Kraak 1992).

Potato production, as many other crops, is challenged by abiotic and biotic factors. Abiotic factors include drought, salinity, temperature, light etc. The biotic factors, on the other hand, are imposed by living organisms like bacteria, fungi, viruses, nematodes and insects. Among the biotic factors, is the Oomycete *Phytophthora infestans* causing the most important disease for potato, late blight. When environmental conditions are conducive to the onset and spread, this disease can devastate potato fields within a couple of weeks (Vossen et al. 2005). The disease attacks the foliage, stems, tubers and fruits, not only of potato but also other *Solanaceae* species like tomato and eggplant (Birch et al. 2012).

Plants have two layers of defence mechanisms against pathogens (Jones and Dangl 2006) firstly by recognizing conserved molecules of the microbes, Pathogen Associated Molecular Patterns (PAMPs) with transmembrane/surface receptors termed PAMP triggered immunity (PTI). In turn, pathogens deploy effectors and manipulate plant defence systems, leading plants into effector triggered susceptibility (ETS). Subsequently, plants respond with their second layer of defence, directly by acting on pathogen virulence factors or via specific recognition of the pathogen effectors, called effector triggered immunity (ETI). This resistance is a fast evolving type of plant response that plants use intracellular receptors, the NB-LRR (nucleotide binding leucine rich repeat) protein products to specifically recognize pathogen effectors. Consequently, pathogens diversify their effectors or mask the

recognized effectors to supress plants' effector triggered immunity, resulting in resistance specificities due to natural selection, leading again to ETS or ETI. (Jones and Dangl 2006) represented this interplay as a zig-zag-zig model between plant defence and pathogen responses resulting in a threshold of plant defence and resistance specificities. Similarly, (Hein et al. 2009) adapted plant pathogen zig-zag-zig model to plant-oomycete continuous interaction and co-evolution as presented in Figure 1 below.
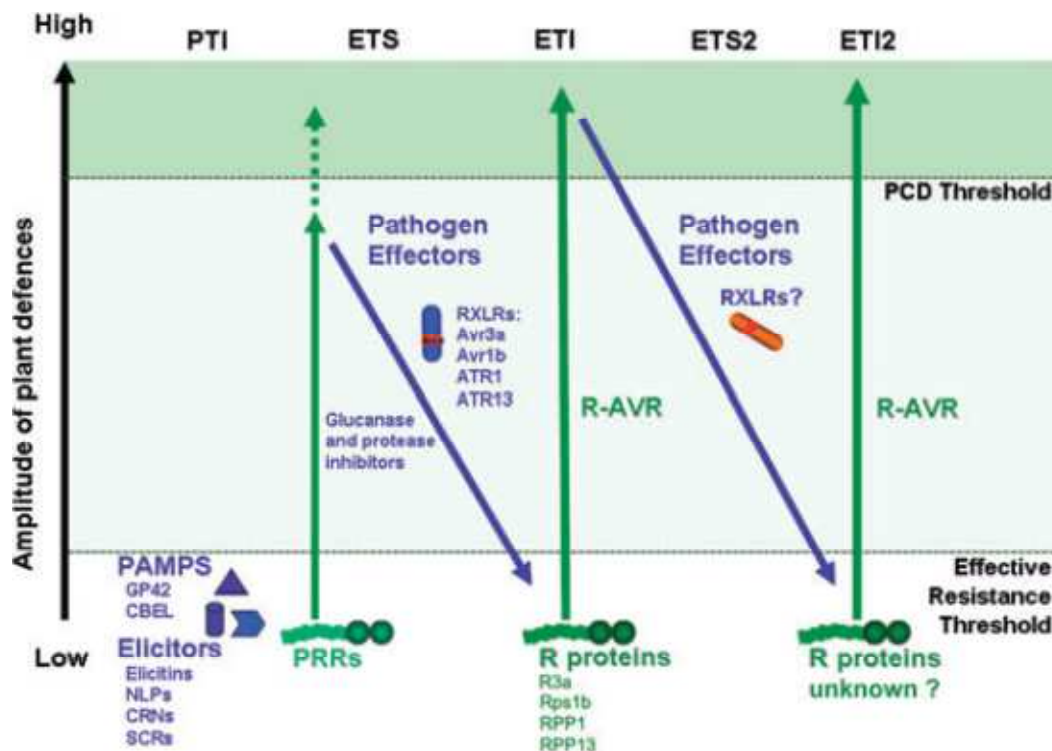
Figure 1: Schematic presentation of oomycete-plant interaction. The scheme shows oomycete PAMPs and effectors triggered susceptibility and Plants' PAMP triggered and effector triggered immunities (taken from Hein et al 2009).

Genetic resistance is considered of paramount importance to sustainably combat the effects of late blight disease. However, due to high genetic variability in *P. infestans* populations, late blight resistance (*R*) genes in host plants can result in the evolution of corresponding virulence races that can breakdown resistance (Jansky 2000). As a result, breeding for durability of resistance is a central point to be taken into account in late blight resistance breeding programs (Colon et al. 1995).

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The evolution of *P. infestans* isolates and rapid breakdown of resistance in potato becomes an intriguing area of research directing attentions into the introgression of multiple *R* genes. Accordingly, insertion of multiple broad spectrum *R* genes (pyramiding) is believed to increase resistance durability. *R* gene pyramiding can be achieved with conventional genetic crossing but, due to high level of heterozygosity in potato, the desired features of varieties cannot be maintained. Breeding by introducing *R* genes through genetic transformation techniques have vital significance to circumvent the heterozygosity problems thereby speeding up the breeding process (Jo et al. 2014).

Multiple late blight *R* genes from wild potato species have been introduced to commercial potato varieties via a single vector, using *Agrobacterium tumefaciens* mediated transformation. In this study, late blight *R* genes; *R8* derived from *Solanum demissum* (Malcolmson and Black 1966), *Rpi-blb2* and *Rpi-blb3* from *S. bulbocastanum* (Vossen et al. 2005, Lokossou et al. 2009), *Rpi-edn2* from *S. edinense* (de Vetten et al. 2011), *Rpi-vnt1.1* from *S. venturii* (Pel et al. 2009) and *Rpi-sto1* from *S. stoloniferum* (Vleeshouwers et al. 2008) were used in combinations of *R8:Rpi-sto*1, *R8:Rpi-edn2, Rpi-blb2:R8, Rpi-blb3:Rpi-edn2, Rpi-vnt1.1:R8,* and *Rpi-blb2:Rpi-edn2*. In principle, each *R* gene in a combination should correspond with differential Avr effectors in order to achieve enhanced resistance against differential isolate collections of the pathogen (Niks et al. 2011). Therefore, such *R* gene combinations were selected based on their differential patterns of reaction for the differential *P. infestans* isolates and believed for providing additive value of durability and level of resistance.

R gene combinations stacked into commercial varieties and tested for their individual functionality to corresponding *P. infestans* isolates were reported as significantly contributing for durable resistance (Zhu et al. 2012, Kim et al. 2012). Reports also showed that stacking of *R* genes derived from wild potato species into susceptible variety Desiree conferred durable resistance at field conditions (Haesaert et al. 2015). (Haesaert et al. 2015) reported that transformants with three stacked *R* genes (*Rpi-sto1:Rpi-vnt1.1:Rpi-blb3*) were more resistant than single gene transformants as well as non-transformant varieties across locations and over years.

This breeding technique by insertion of genes from the same or crossable species is termed as cisgenesis and considered at least as safe as conventional introgression breeding (ORGANISMS 2012). Accordingly, cisgenic techniques could be best option to be exempted from GM product legislation as the end product is indistinguishable from conventional crossing products. However, researches also showed that in *Agrobacterium*-mediated transformation, vector backbone sequences linked outside the right and left border (RB and LB) of T-DNA may also integrate within the genome of transformed plants in addition to sequences between right and left border of T-DNA (Kononov, Bassuner, and Gelvin 1997). Similarly, (De Buck et al. 2000) reported that at least 1000 bp of vector backbone sequences found linked to both borders of integrated T-DNA. Vector backbone sequences integration into the genome of transformed plants raises many societal concerns relating to GM issues. In Europe, genetic transformation of plants and their propagation in the field is subject to strict regulations. GM regulation for field trial requires that transformants should be free from bacterial vector backbone sequences. Also, a low number of T-DNA copies are preferred in order to describe the genomic integration site.

In genetic transformation, *A. tumefaciens* mediated transformation is a commonly used technique (Zhu et al. 2013), but also known to result in single or higher copy numbers of T-DNA integrated in the host genome. The higher copy numbers of transgenes theoretically would lead to higher transgene expression level but, may also change the interaction with host genome, resulting in changes in chromosomal arrangements and transcriptional level at the integration locus or even gene silencing (Tenea and Cucu 2006).

This project investigated three research questions, the first was to find possible reasons why *R* genes were not integrated in the genome or were not sufficiently expressed in a previous study where pyramiding of two *P. infestans R* genes was pursued using transformation of a susceptible potato variety (Desiree) with a single T-DNA vector. A potential solution is sought in the transformation of different constructs harbouring the same *R* genes delivered through two different *A. tumefaciens* strains. A second research question was to examine the frequency of transformation events functionally expressing both late blight *R* genes by carrying out bioassays. A third research question was to investigate how often late blight resistant events with no vector backbone gene could be selected by PCR.

Throughout this research, partial and complete gene deletion was identified from the problematic construct and transformation by reversing gene constructs with two *A.*

tumefaciens strains was initiated to find better combinability and stability. From the other five transformation series, 32 transformant events (7, 6,6,7 and 6 respectively from *R8:Rpi-edn2, Rpi-blb2:R8, Rpi-blb3:Rpi-edn2, Rpi-vnt1.1:R8, and Rpi-blb2:Rpi-edn2*) resistant to corresponding *Pi* isolates, no vector backbone gene integration and true to type for variety Desiree were maintained for further T-DNA copy number analysis. These transformant events performed better resistance against the corresponding *Pi* isolates than single *R* gene transformants, as well as the non-transformed variety Desiree and recommended for field condition resistance tests.

2. Materials and methods

2.1. Plant materials

In this study, both transgenic and non-transgenic events of variety Desiree were used. Transgenic events with combinations of two *R* genes including the series A114 (R8:sto1), A115 (R8:edn2), A116 (*blb2:R*8), A118 (*blb3:edn2*), A119 (*vnt1.1:R8*), and A61 (*blb2:edn2*) were the experimental units. Non-transgenic Desiree and transgenic events with a single *R* gene were A74.8-14 (*R8), A*73.1-44 (*Rpi-edn2), A02-33 (Rpi-blb2),* A03-142 (*Rpi-blb3*) and A13-13 (*Rpi-vnt1.1*), which were used as control treatments. Letter "A" stands for potato variety Desiree, and the numbers represent transformation number.

2.2. Plant transformation - Desiree variety

Resistance gene combinations of *Rpi-sto1:R*8 and *Rpi-blb3:Rpi-sto1* were constructed in binary vector pBINPLUS-PASSA. An *A. tumefaciens* mediated transformation method was used to transfer these *R* gene constructs into the late blight susceptible variety, Desiree. Two *A. tumefaciens* strains (Agl-1(virG) and Agl-0) were used to identify suitable strains for particular gene combination that can confer efficient transformation efficiency.

Only tops of Desiree variety were propagated on MS20 medium containing 4.4g/l MS with vitamins, 20g/l sucrose, dissolved in MQ (pH adjusted to 5.8) and 8g/l micro agar added

(Murashige and Skoog 1962). These cuttings were grown *in vitro* for 4 weeks at 24^0C under 16 hour light and 8 hour dark photoperiods (16/8 hour) to induce roots and internodes. After 4 weeks of *in vitro* growth, 2-5mm cuttings of top internodes were prepared. The cuttings were placed on petri dishes containing R3B medium (4.4g/l MS including vitamins, 30g/l sucrose, 8g micro agar, 2mg/l 1-Naphthaleneacetic acid (NAA), 1mg/l 6-Benzylaminopurine (BAP), pH 5.8) with two sterile filter papers on top, saturated with 1.5 ml liquid PACM medium (4.4g/l MS including vitamins, 30g/l sucrose, 2g/l casein hydrolysate, 1mg/l 2,4-Dichlorophenoxyacetic acid (2,4-D), 0.5 mg/l kinetine, pH 6.5). The internode cuttings on R3B medium containing PACM saturated filter papers on top were incubated for two days at 24^0C under 16/8 hour photoperiods.

A. tumefaciens strains, Agl-0 and Agl-1(virG) each with gene constructs of *Rpi-blb3:Rpi-sto1* and *Rpi-sto1:R8* including the selectable marker gene, *NPTII*, in the binary vector pBINPLUS-PASSA- were cultured in Liquid Broth (LB) medium containing antibiotics and selection agent (Table 2.1). The strains were grown overnight at 30^0C ±140 rpm on shaker. The optical density (OD) of each culture was measured at 600nm and diluted to achieve a final value of 0.5 to 0.8 OD.

Table 2.1: Transformation of R gene combinations with two *A. tumefaciens* strains and antibiotic requirements

Bacterial stock nr	Gene construct	A. tum Strain	Antibiotics included	Final OD	
				Transformation 1	Transformation 2
337	*Rpi-sto1*:R8	AGL-1	Carb + CAM + Kan	0.62	0.54
344	*Rpi-sto1*:R8	AGL-0	Rif + kan	0.52	0.66
339	*Rpi-blb3: Rpi-sto1*	AGL-1	Carb + CAM + Kan	0.47	0.43
345	*Rpi-blb3: Rpi-sto1*	AGL-0	Rif + Kan	0.55	0.48

*Concentrations of antibiotics: 75μg/ml carbenicillin (Carb), 12.5μg/ml chloramphenicol (CAM), 100μg/ml kanamycin (Kan) and 25μg/ml rifampicin (Rif).

Internode cuttings of variety Desiree, after now called explants, were co-cultivated for 5 to 10 minutes with respective *A. tumefaciens* LB cultures. The explants were removed from the solution by using sterile sieve and dried on sterile filter paper for about 10 to 15 seconds. These explants were grown in ZCVK medium (MS20 medium, 1mg/l Zeatine, 200mg/l

Cefotaxime, 200mg/l Vancomycin and 100mg/l Kanamycin filter sterilized, pH 5.8) under 16/8 hours day and night in 24^0C climate room. After two weeks of infection, explants were transferred to fresh ZCVK medium and transferred every three weeks to fresh ZCVK plates until sufficient numbers of shoots were harvested. Explants which developed green callus were counted in a monthly interval to estimate transformation efficiency. Shoots emerging from the calluses were harvested and transferred to fresh MS20 medium containing only Cefotaxime and Kanamycin antibiotics (200mg/l Cefotaxime and 100mg/l Kanamycin) for further shoot growth and root induction. For this particular thesis research, transformation success was evaluated mainly based on formation of green callus, while normal shoot growth, root induction and green stem colour are proposed to select final well transformed events.

2.3. Validation of *R* gene transformation events based on climate cell assays

Detached leaf assay (DLA) and whole plant assay phenotyping approaches were carried out to investigate the resistance conferred by *R* gene combinations against differential *P. infestans* isolates. In this particular study, NL12226 and USA618 *P. infestans* isolates were used for both DLA and whole plant assay in A61 and few events of A115 transformants. For other transformant series (A114, A115, A116, A118 and A119), climate cell assays were performed before the start of this thesis and the data was taken and analysed in this thesis research. The *R* gene combinations for all transformation series were selected based on differential reaction pattern to the corresponding isolates described in Table 2.2. The isolates were grown on new RSA (Rye-Sucrose-Agar) medium plates by transferring approximately 5mm by 5mm slices from stock plates. These inoculum plates were incubated for ± two weeks at 15^0C to produce sporangia. After two weeks, sufficient sporangia were produced and suspensions of the sporangia were washed-off using 15ml ice-cold tapwater and a driglaski-spatula. This suspension was incubated at 4^0C for two hours to produce zoospores. Thereafter, the amount of zoospores was counted in a counting chamber using a microscope. For inoculation, the density of zoospores was adjusted to approximately 10 zoospores per large square of the counting chamber by diluting with ice-cold water.

Table 2.2: *P. infestans* isolates used in climate cell assays with their (a)-virulence spectrum

Transformants	Gene construct	*P. infestans* isolate			*P. infestans* isolate		
		Isolate 1	active effector	inactive effector	Isolate 2	active effector	inactive effector
A115	*R8:edn*2	NL12097	Avr8	avredn2	IPO-C	Avredn2	avr8
A116	*blb*2:*R8*	US090017	Avrblb2	avr8	NL12003	Avr8	avrblb2
A118	*blb*3:*edn*2	NL12097	Avrblb3	avredn2	IPO-C	Avredn2	avrblb3
A119	*vnt*1.1:*R8*	US090017	Avrvnt1.1	avr8	Ec-1	AvrR8	avrvnt1.1
A61*	*blb*2:*edn*2	USA618	Avrblb2	avredn2	NL12226	Avredn2	avrblb2
A115**	R8:*edn*2	USA618	Avr8	avredn2	NL12226	Avredn2	avr8

*DLA and whole plant assay in November 2015

**DLA in November 2015

2.3.1 Detached leaf assay (DLA)

The activity of individual *P. infestans R* genes in events transformed with two-*R* gene constructs was tested by inoculation with differential *P. infestans* isolates. *R* gene combinations tested by DLA in this study are *Rpi-blb2:Rpi-edn2* (A61) and *R8:Rpi-edn2* (A115). For *Rpi-blb2:Rpi-edn2* combination, isolate USA618 and NL12226 were used so that *Rpi-blb2* can recognize USA618 and *Rpi-edn2* by NL12226 (Table 2.2). For A115 transformants (*R8:Rpi-edn2*), the same *P. infestans* isolates as for A61 were used where USA618 can be recognized by *R*8 and isolate NL12226 by *Rpi-edn2*.

A115 and A61 transformant events were grown *in vitro* for two weeks under 16/8 hour light and dark photoperiods in 24⁰C climate room during November 2015. These plants were transferred to greenhouse and grown for ± 8 weeks on standard soil media under 16/8 hour light and dark photoperiods. A74.8-14 (*R*8), A73.1-44 (*Rpi-edn2*) and A02-33 (*Rpi-blb2*) events were used as positive controls for the performance of individual *R* genes and non-transformed Desiree as a susceptible control. After ±8 weeks, two young and well stretched leaves from each event were excised. The excised leaves were inserted with their petioles into water saturated foam in upside down leaf positions inside trays with moist paper at bottom. Three drops of 10µl suspension from each two isolates were pipetted on the main leaf and two drops of 10µl suspension on the side leaves (USA618 on left side and NL12226 on right hand side, Fig. 2 A).

The leaves inside the tray were then covered with plastic sheet to maintain 100% humidity (Fig. 2 B) and kept in 16/8 hour light and dark photoperiods in 18^{0}C climate room (Fig. 2 C).

Disease symptoms were scored 6 day post inoculation by using a method developed like in Table 2.3. Resistance scoring was performed by comparing the resistance level of each *R* gene with control events in relation to HR size and development of water soaked lesions and/or sporulation of the pathogen on the leaves (Table 2.3).
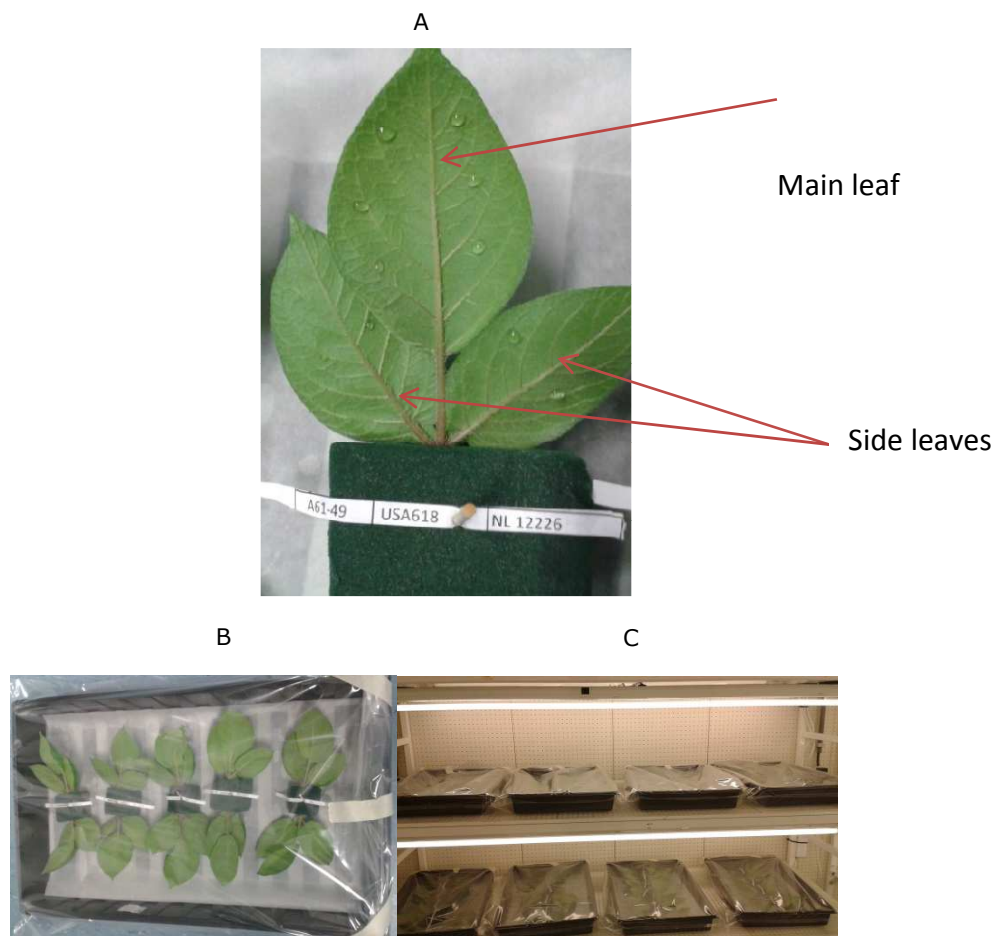

Figure 2.1: Detached leaf assay inoculation. A) Leaf parts and inoculated spots, B) inoculated leaves within tray sealed by plastic bag, C) trays with inoculated leaves in climate cell

Table 2.3 Scoring methods of disease symptom for DLA and whole plant assay

Disease scale	Quantitative resistance index	Score	Symptom
R9	4	R	ER When there is no visible symptom
R8	3.5	R	Very small HR
R7	3	R	HR
R6	2	I	Big HR
V5	1.5	I	Big HR + water soaking
V6	1	S	Water soaked lesions but no sporulation
V7	0.5	S	Water soaked lesions + sporulation on inoculated leaf side
V8	0	S	Water soaked lesions + sporulation on both leaf side

R: resistant, I: intermediate resistance, S: susceptible, HR: hypersensitive response, ER: extreme resistance

 ER and HR are the resistant group while big HR is the intermediate group and Sporulation is the susceptible group.

Resistance was determined based on disease symptoms quantitative index (Table 2.3). Resistant (R) is scored when the score is ≤ 4.0 and ≥ 3.0, intermediate (I) when < 3.0 and ≥ 1.5 and susceptible (S) when ≤ 1.0 and ≥ 0.0. For plants expressing multiple levels of symptoms, the average of the scores was taken.

2.3.2 Whole plant assay

Intact potted plants were directly challenged by inoculation of leaves with zoospore suspensions of selected isolates. In this thesis research, only A61 (*Rpi-blb2:Rpi-edn2*) events were tested via whole plant assay. Transformants were first propagated *in vitro* for two weeks under 16/8h day/night photoperiods in 24^0C climate room. After rooting and shoot growth under *in vitro* conditions, the plants were taken out to greenhouse for another two weeks growth on standard soil medium with 16/8h day/night photoperiods. Potted plants were then transferred to climate cell room with photoperiods of 16/8h day/night and 15^0C temperature. Suspensions of *P. infestans* isolates was prepared as explained in section 2.4.

Depending on plant size and leaf availability, three to five leaves of each plant were inoculated with isolates USA618 on left and NL12226 on right side of leaves.

Figure 2.2: whole plant inoculation assay. Intact potted plants covered with plastic in 15^{0}C climate cell after inoculation

Two to three drops (depending on leaf/plant size) of 10µl suspension from selected isolates was applied within two days of climate cell transfer and 100 % relative humidity was maintained by covering with plastic sheet (Fig. 2.2). Disease symptom scoring was also done in a similar manner as performed for DLA.

2.4. Integration of T-DNA and vector backbone genes, and T-DNA copy number analysis

In a previous study, *R* gene combinations of *R8:Rpi-edn2, Rpi-blb2:R8, Rpi-blb3:Rpi-edn2, Rpi-vnt1.1:R8 and Rpi-blb2:Rpi-edn2* were transformed to Desiree variety (transformant series A115, A116, A118, A119 and A61) and maintained in the lab. Except A61 (*Rpi-blb2:Rpi-edn2*) events, DNA was isolated before the start of this project from *in vitro* grown transformants. During this thesis research, additional events of transformation series A115, A118 and A119 were propagated to expand the chance of selecting desirable plants. These additional events and A61 events were grown *in vitro* for two weeks on MS20 medium under 16/8 hours light

and dark in 24^{0}C climate room and further grown in greenhouse for ± 2 weeks for DNA isolation.

DNA isolation was performed based on Fulton plant DNA extraction protocol. The top young tender leaves were harvested to ensure high quality and quantity of DNA. After isolation, 1µl of direct isolated DNA, 5µl of MQ and 2µl of loading buffer (6x orange loading dye) was mixed for loading to gel. From this mix, 7µl was loaded to a 1% agarose gel to monitor the quality and concentration of DNA based on band intensities. Based on the band intensities, DNA for working samples (PCR analysis) was diluted 20 times (1/20 from the stock DNA) by MQ water (with 1µl RNAse [20µg/ml]/ml MQ). 5µl of diluted DNA, 5µl of MQ and 2µl of loading buffer was mixed for loading 10µl of it to gel. By looking at the uniformity of band strength, DNA was added for those showing less band intensity (too low concentration) and MQ for those showing strong band intensity (too high concentrations). With this estimate, the concentration was better balanced to perform further PCR analysis and isolated DNA was stocked in -20^{0}C freezer.

Integration of T-DNA and vector backbone genes in the genome of each transformation event were analysed by PCR (polymerase chain reaction) upon the DNA isolated from leaves of each event. For each T-DNA and vector backbone gene, PCR were performed according to specificity conditions of respective markers (Table 2.4). Total PCR reaction volume of 15µl comprising 10.47µl MQ, 1.5µl 10x DreamTaq buffer (20mM MgCl2,Thermo-Scientific), 0.8µl dNTP mix (0.2mM each), 0.6µl forward primer (100µM), 0.6µl reverse primer (100µM), 0.03µl DreamTaq DNA Polymerase (5U/µl, Thermo-Scientific) and 1µl DNA ([50ng/µl] DNA with RNase) were used for amplification.

Table 2.4: Specificity and PCR conditions of primer pairs used for T-DNA and vector backbone gene analysis

Target Gene	Primer code	Sequence (5'-3')	Annealing temperature (^{0}c)	Annealing time (sec)	Product length (bp)	Type
Rpi-blb2	Lk51+LK68	GGACTGGGTAACGACAATCC	58	30	±800	T-DNA
		AGCACGAGTTCCCCTAATGC				
Rpi-blb3	LK55 + LK56	AGCTTTTTGAGTGTGTAATTGG	58	30	305	T-DNA
		GTAACTACGGACTCGAGGG				
Rpi-vnt1.1	LK70 + LK69	ATGAATTATTGTGTTTACAAGACTTG	53	30	±1100	T-DNA
		AGCATTGGCCCAATTATCATTAAC				
Rpi-sto1	LK11+LK12	ACCAAGGCCACAAGATTCTC	65	30	890	T-DNA
		CCTGCGGTTCGGTTAATACA				
R9a Rpi-edn2	MA447 + MA446	CTTTGATGTGGATGGATGGTG	58	30	400	CAPS (HpyCH4IV)
		GCATCATGTCTGCACCTATG				
R8-GC	Sw5-C_F + Sw5-G_R	CTGGATTTCTTCAAGATTCGTCGT	54	30	±700	CAPS (HpyCH4IV)
		AGTAAACTTTGACACCTTTAGTTCACCAT				
	R8-CT220F_F1 + R8-CT220F_R1	CAAGTTCCTGACCATTACAAAAGT				Internal controls
		CAACGATGGTACCGATGGAT				
tetA	AL1+AL2	TCATTGGGCTGTCGGTCTTC	64	30	525	Vector backbone
		TAAAGGTGAGCAGAGGCACG				
nptIII	AL3+AL4	AGACGGAAAAGCCCGAAGAG	62	30	113	Vector backbone
		GCCGCTTCTCCCAAGATCAA				
insB	AL 5+AL6	CGAACGACCTACACCGAACT	65	30	246	Vector backbone
		GCTGGCGTTTTTCCATAGGC				
oriV	AL7+AL8	CGCGAGTTTCCCACAGATGA	64	30	232	Vector backbone
		AAAGACAGGTTAGCGGTGGC				
Traj	AL9+AL10	GTCGGTGAGCCAGAGTTTCA	62	30	194	Vector backbone
		GCCTTCCAGACGAACGAAGA				
trfA	AL11+AL12	GGTCGATCAATGGCCGGTAT	65	30	243	Vector backbone
		GCTTGCGGTACTTCTCCCAT				
tetR	AL13+AL14	GTCTGACGACACGCAAACTG	58	30	125	Vector backbone
		CGTATGATTCTCCGCCAGCA				
NPTII end R + NPTII begin R	AL15+ AL16	CTGGGGTTCGAAATGACCGA	54	360		*R* gene deletion
		GATGGATTGCACGCAGGTTC				
R8-C-F + R8-G-R	AL17+ AL18	CTGGATTTCTTCAAGATTCGTCGT	54	360		*R* gene deletion
		AGTAAACTTTGACACCTTTAGTTCACCAT				
P1. R8 end F + NPTII end1 R	AL19 + AL20	CCGACCCAATACCCAACCAT	54	360	1737	*R* gene deletion
		CTGGGGTTCGAAATGACCGA				
P2. R8 end F + NPTII end2 R	AL21 +AL22	GCTCCAACCAAACAACCCCT	54	360	1420	*R* gene deletion
		GGGTTCGAAATGACCGACCA				
P1. R8 half F + NPTII end1 R	AL23	TCTCAAAGTGGGATGCCTCG	54	360	3167	*R* gene deletion
P2. R8 half F + NPTII end3 R	AL25 + AL26	GAAGAGTGGTGCCTTGGAGA	54	360	3233	*R* gene deletion
		TGGGGTTCGAAATGACCGAC				

PCR programs run at 94^0C initial denaturation for 4 minutes followed by 35 cycles of 30 seconds denaturation at 94^0C, 30 seconds of annealing at corresponding annealing temperature (Table 2.4) and 1 minute extension at 72^0C, and 10 minutes of final elongation at 72^0C. PCR products were analysed with gel electrophoresis by loading total volume of 10µl, consisting of 4µl PCR product, 2µl of loading dye (6x orange loading dye) and 5µl of MQ water on 1- 2% agarose gel, depending on the respective primers product length. Internal controls of CT220 T-DNA fragments were also amplified to prove the PCR competence and suitability of isolated DNA for analysis.

3. Results

3.1 Comparison of T-DNA integration efficiency among two-*R* gene constructs

3.1.1 Total T-DNA gene integration

The six transformation series (A114, A115, A116, A118, A119 and A61) with *R* gene combinations of *R*8:*Rpi-sto1*, *R*8:*Rpi-edn2*, *Rpi-blb2*:*R*8, *Rpi-blb3*:*Rpi-edn2*, *Rpi-vnt1.1*:*R8* and *Rpi-blb2:Rpi-edn2* respectively, were investigated to evaluate important features of transformation, like T-DNA gene integration, *R* gene activity and plant true to typeness. From these transformation series, a total of 189 events were studied to screen events containing complete integration of *R* gene combinations among other features (Table 3.1). Specific markers used for each individual T-DNA gene integration analysis are listed in table 2.4.

Table 3.1: Total available events and T-DNA gene integration in two-R gene transformants with PCR analysis

Transformant series	Gene constructs	Available plants	gene 1 integration	gene 2 integration	Both genes integration
A114	*R8:Rpi-sto1*	21	18	0	0
A115	*R8: Rpi-edn2*	49	40	44	35
A116	*Rpi-blb2:R8*	21	21	13	13
A118	*Rpi-blb3: Rpi-edn2*	21	21	18	18
A119	*Rpi-vnt1.1:R8*	21	20	19	18
A61	*Rpi-blb2: Rpi-edn2*	56	29	26	24
Total		**189**	149	120	**108**

Individual *R* gene integration in the genome of transformants ranged from 120 for the first *R* gene to 149 events for the second *R* gene (Table 3.1). While most of independent events in the five transformation series exhibited integration of each of the respective T-DNA genes, none of the A114 events contained the *Rpi-sto1* gene. However, the first gene (*R*8) of A114 transformation series was well integrated in most events.

3.1.2 *R* gene deletion analysis

In a previous study, A114 transformants with *R* gene combination of *R8:Rpi-sto1* were all susceptible to *P. infestans* isolates of NL09066 and US090017. Furthermore, all the events lacked integration of *Rpi-sto1* T-DNA gene (Table 3.1). Accordingly, this study investigated the possible causes for the absence of *Rpi-sto1* gene in the genome of transformants and the failure of *R8* gene to confer resistance. A potential reason was sought by a detailed T-DNA gene deletion analysis based on amplicon size by using *R8* and *NPTII* gene primers that flanked the proposed deleted *Rpi-sto1* gene. Multiple primers were designed at the beginning, half and end of the *R8* gene pairing with primers at the beginning and end of the *NPTII* gene.

First, the performance of six primers in the *R8* gene, in combination with the primers in the *NPTII* gene was tested using A74.8-14, which contain only the *R8* and the *NPTII* gene, as a control. One primer pair (*R8* end2-F in combination with *NPTII* end2-R) amplified a fragment of expected size in A74.8-14 (Fig 3.1 A). The *NPTII* end2-R primer was also found to match with two other forward primers from *R8* sequences (Fig 3.1 B and C) amplifying a fragment of expected size from the A74.8-14 control transformant. From A114 events having partial *Rpi-sto1* gene integration, we expected to amplify a band greater than the size of this control. Since the size of *Rpi-sto1* gene is 6.6 kb in between *R8* and *NPTII* genes in the plasmid vector, amplification of whole *Rpi-sto1* gene was not expected due to the fact that PCR cannot amplify very large fragments.

With these three primer pairs, the bands of six events, A114-3, A114-5 and A114-6 (Fig. 3.1 A), A114-8 (Fig 3.1 B), A114-16 and A114-22 (Fig 3.1 C) were the same as the *R8* control plant (A74.8-14), showing that the entire *Rpi-sto1* gene was deleted. One event, A114-14 (Fig 3.1 B and C) showed less size than the control, showing that also the 3' end of *R8* was deleted. Three events, A114-16, A114-17 and A114-18 (Fig 3.1 B) amplified a fragment that was slightly greater than the *R8* control plant (A74.8-14), which indicated that part of the *Rpi-sto1* gene was still present but that the majority of this gene was deleted. A114-16 event in Fig. 3.1 C showed amplification of a fragment equal to the *R8* control indicating complete deletion of the *Rpi-sto1* gene. However, in Fig. 3.1 B, it amplified slightly greater than the control indicating partial deletion of the *Rpi-sto1* gene. In either case, it indicated that there

is deletion of the *Rpi-sto*1 gene in this transformation series. Events with no amplicon at all cannot tell us about the deletion size, because this might be due to the fact that PCR could not amplify large sized DNA, or because the *R8* forward primer annealing site was deleted.

The partial amplifications on the bacterial construct (278 bact nr) also indicated that partial deletion had occurred already in the bacterial construct (Fig. 3.1 B and C).

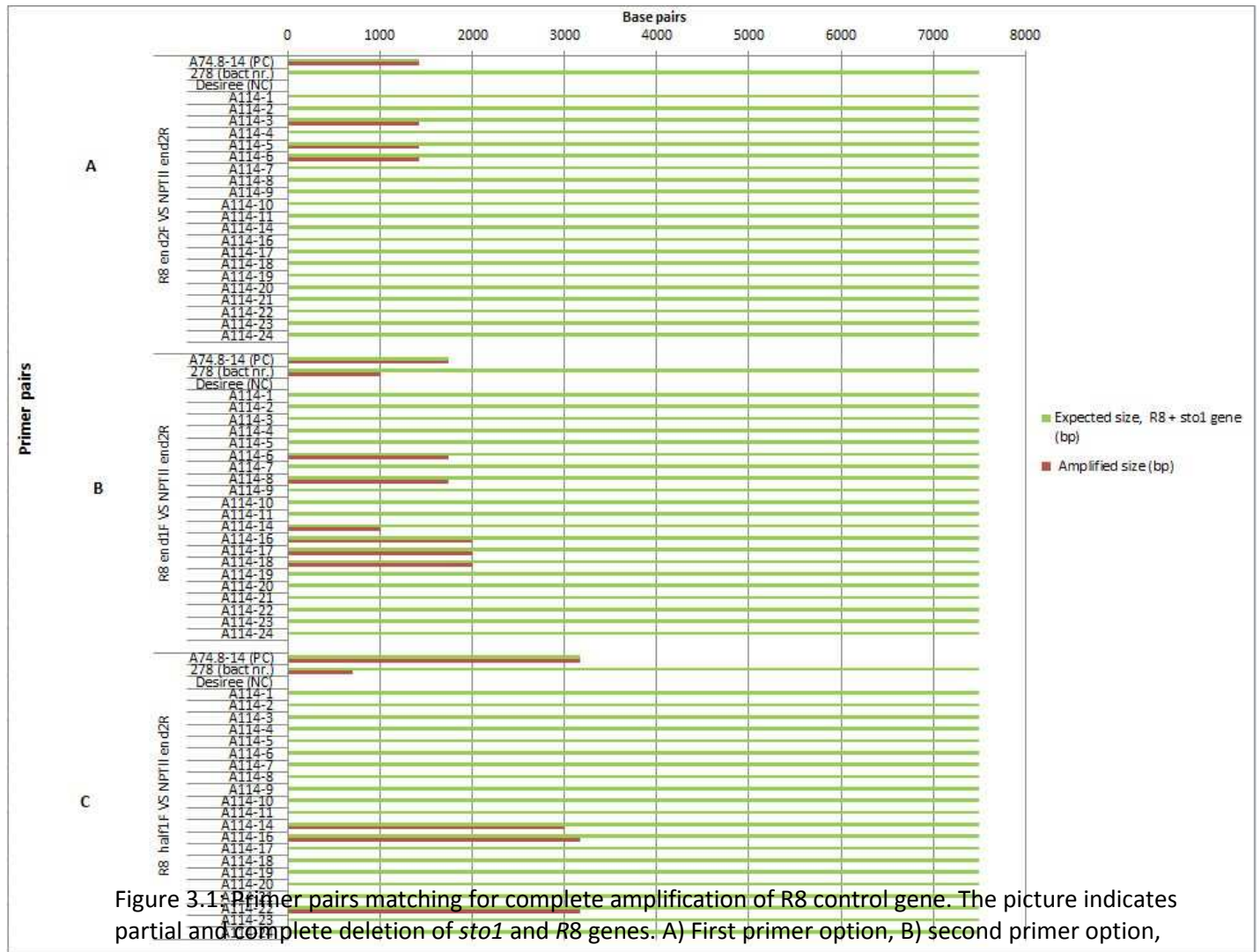Figure 3.1: Primer pairs matching for complete amplification of R8 control gene. The picture indicates partial and complete deletion of *sto1* and *R8* genes. A) First primer option, B) second primer option, C) third primer option

*A74.8-14 (PC) = positive control containing the *R8* and *NPTII* gene sequences

*278 (bact nr) bacterial construct number containing the *R8:Rpi-sto1* binary vector

*Desiree (NC) = negative control.

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3.2 Selection of well transforming constructs and suitable *A. tumefaciens* strains

Instability of the *R*8:*Rpi-sto1* gene combination was revealed by performing PCR based gene deletion analysis (Fig. 3.1). Accordingly, reverse position of these genes (*Rpi-sto1:R8*) was constructed in a binary pBINPLUS PASSA vector and new transformation in two rounds was performed to evaluate the potential combinability of the reverse gene combination. In addition, the suitability of two different *A. tumefaciens* strains (Agl-1+VirG and Agl-0) was studied. Similarly, another gene combination (*Rpi-blb3:Rpi-sto1*) which previously showed low transformation efficiency was also transformed with *A. tumefaciens* strains Agl-1+VirG and Agl-0. A total of ±160 explants in the first round and ±120 explants in the second round were used for each gene construct to compare transformation efficiencies and to select desirable transformants.

Transformants were selected based on resistance to selection agent kanamycin, rendered by the *NPTII* (neomycin phosphotransferase) gene resulting in induction of green callus and subsequent shoots and rooting. Each specific combination of *R* gene constructs and bacterial strain were monitored during the study period. The non-transformed explants (which was not co-cultivated with bacterial solution of interest) grew into shoots on ZCV medium (Fig.3.2 A) but didn't induce shoots or green callus on ZCVK medium (Fig.3.2 B). Explants treated with the *A. tumefaciens* cultures were able to develop green callus and subsequently regenerated green shoots under kanamycin containing medium (Fig. 3.2 C & D). Similarly, the growth and antibiotic controls, as well as treatments under investigation also showed proper progress in the second round transformation (Fig. 3.3) indicating the appropriateness of the experimental set up.
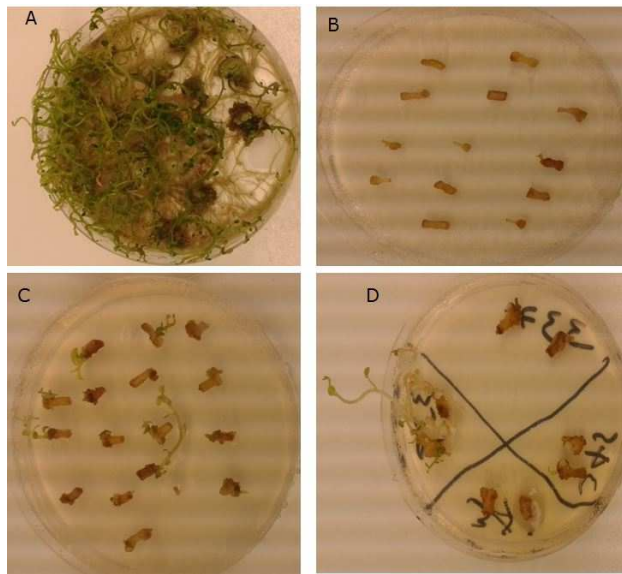
Figure 3.2: 1st round transformation (10 weeks after transformation). A = no incubation with *A. tumefaciens* on ZCV medium (growth control); B = no incubation with *A. tumefaciens* on ZCVK (antibiotic control); C= incubation with *A. tumefaciens* construct 345 (*Rpi-blb3:Rpi-sto1*) on ZCVK medium; D = incubation with *A. tumefaciens* constructs 337 (North), 345 (east), 344 (south) and 339 (west) and growth on ZCVK medium.
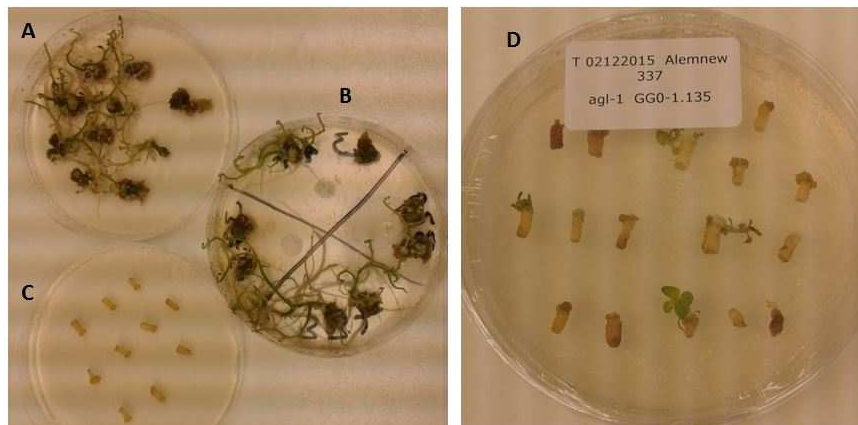
Figure 3.3: 2nd round transformation (8 weeks after transformation). A = no incubation with *A. tumefaciens* and growth on ZCV medium (growth control); B = incubation with *A. tumefaciens* constructs 344 (North), 339 (east), 337 (south) and 345 (west) and growth on ZCVK medium; C = no incubation with *A. tumefaciens* and growth on ZCVK (antibiotic control); D = incubation with *A. tumefaciens* construct 337 (*Rpi-sto1:R8)* on ZCVK medium.

Gene combination of *Rpi-sto1:R*8 (construct 337) showed 62.5 % and 85% green callus development respectively in the first and second rounds of transformation with Agl-1 *A. tumefaciens* strain while only 5% and 43% (construct 344) with Agl-0 strain. Similarly, *Rpi-blb3:Rpi-sto1* (construct 345) performed 84.3% and 56% green callus induction for the first and second round transformation respectively with Agl-0 but only 47% and 27% (construct 339) with Agl-1 *A. tumefaciens* strain (Fig. 3.4). So, although the efficiency of transformation varied in the two rounds, the rate of transformation and strains suitability was consistent. An example of observed resistance of explants to the selection agent Kanamycin in each of the four constructs for the first round transformation is presented in Fig. 3.5.
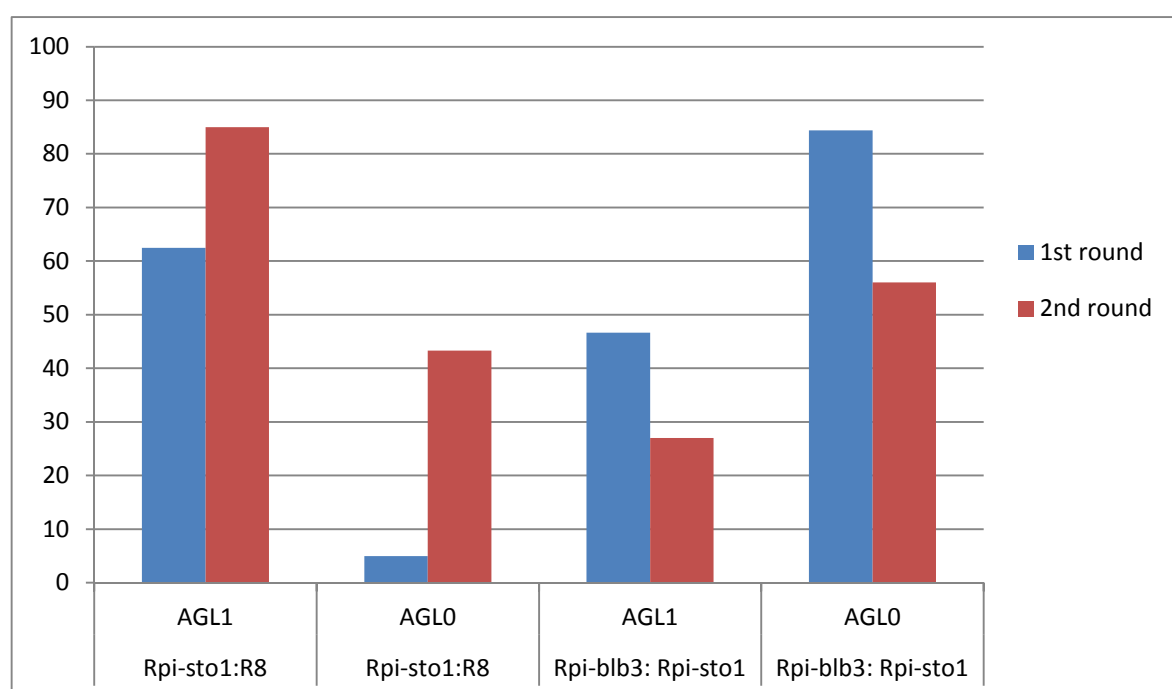
Fig. 3.4: Transformation efficiency of R gene constructs with two *A. tumefaciens* strains based on green callus induction
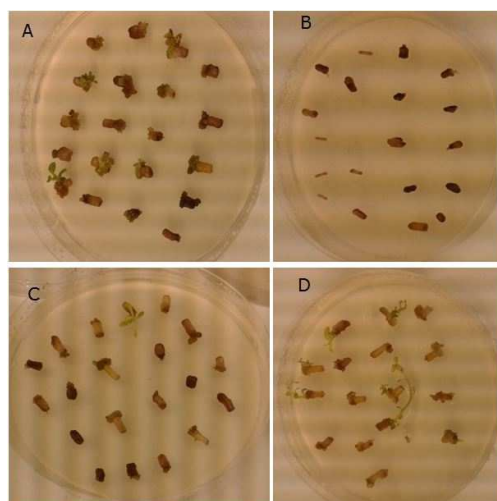
Fig. 3.5 Comparison of gene constructs and *A. tumefaciens* strains (First round transformation). A: construct *Rpi-sto1:R*8 with Agl-1; B: construct *Rpi-sto1:R*8 with Agl-0; C: construct *Rpi-blb3*: *Rpi-sto1* with Agl1; D: construct *Rpi-blb3: Rpi-sto1* with Agl-0.

This experiment indicated that different *R* gene combinations required specific *A. tumefaciens* strains and the two *A. tumefaciens* strains are suitable for different *R* gene combinations. Therefore, the combinability of *R* genes constructs might depend on the *A. tumefaciens* strain used or conversely, the influence of *A. tumefaciens* strains in facilitating efficient transformation to regenerate transformants could also depend on the *R* gene combination used.

3.3   Selection of well performing events

3.3.1   Selection based on both *R* genes integration

Selection of desirable transformation events based on T-DNA gene integration was performed by excluding events which contained only one of the T-DNA genes. A total of 108 transformation events were found containing both introduced *R* genes out of 189 events examined (Table 3.1). Transformation series A118 (*Rpi-blb3:Rpi-edn2*) and A119 (*Rpi-Vnt1.1:R8*) showed the highest frequency of events where both *R* genes were integrated. The lowest frequency was in A61 and A114 series as mentioned before (Fig. 3.6). Events harbouring both T-DNA genes were candidates to proceed into the next screening process depending on the resistance expression in the phenotyping analysis.
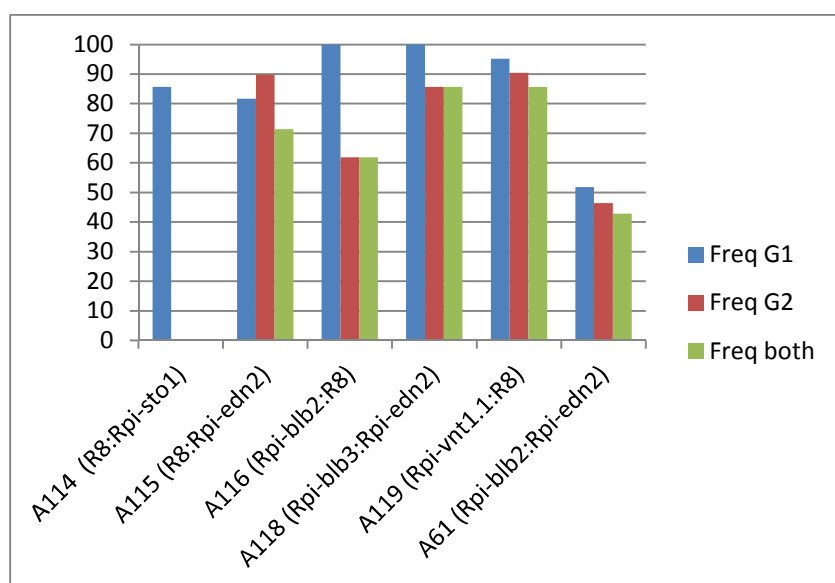
Fig. 3.6 Frequency of T-DNA integration in two *R* gene transformants based on PCR analysis (%).

G1: gene 1, G2: gene 2, Freq: frequency

3.3.2   Selection based on activity of integrated *R* genes

Functional expression of integrated *R* genes was investigated by challenging transformants with corresponding isolates of *P. infestans* in two phenotyping approaches, detached leaf assay (DLA) and whole plant assay. Appropriate *P. infestans* isolates (Table 2.2) were employed for all transformation series, A114, A115, A116, A118, A119, and A61. Resistance expression of individual *R* genes ranged 103 events for the first gene and 97 events for the second gene (Table 3.2). A total of 83 events harbouring both introduced *R* genes (PCR) and matching for active resistance expression in both climate cell assays (DLA and whole plant assay) were maintained as candidates for vector backbone gene and T-DNA copy number analysis. DLA and whole plant assay phenotyping were performed independently to elucidate the resistance expression of introduced *R* genes and the consistency of resistance in both assays. The outcomes of both DLA and whole plant inoculation assays are presented separately in the following sections.

Table 3.2: Active resistance expression in two-*R* gene transformants based on climate cell assays

Transformant series	Gene construct	Total present genes*			Active genes**		
		gene 1	gene 2	Both genes	gene 1	gene 2	Both genes
A114	*R8*:*Rpi-sto*1	18	0	0	0	0	0
A115	*R8*:*Rpi-edn*2	40	44	35	32	37	27
A116	*Rpi-blb*2:*R8*	21	13	13	19	12	12
A118	*Rpi-blb3:Rpi-edn*2	21	18	18	10	10	10
A119	*Rpi-vnt1.1:R8*	20	19	18	19	16	15
A61	*Rpi-blb*2:*Rpi-edn*2	29	26	24	23	22	19
Total				108	103	97	83

* PCR analysis, ** Climate cell phenotyping

3.3.2.1 Detached leaf assay (DLA)

The response of transformant events against the corresponding *P. infestans* isolates was categorized into three groups. Accordingly, resistant, intermediate and susceptible events were identified based on HR size and development of water soaked lesions and/or sporulation of the pathogen on the leaves. For example, events A and B were identified as resistant while C is as susceptible (Fig. 3.7). From this particular DLA analysis, 43 A61 (Table 3.3) and 21 A115 events (data not shown) showed resistance to USA618, suggesting that *Rpi-blb2* gene in A 61 and *R*8 gene in A115 transformants were active. Likewise, 40 A61 and 20 A115 events showed resistance to NL12226, suggesting that *Rpi-edn2* was active in both transformation series.
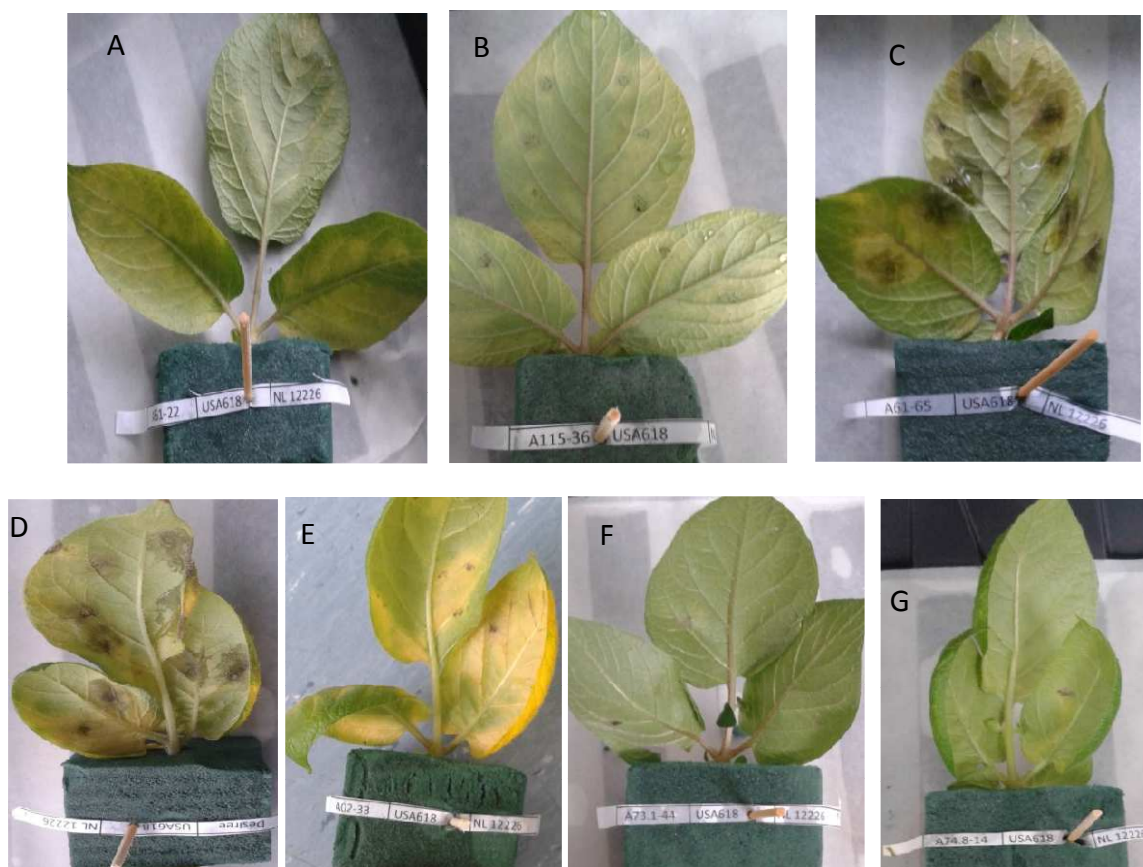
Fig.3.7: Observations on Detached leaf assay inoculation with *Pi* isolates; Left half was inoculated with USA618 and right half was inoculated with NL12226. A) A61-22 (*Rpi-blb2*:*Rpi-edn2*) with score of *R*9 for both isolates, B) A115-36 (*R8*:*Rpi-edn2*) with score of *R*8 for both isolates, C) A61-65 (*Rpi-blb2*:*Rpi-edn2*) with score of V*8* for both isolates, D) Desiree (Negative control) with scores of V8 for both isolates, E) A02-33 (*Rpi-blb2*) with scores of *R*8 for USA618 and *R*6 for NL12226, F) A73.1-44 (*Rpi-edn2*) with scores of R7 for USA618 and *R*9 for NL12226, G) A74.8-14 (*R*8) with scores of *R*9 for USA618 and *R*8 for NL12226. *For the values of *R* and *V*, refer to Table 2.3.

Transformants expressing both *R* genes (R/R) are categorized resistant, while transformants expressing only one of the *R* genes (R/S, R/I,) or none of them (I/I, I/S, S/S) are categorized susceptible and could not be selected for further analysis. For example, based on the *R* genes activity in DLA of November 2015, 40 events of A61 transformants were categorized in the resistant group while 3 events categorized under the susceptible group (Table 3.3). In other 3 events, one of the *R* genes (*Rpi-blb2*) rendered resistance while the second one (*Rpi-edn2*) performed intermediate.

Similarly, other 6 events showed intermediate resistance for both *Rpi-blb2* and *Rpi-edn2* genes. In such cases, the 9 (3+6) events were categorized under intermediate resistant. However, not all the resistant events of A61 were PCR positive for both T-DNA genes. Accordingly, selection of these events for further screening was based on the match of an event between T-DNA gene integration and active resistance expression.

Table 3.3: Example for resistant, intermediate and susceptible events of A61 transformants against corresponding *P. infestans* isolates with DLA.

		NL12226 VS *Rpi-edn2*		
	Level	Resistant	Intermediary	Susceptible
USA618 VS *Rpi-blb2*	Resistant	40	3	0
	Intermediary	0	6	0
	Susceptible	0	0	3

3.3.2.2 Whole plant assay

In addition to DLA, whole plant inoculation assay was performed to test the activity of individual *R* genes in A61 (*Rpi-blb2:Rpi-edn2*) events. With whole plant inoculation assay, all events of A61 transformants didn't show any disease symptom except the only one event A61-65, which showed susceptibility to USA618. In DLA analysis, at least three events were susceptible for both isolates (Table 3.3).

The whole plant assay was performed to distinguish if differences in resistance expression of events from the DLA analysis would be observed. This could help to select events with consistent resistance expression of introduced genes in both assays. In whole plant assay, intact plants were incubated in a relative humidity which might be lower than in DLA, which might lower the infection process of the pathogen. On the other hand, in DLA analysis, closed trays could result in higher relative humidity that might increase the infection process of the pathogen which in turn could lower the resistance expression.

In whole plant assay, the negative control (non-transformed Desiree) and other control transformants for individual *R* gene activity (figure not shown) didn't show consistency when the assay was repeated (Fig.3.8).
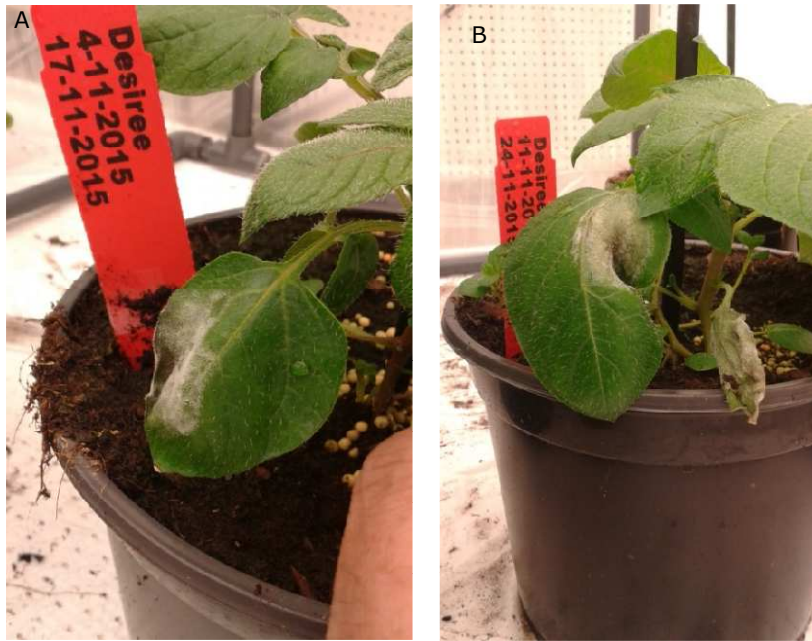
Fig. 3.8: Observed results of whole plant assay inoculation; Non-transformed Desiree variety with USA618 isolates on the left and NL12226 on the right side: A) first assay, B) repeat of the assay.

The non-transformed variety Desiree showed resistance to NL12226 in the first round while susceptible for the other isolate USA618 (Fig. 3.8 A). The positive control for USA618, *Rpi-blb*2 transformant (A02-33) was resistant and the other single gene transformant with *Rpi-edn2* gene (A73.1.44) was slightly susceptible for this isolate (Figure not shown). Therefore, only conclusions could be drawn about the activity of *Rpi-blb*2, suggesting that *Rpi-blb*2 was active. When this assay was repeated, the non-transformed Desiree performed the other way round, showing resistance for USA618 isolate and susceptible to NL12226 (Fig. 3.8 B). The positive control (*Rpi-edn2* transformant) for NL12226 isolate was also resistant but also the other single gene transformant, A0-33 (Figure not shown). Therefore, conclusions could not be drawn about the activity of *Rpi-edn2* gene.

In general, as explained for the DLA, the resistant events of A61 in whole plant assay were not all PCR positive for the T-DNA genes. Consequently, events were selected based on the match in resistance and integration of both introduced *R* genes.

This experiment showed that A61 transformants had low T-DNA gene integration (Fig. 3.6) but higher resistance expression of integrated genes (Fig. 3.9).
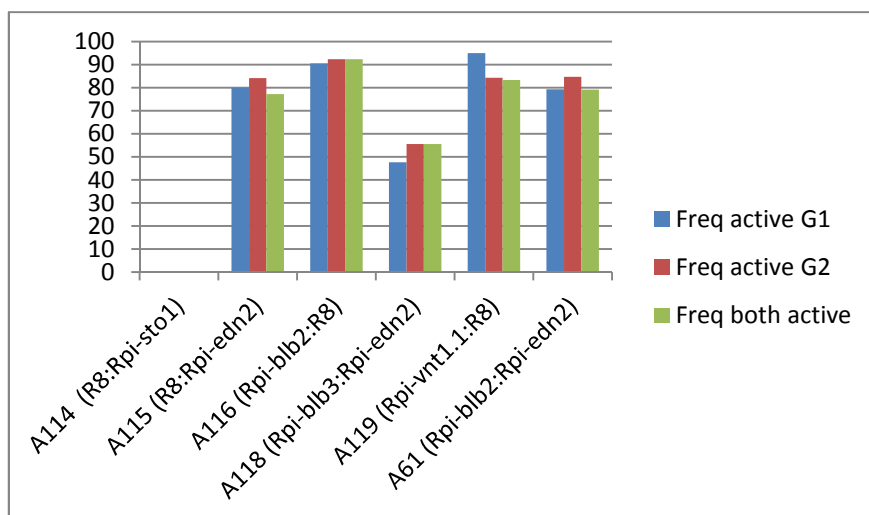
Fig. 3.9: Frequency of resistance expression in individual and both integrated T-DNA genes with climate cell assays (%).

3.3.3 Selection based on true to type for variety Desiree

Besides presence and resistance expression of introduced genes, transformants were also characterized for normal potato plant morphological appearance and growth characteristics. Accordingly, 165 regenerated events showed true to type for variety Desiree, out of the total 189 events propagated from the six transformation series (Table 3.4). High dropouts of regenerant events from true potato morphology was observed in A118 (*Rpi-blb2:Rpi-edn2*) transformants with frequency of 28.5%. In other transformants, as low as 8% dropouts from true to type was observed. The highest frequency of regenerating true to type for variety Desiree was obtained in A115 (*R8:Rpi-edn2*) and A61 (*Rpi-blb2:Rpi-edn2*) transformants with approximately 92% in both transformation series (Fig. 3.10).

Table 3.4: Growth and morphological appearance of transformants under greenhouse growing condition

Transformation series	Gene construct	# Total plants	# Plants true to type (good)	# Plants abnormal
A114	*R8:Rpi-sto*1	21	19	2
A115	*R8:Rpi-edn*2	49	45	4
A116	*Rpi-blb*2:*R8*	21	18	3
A118	*Rpi-blb3:Rpi-edn*2	21	15	6
A119	*Rpi-vnt1.1:R8*	21	17	4
A61	*Rpi-blb*2:*Rpi-edn*2	56	51	5
Total		189	165	24
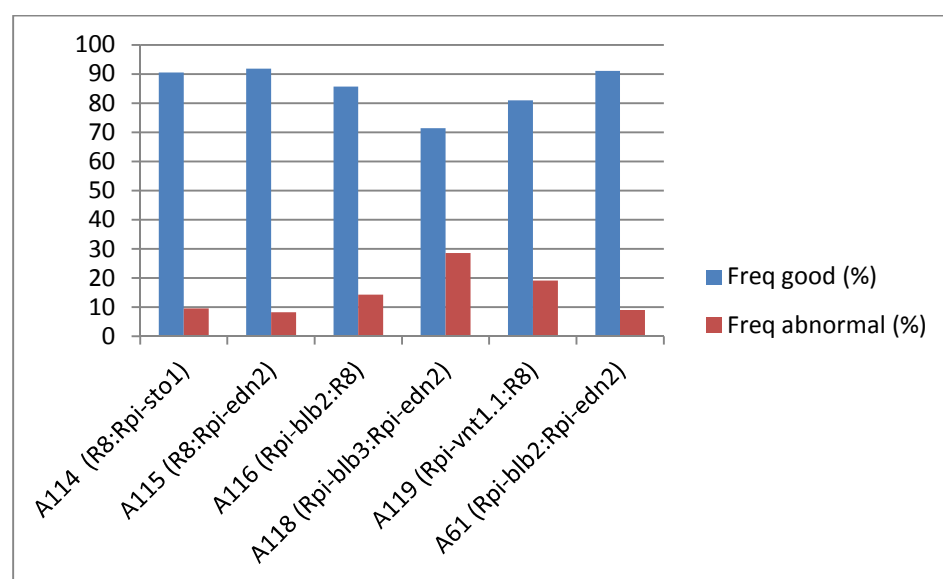
Fig. 3.10: Frequency of events expressing true to type (good) and abnormal for variety Desiree (%)

In general, transformants expressing resistance in both bioassays and confirmed for stable integration of both T-DNA genes with molecular analysis, and exhibiting true to type for variety Desiree were continued for vector backbone gene and T-DNA copy number analysis.

3.3.4 Selection against vector backbone genes

Integration of vector backbone genes into the genome of transformants is one of the problems to release genetically engineered products into the environment. Therefore, selection against these vector backbone containing events is important to carryout field trials with these plants. 57 events from the five transformation series harbouring complete T-DNA gene integration with active resistance expression and including other desirable phenotypic characteristics were grown in greenhouse and tested for vector backbone gene integration. In this research, the presence of seven vector backbone genes namely *tetA, trfA*, *nptIII*, *insB*, *oriV*, *traJ* and *tetR* in the order of LB to RB were examined in the 57 transformant events by PCR analysis.

3.3.4.1 Designing new primers and optimization of specificity conditions

For the vector backbone genes analysis, few primers were available in the lab. However, the previous primers were not sufficiently specific and new primers were designed to get more specific primers. Specificity conditions (annealing temperature) for newly designed primers were optimized (Table 2.4) by performing temperature gradient analysis on control plants.

The new primers were compared with previous primers for their specificity and amplicon qualities. Accordingly, clearer amplification (strong band intensity), low primer dimmer and better specificity was obtained with the new primers (Fig. 3.11 A), while high primer dimmer, weak amplification (many faint bands) and non-specificity was observed with previous primers (Fig. 3.11 B). Therefore, the new primers were used for vector backbone gene analysis and improved assays were achieved for each backbone genes.
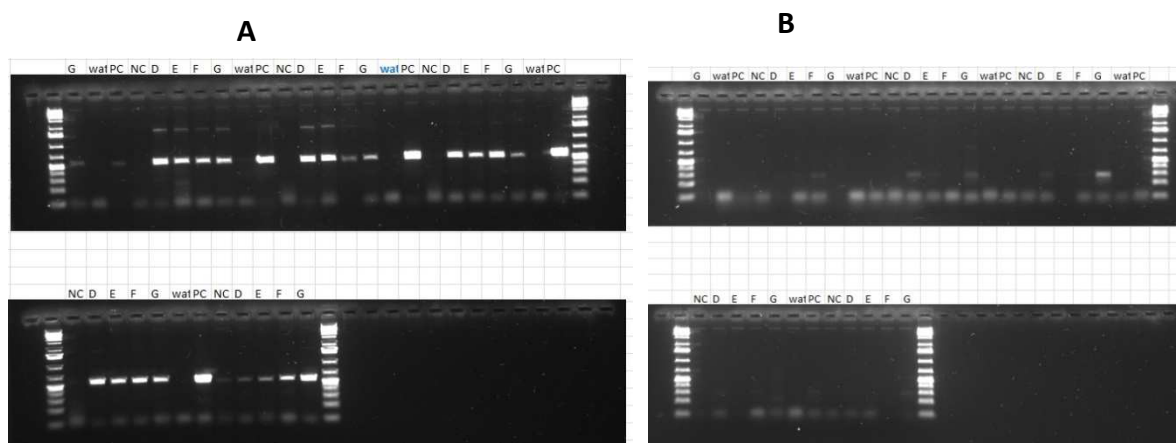
Fig. 3. 11: Comparison of old and new primers and optimizing primer specificity conditions in control plants (*tetA*): A) nice amplification quality- new primer B) poor amplicon quality- previous primer

3.3.4.2 PCR analysis for individual vector backbone genes

PCR programs were performed based on specificity conditions of the primers (Table 2.4) to all the reactions of *R8:Rpi-edn2* (A115), *Rpi-blb2:R8* (A116), *Rpi-blb3:Rpi-edn2* (A118), *Rpi-vnt1.1:R8* (A119) and *Rpi-blb2:Rpi-edn2* (A61). The results from DNA isolated in *in vitro* grown events showed lots of false positives for vector backbone gene integration, roughly more than 95% of tested events were positive for a backbone gene (Fig. 3.12 A). These results were unacceptable that maximum positive events for a vector backbone gene integration studied yet is 75%.
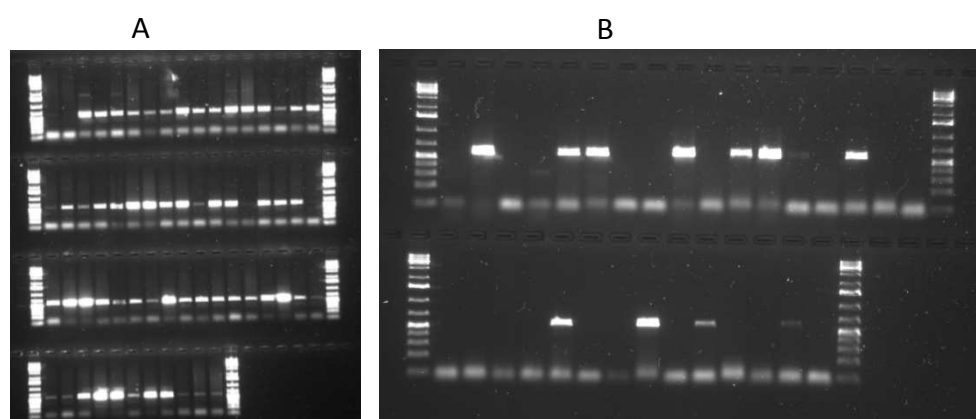
Fig. 3. 12: Examples of *tetA* backbone gene analysis with DNA from: A) *in vitro* plants, B) greenhouse grown plants.

As a potential solution, we assumed that such circumstances could be due to the bacterial genes which might not be completely disappeared at *in vitro* level. For this reason,

transformant events which were further grown in greenhouse for expanding the selection were tested for the frequency of backbone gene integration. Consequently, reliable percentages of positive and negative events for vector backbone genes have been obtained from these events (Fig. 3.12 B). As a result, we realized that transformants could have bacterial contamination at *in vitro* level and propagating further in greenhouse could completely disappeared the bacterial genes. Accordingly, previously tested events of A115, A116, A118, A119 transformation series were again propagated *in vitro* for two weeks and further grown for ±2 weeks in greenhouse on standard soil medium. After two weeks of greenhouse growth, DNA was isolated from these plants. Consequently, PCR analysis for the integration of each seven vector backbone genes was performed on this DNA.

From this analysis, 32 vector backbone gene free events were obtained out of the 57 events of the five transformation series (Table 3.5). High frequency of backbone gene free events were found in A118 (*Rpi-blb*3:*Rpi-edn*2) followed by A115 (*R8*:*Rpi-edn*2) and A116 (*Rpi-blb2:R*8) transformants (Fig. 3.13).
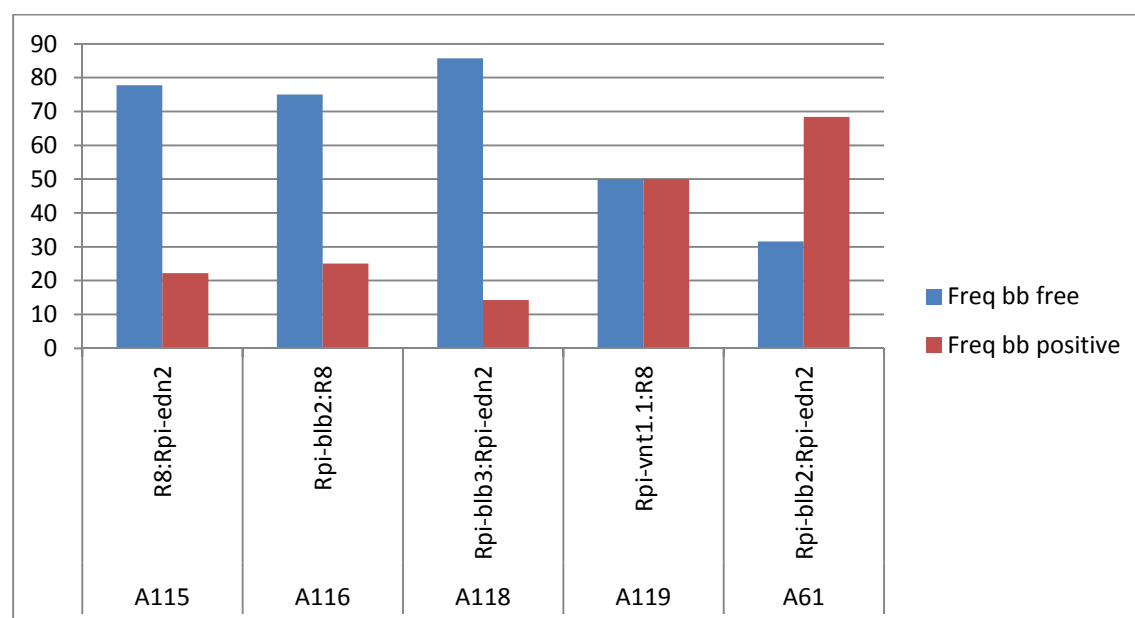
Fig. 3.13: Frequency of vector backbone gene free transformants (%): Freq = frequency, bb = backbone.

Table 3.5: List of plants selected as vector backbone gene free based on PCR analysis

Transformant event	Gene construct	Vector backbone genes					
		tetA	trfA	NPTIII	oriV	traJ	tetR
A115-2	R8:edn2	-	-	-	-	q	-
A115-3	R8:edn2	-	-	-	-	-	-
A115-12	R8:edn2	-	-	-	-	-	-
A115-13	R8:edn2	-	-	-	-	-	-
A115-17	R8:edn2	-	-	-	+	-	-
A115-18	R8:edn2	-	-	-	-	-	-
A115-24	R8:edn2	-	-	-	-	-	-
A116-1	blb2:R8	-	+	-	-	-	-
A116-5	blb2:R8	-	-	-	-	-	-
A116-19	blb2:R8	-	-	-	-	-	-
A116-22	blb2:R8	-	-	-	q	-	-
A116-26	blb2:R8	-	+	-	-	q	-
A116-28	blb2:R8	-	-	-	q	-	-
A118-9	blb3:edn2	-	-	-	q	-	-
A118-11	blb3:edn2	-	-	-	-	-	-
A118-14	blb3:edn2	-	+	-	-	-	-
A118-18	blb3:edn2	-	+	-	-	q	-
A118-22	blb3:edn2	-	-	-	-	-	-
A118-23	blb3:edn2	-	-	-	-	-	-
A119-1	vnt1.1:R8	-	-	-	q	-	-
A119-8	vnt1.1:R8	-	-	-	q	-	-
A119-10	vnt1.1:R8	-	-	-	-	-	-
A119-11	vnt1.1:R8	-	-	-	-	q	-
A119-13	vnt1.1:R8	-	-	-	-	-	-
A119-17	vnt1.1:R8	-	-	-	-	-	-
A119-21	vnt1.1:R8	-	-	q	q	q	-
A61-12	blb2:edn2	-	-	-	-	-	-
A61-44	blb2:edn2	-	-	-	-	-	-
A61-47	blb2:edn2	q	-	+	-	q	-
A61-49	blb2:edn2	-	-	-	-	-	-
A61-56	blb2:edn2	-	-	q	-	-	-
A61-63	blb2:edn2	-	-	+	-	-	-

"-"indicates no integration of the respective vector backbone gene

"q" indicates presence of invisible bands which didn't counted as positives

"+" indicates false positives for vector backbone gene integration

The pattern of backbone gene integration was mostly concentrated around the left border of the T-DNA, especially for A61 and A119 transformation series. On the other hand, little interruption of backbone gene integration was observed in A116 and A118 transformations but insignificant interruption pattern was observed in other transformation series (supplementary information in appendix B). In addition, the *nptIII* backbone gene was found in most events of A61 transformants while very low number of events of other transformation series harboured this backbone gene. In general, from all the tested transformation events, the pattern of vector backbone gene integration frequency was decreasing from the left border to the right border. The closest backbone gene to the left border, *tetA*, showed incidence of 40% integration followed by 37% for *trfA*, the next backbone gene to *tetA,* whereas at the closest right border, *tetR* showed only 17% incidence in the overall studied transformants (data in appendix B).

PCR results for *insB* backbone gene was also uncertain that all tested events were positive for this backbone gene including the negative control, non-transformed Desiree. Repeated PCR reactions were performed carefully to avoid possible contamination during reaction mixes. Though such efforts were performed, the results persistently showed that all tested event were positive for this backbone gene. This backbone gene is found in the middle of the T-DNA region, where there would not be convincing reason that only this gene is present for an event while all other backbone genes (on both borders of the T-DNA) were absent. Due to such reasons, the data of this gene was avoided for selecting backbone gene free events because of the assumption that the primers might be contaminated initially. Similarly, few events which showed positive only for a particular backbone gene where all other backbone genes were absent were selected as backbone free, because these were assumed as false positives (Table 3.5). With the same justification, appearance of very invisible bands (mentioned as q in Table 3.5) were taken as negative referring the absence of other adjacent backbone genes.

4. Discussion

4.1 Plant genetic transformation, T-DNA gene integration efficiency and (in) stability

The ability of crop plants expressing foreign genes after genetic transformation opened up new era of transforming plants to advance their defence against biotic and abiotic stresses, to produce improved products for agricultural and pharmaceutical purposes as well as for industrial values (Bhat and Srinivasan 2002). Genetic transformation for resistance breeding in potato against late blight disease has gained eminent concern following Irish potato famine. Subsequently, during the 20th century, resistance breeding against late blight focused on introducing major dominant *R* genes from *Solanum demissum* via genetic transformation. However, introgression of dominant *R* genes into susceptible varieties has been confronted by evolution of corresponding virulent *Phytophthora infestans* races (Bradshaw et al. 2006). For that reason, pyramiding combinations of broad spectrum *R* genes having synergistic resistance against particular disease or for different diseases, into single variety via genetic transformation is deemed to achieve broad spectrum durable resistance (Jain, Brar, and Ahloowalia 2010). In addition, (Tan et al. 2010) reprted that both durable and high level of resistance to potato late blight could be built up from the combined additive effect of two *R* genes pyramiding. Similarly, (Haesaert et al. 2015) studied that stacking of multiple *R* genes provided high level of resistance while these *R* genes showed lower level of resistance individually. This confirm that the advantage of the *R* genes' combined effect for building up durable and high level of resistance.

This research studied genetic transformation of potato variety Desiree, with two *P. infestans R* genes cloned from wild relative (crossable) species, using *Agrobacterium* mediated transformation. Six transformation series (A114, A115, A116, A118, A119 and A61) which were transformed before the start of this thesis were investigated with gene specific PCR for complete integration and stability of both introduced T-DNA genes. Different outcomes of T-DNA genes integration was achieved among the six transformation series, ranging from 42.8% in A61 (*Rpi-blb2:Rpi-edn2*) to more than 85% in A118 (*Rpi-blb3:Rpi-edn2*) and A119 (*Rpi-vnt1.1:R8*) transformants. Low frequency of the two T-DNA genes integration was observed in A61 (*Rpi-blb2:Rpi-edn2*) events, while A115 (*R8:Rpi-edn2*) and A116 (*Rpi-blb2:R8*) transformation series succeeded up to 61.9% and 71.4% respectively (Fig. 3. 6).

In two *R* gene pyramiding, (Jo et al. 2014) reported that 100% integration of both T-DNA genes was attained based on PCR positive events for both T-DNA genes in relation to rooted regenerants. In triple *R* genes pyramiding, (Zhu et al. 2012) also reported 82% (23 out of 28 events) achievement of T-DNA integration with PCR analysis. Accordingly, our T-DNA gene integration in A118 (*Rpi-blb3:Rpi-edn2*) and A119 (*Rpi-vnt1.1:R8*) transformants was in agreement with the study of (Zhu et al. 2012).

In this study, molecular (PCR) analysis confirmed that *Rpi-sto1* T-DNA gene in A114 events was not stably integrated, while most events were positive for the other (*R8*) gene. Furthermore, both the genes (*Rpi-sto1* and *R8*) did not conferred resistance to the corresponding *P. infestans* isolates in all events during climate cell phenotyping. The regenerants selected based on kanamycin resistance could indicate that initially the gene of interest was transformed successfully. The absence of this gene later during molecular analysis and the failure to confer resistance could be indicative of the instability of the gene within the genome of transformants. The gene deletion analysis with *R8* and *NPTII* primers proved that there had been deletion of *Rpi-sto*1 gene. Some events found amplified slightly greater than the *R*8 control (indicating partial deletion) and few others exactly equal to the *R*8 (complete deletion) while one another less than *R*8 (partial deletion of *R*8 and complete deletion of *Rpi-sto*1) as mentioned in the results section (Fig. 3.1 A, B and C). These analyses indicated that the construct was unstable and concluded that scattered partial and complete deletions occurred with these genes combination.

Transformation of potato, variety Desiree, was carried out by using two *A. tumefaciens* strains to study the combinability and stability of the reverse gene position (*Rpi-sto1:R8*) of problematic construct and another gene combination, *Rpi-blb3*:*Rpi-sto1*. After two months of transformation, the reverse gene combination of A114 construct, *Rpi-sto1:R8* induced greater green callus with AGL-1 *A. tumefaciens* strain than AGL-O strain. Conversely, the other construct, *Rpi-blb3*:*Rpi-sto1* induced greater green callus with AGL-O than AGL-1 strain. In previous studies, AGL-1 strain was reported as suitable to host plant genome and to inducing greater number of transformed callus than LBA4404 and AGL-O strains (Petti et al. 2009, Lazo, Stein, and Ludwig 1991). (Petti et al. 2009) also stated that transformation rate is influenced by the genotype of *A. tumefaciens* strain used and particularly, AGL-1 was stated as hyper virulent to develop transformed callus. However, in our experiment, both

AGL-1 and AGL-O strains behaved differently to the different gene combinations and both strains were suitable for the different gene combinations.

In a triple late blight *R* genes pyramiding experiment with Desiree variety, (Zhu et al. 2012) reported that five and nine months were counted to achieve a transformation efficiency of 14% and 59% respectively with complete root induction and shoot regeneration. (Jo et al. 2014) also reported that the size of T-DNA constructs could delay time of transformant regeneration. The size of constructs, under study in this research ranged from 13.7kb in *Rpi-sto1:R8* construct to 15.1kb in *Rpi-blb3:Rpi-sto1* construct. Accordingly, the regeneration time may vary depending on the size of the constructs.

This study indicated that different gene constructs required specific *Agrobacterium* strain and the suitability of the strains might depend on the type of gene constructs. Nevertheless, whether the combinability of *R* genes depends on the *Agrobacterium* strain used or conversely, the influence of the strains for efficient transformation depends on the type of gene combination used, should be investigated further. It should also be noted that our transformation was with Desiree variety and transformation rate of potato is variable depending on genotype of plants used (Heeres et al. 2002).

Green callus induction in this experiment was considered as indicator for transformation of the genes of interest. However, since all the shoots were not completely harvested and tested for root induction, it could not be possible to conclude that the above transformation efficiency is fully achieved. Shoots that grow further and develop roots in MS20 CK medium were transferred to fresh standard MS20 medium for permanent maintaining in the lab. Further Follow ups for shoot growth and root induction could not be fully performed with this thesis project due to time period completion. Subsequent monitoring activities for complete root induction and shoot regeneration, selection of well transformed events, PCR for T-DNA and vector backbone gene integration, analysis of *R* gene activity and T-DNA copy number were recommended as a continuation study.

4.2 Validation of *R* gene activity among two-*R* gene transformants

Functional expression of genes being PCR positive for T-DNA integration into host genome may be affected by silencing or other interfering factors (Butaye et al. 2004, Muskens et al. 2000). In order to determine the functional expression and activities of introduced *R* genes, inoculation assays were carried out in climate cells. Based on PCR analysis, 108 events from all transformation series studied were positive for two of T-DNA genes but with climate cell assays, only 83 of these events (76.8%) showed active resistance expression of both integrated genes against the corresponding *P. infestans* isolates (Table 3.2). Transformant events which were positive for both T-DNA genes but susceptible during climate cell assays were avoided from further backbone analysis.

In terms of each transformation series, different percentage of resistance expression was observed ranging from 55.56% in A118 (*Rpi-blb3:Rpi-edn2*) to 92.31% in A116 (*Rpi-blb2:R8*) transformants (Fig 3.9). Remarkably, A118 (*Rpi-blb3:Rpi-edn2*) transformants were the highest with respect to total T-DNA gene integration (85.7%) but lowest (55.56%) in terms of resistance expression of these genes. This could be justified in the sense that random integration of T-DNA at different chromosomal locations can result differences in functional expression of the transgenes. (Pröls and Meyer 1992) studied that T-DNA genes integrated at subtelomeric position would be actively expressed while those integrated at heterochromatic region could be supressed. Accordingly, individual tranformant events harbouring the same set of T-DNA genes could perform in a different way based on the position of their integration in the host genome. Similarly, variability in the level of resistance expression in inter-transformants could be caused by gene silencing phenomena. This gene silencing might be influenced by various factors like epigenetic and copy number of introduced genes (Butaye et al. 2004, Muskens et al. 2000). However, in terms of T-DNA copy number influence for transgene silencing, having single copy is not exclusively guarantee such that it could also occasionally occur in single transgene harbouring events (Meza et al. 2002). Thus, investigating individual independent events from each transformation series is essential in order to select events with complete integration and expression of the introduced genes as well as with all the desired characteristics. A61 (*Rpi-blb2:Rpi-edn2*) transformants showed extreme difference in terms of total T-DNA gene integration (42.86%) and in terms of active resistance expression (79.17%). Furthermore,

most A61 transformant events showed resistance while T-DNA genes were not detectable with PCR analysis. However, if the events were PCR positive for T-DNA genes but susceptible during climate cell assays, it could be explained as there might be silencing or other interferences for resistance expression of the genes. The inability to detect T-DNA genes while the events were resistant for the *P. infestans* isolates might be due to the T-DNA markers were les strong enough for detecting the respective genes.

Additionally, control events during climate cell inoculation assays didn't showed appropriate response to the corresponding *P. infestans* isolate. For example, in whole plant assay, the single *R* gene transformant A02-33 (*Rpi-blb2*) was resistant for both USA618 and NL12226 *P. infestans* isolates. Besides, A73.1.44 (*Rpi-edn2*) was resistant for NL12226 isolate but only slightly susceptible to USA618. Similarly, in DLA phenotyping where the plants were eight weeks old in greenhouse, the *Rpi-edn2* gene (A73.1.44) gave resistance for both *P. infestans* isolates. This could be indicative for the thoughts that *Rpi-edn2* gene is more active with broad spectrum resistance in older plants than in younger plants (Jack Vossen; personal communication). Such situation might also arose due to absolute resistance of individual *R* genes and not appropriate to identify the effect of individual genes in *R* gene pyramiding experiments (Tan et al. 2010). Furthermore, whole plant assay in A61 transformants was not reproducible that non-transformed variety Desiree showed resistance to one of the isolate while susceptible for the other isolate, but performed the other way round when the assay was repeated. However, the non-transformed Desiree was susceptible for both isolates during DLA analysis. Based on these scenarios, validation of resistance expression of *R* genes in A61 (*Rpi-blb2:Rpi-edn*2) transformant events might not be certain enough. Accordingly, it could not be wise to draw concrete conclusions in this particular experiment about the frequency of resistant expression in A61 (*Rpi-blb2:Rpi-edn2*) transformants events. Therefore, repeating climate cell inoculation assays for A61 transformants will be valuable to clearly determine the frequency with which this construct had performed. One option to be suggested for repeating this experiment is carrying out DLA analysis from the same leaves for whole plant assay with the same *P. infestans* growth and suspension conditions. Another option would be suggested to analyse integration of the T-DNA genes with more specific and strong T-DNA markers.

4.3   Selection of well performing events with desirable characteristics

Generally, this study investigated the combinability and functionality of pyramiding two late blight *R* genes and selection of well performing transformants, signifying all desirable variety Desiree characteristics. In addition to investigating the resistance spectra of stacked *R* genes, other societal ethics of plant transformation like integration of vector backbone genes and plant true-to-typeness was also studied.

This study began by pyramiding two late blight *R* genes in six distinct transformation series (Table 3.1); out of which 189 rooted transformant events were regenerated. Selection of desirable transformant events was started by narrowing down events based on integration of the two T-DNA genes (PCR). Subsequent narrowing was made based on functional expression of stacked R genes (climate cell assays) and plant morphology for true to type. Based on these characteristics, 57 events (Appendix B) were handed for further vector backbone gene and T-DNA copy number analysis. Selection of events against vector backbone genes was crucial factor, firstly because of European Directive 2001/18/EC field trial regulation, these events should be free from vector backbone genes to be tested in field conditions. This would also increase the chance of being exempting cisgenic products from the strict GM regulation (Schouten, Krens, and Jacobsen 2006). Secondly, (Iglesias et al. 1997) reported that inclusion of vector DNA in transformed plants could result in unstable expression of integrated genes of interest, possibly due to recognition of foreign prokaryotic vector DNA and consequent methylation together with the genes of interest. In addition, (Zhu et al. 2013) reported that transformants with three stacked *R* genes without vector backbone integration showed stable inheritance and functional expression of introduced genes in subsequent generations. This study also supports the idea that vector backbone genes can influence resistance expression and stability of introduced genes.

In this study, variable percentage of vector backbone gene integration was recorded among the five transformation series. In A118 (*Rpi-blb*3:*Rpi-edn*2) transformants, 85% of tested events were backbone gene free while the least was in A61 (*Rpi-blb*2:*Rpi-edn*2), only 31.58% of tested events were free from backbone gene (s). Therefore, this research is in agreement with (Zhu et al. 2013) who studied three late blight *R* genes pyramiding and achieved a success of 45% vector backbone gene free events among *R* gene containing transformants.

(Iglesias et al. 1997) stated that vector backbone genes could interfere with stable expression of transgenes. However, in this study, A118 (*Rpi-blb*3:*Rpi-edn*2) transformants showed high frequency of vector backbone gene free events but also low frequency of resistance expression. Nevertheless, events where the genes did not expressed active resistance were thrown away first and were not included for backbone gene analysis. This might lead the comparison in the way that low percentage of resistance expression and also low percentage of vector backbone gene integration among the selectively tested events. Moreover, A61 (*Rpi-blb*2:*Rpi-edn*2) transformation series showed both high percentage of resistance expression and high percentage of backbone gene integration, which seemed contradicting with (Iglesias et al. 1997). However, as mentioned earlier, climate cell inoculation assays of A61 transformants was not reproducible and the controls were not responsive in accordance to the expectations for the corresponding *P. infestans* isolates.

(De Buck et al. 2000) also indicated that variation in vector backbone gene integration among transformation series could arose neither due the plant species nor to the explant types used or the transformation employed. Rather, (De Buck et al. 2000) explained it as due to initiation and inefficient termination of T-DNA transfer at the right border and continued copying with inefficient termination at the left border. The pattern of vector backbone genes integrations in this experiment could be supported by (De Buck et al. 2000) explanation. That is, the right border backbone gene integration was only 17% (*tetR* gene) while in the left border, up 40% (*tetA*) backbone gene integration was observed from total transformants studied.

On the other hand, (Petti et al. 2009) reported that integration of vector backbone genes is influenced by the genotype of *Agrobacterium* strain used for transformation and AGL1 was found with low frequency of non-T-DNA sequences integration than LBA4404. However, in this study, the *Agrobacterium* strain used for all the five transformation series was AGL1 + virG and the variation in vector backbone gene integration could not be accounted for the genotype of *Agrobacterium* employed. Hence, variation in vector backbone gene integration among transformation series is common phenomena and the basis for the variation within same *Agrobacterium* genotype is yet to be investigated. Vector backbone gene integration in this study was ranged from 22% in A115 (*R8*:*Rpi-edn*2) to 68% in A61 (*Rpi-blb*2:*Rpi-edn*2) transformant events.

These results were in line with (De Buck et al. 2000) who indicated that 20% to 80% of transformant events could be contaminated with vector backbone genes and (Kononov, Bassuner, and Gelvin 1997) also reported 75% of vector backbone gene integration in transgenic tobacco. In general, with this selection processes, 32 independent transformants; seven, six, six, seven, and six events in A115, A116 A118, A119, and A61 series respectively were kept. These events were resistant for corresponding *P. infestans* isolates at laboratory level and free from vector backbone genes. Accordingly, the events were maintained and recommended for T-DNA copy number analysis and subsequent field trial to test resistance expression at field conditions (Table 3.5).

T-DNA copy number analysis could not be performed in this study due to the time limit of the thesis period. The copy number of T-DNA genes inserted to the genome of transformant events can be determined with two approaches, directly by using the respective T-DNA markers or indirectly by the copy number of *NPTII* and *Ef*-1a housekeeping genes with Real-time PCR (qPCR) detection system. This is because, the copy number of *NPTII* and *Ef*-1a housekeeping genes is believed equal to the copy number of T-DNA genes.

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Appendix A: List of plants studied, T-DNA integration and *R* genes activity

Transformant events	PCR result T-DNA genes		activity of sto1	activity of R8
	sto1	R8-GC		
A114-1	-	+	R	S
A114-2	-	+	S	S
A114-3	-	+	S	S
A114-4	-	+	R	S
A114-5	-	+	R	S
A114-6	-	+	S	S
A114-7	-	u	S	S
A114-8	-	+	S	S
A114-9	-	+	S	S
A114-10	-	+	S	S
A114-11	-	+	S	S
A114-14	-	u	S	S
A114-16	-	+	S	S
A114-17	-	+	S	S
A114-18	-	+	R	S
A114-19	-	+	S	S
A114-20	-	+	S	S
A114-21	-	+	R	S
A114-22	-	+	S	S
A114-23	-	u	S	S
A114-24	-	+	R	S

	PCR for T-DNA genes		activity of R8	activity of edn2
	R8-GC	edn2		
A115-1	+	+	S	R
A115-2	+	+	R	R
A115-3	+	+	R	R
A115-4	u	+	S	S
A115-5	+	+	R	R
A115-6	-	+	R	R
A115-7	u	+	S	R
A115-8	u	+	S	S
A115-9	u	+	S	R
A115-12	+	+	R	R
A115-13	+	+	R	R
A115-15	+	+		
A115-16	+	+	R	R
A115-17	+	+	R	R
A115-18	+	+	R	R
A115-20	+	+	R	S
A115-22	+	+	R	R
A115-23	+	-	S	R

	blb2	R8-GC	activity of blb2	activity of R8
A115-24	+	+	R	R
A115-27	+	+	S	R
A115-29	+	-	S	R
A115-32	+	+	Q	R
A115-33	+	+	R	R
A115-34	+	+	R	R
A115-35	+	+	S	S
A115-36	+	+	R	R
A115-37	U	+	R	R
A115-38	+	+	S	S
A115-39	+	+	R	R
A115-40	+	+	R	R
A115-41	+	+	R	R
A115-42	+	+	R	R
A115-43	+	-	R	R
A115-44	+	+	R	R
A115-45	+	+	R	R
A115-46	-	+	R	R
A115-47	+	+		
A115-49	U	+	R	R
A115-50	-	+	R	R
A115-51	+	+	R	R
A115-52	+	+	R	R
A115-53	+	+	R	R
A115-54	+	+	R	R
A115-55	+	+	R	R
A115-56	+	+	R	R
A115-57	+	u	R	R
A115-58	+	+	R	R
A115-59	+	+	R	R
A115-60	-	+	Q	R

	PCR for T-DNA genes			
	blb2	R8-GC	activity of blb2	activity of R8
A116-1	+	+	R	R
A116-2	+	+	R	R
A116-3	+	-	S	S
A116-4	+	+	R	R
A116-5	+	u	R	R
A116-6	+	-	R	R
A116-8	+	+	R	R
A116-10	+	+	R	R
A116-11	+	+		

A116-12	+	-	R	R
A116-13	+	+	R	R
A116-17	+	u	R	R
A116-18	+	-	R	R
A116-19	+	+	R	R
A116-20	+	-	R	R
A116-21	+	-	R	R
A116-22	+	+	R	R
A116-23	+	-	R	R
A116-26	+	+	R	R
A116-28	+	+	R	R
A116-29	+	-	R	R

	PCR for T-DNA genes			
	blb3	edn2	activity of blb3	activity of edn2
A118-1	+	+	S	S
A118-2	+	+	R	R
A118-3	+	+	R	R
A118-4	+	-	S	S
A118-5	+	+	R	R
A118-7	+	-	S	S
A118-8	+	+	S	S
A118-9	+	+	R	R
A118-10	+	+	R	R
A118-11	+	+	R	R
A118-12	+	+	S	S
A118-13	+	+	S	S
A118-14	+	+	R	R
A118-17	+	+	S	S
A118-18	+	+	R	R
A118-19	+	+		
A118-20	+	+	S	S
A118-21	+	+	S	S
A118-22	+	+	R	R
A118-23	+	+	R	R
A118-24	+	-	R	S

	PCR for T-DNA genes			
	vnt1.1	R8	activity of vnt1.1	activity of R8
A119-1	+	u	R	R
A119-2	+	+	R	R
A119-3	+	+	R	R
A119-4	+	+	R	R
A119-5	+	+		
A119-6	+	-	R	R
A119-7	+	+	R	R

A119-8	+	+	R	R
A119-9	+	+	R	R
A119-10	+	+	R	R
A119-11	+	+	R	R
A119-12	+	+	R	R
A119-13	+	+	R	R
A119-14	+	+	R	R
A119-15	+	+	R	R
A119-16	+	+	R	R
A119-17	+	+	R	R
A119-18	+	+	R	S
A119-19	-	+	S	R
A119-21	+	+	R	R
A119-22	+	+	R	R

	PCR for T-DNA genes			
	blb2	edn2	activity of blb2	activity of edn2
A61-1	u	-	R	R
A61-3	-	-	R	R
A61-4	-	-	R	R
A61-5	-	-	R	R
A61-6	-	-	Q	Q
A61-7				
A61-8	-	-	R	R
A61-9	u	+	R	R
A61-11				
A61-10	-	-	R	R
A61-12	+	+	R	R
A61-14	-	-	R	Q
A61-18	+	+	S	S
A61-19	u	-	Q	Q
A61-20	-	-	R	R
A61-21	-	-	R	R
A61-22	+	+	R	R
A61-23	+	+	R	R
A61-24	-	-	R	R
A61-26	+	+	Q	Q
A61-28	-	-	R	R
A61-29				
A61-30	+	-	R	R
A61-31	+	-	Q	Q
A61-32	u	+	R	R
A61-33	+	+	R	R
A61-34	+	+	R	R
A61-35	+	+	R	R
A61-36	u	-	R	Q

A61-38	+	-	R	Q
A61-39	u	-	R	R
A61-41	-	-	R	R
A61-42	-	-	R	R
A61-43	+	+	Q	Q
A61-44	+	+	R	R
A61-45	+	+	R	R
A61-46	+	+	R	R
A61-47	+	+	R	R
A61-48	-	-	R	R
A61-49	+	+	R	R
A61-50	+	U	R	R
A61-51	+	+	S	S
A61-52	+	-	Q	Q
A61-53			R	R
A61-54			R	R
A61-55	+	+	R	R
A61-56	+	+	R	R
A61-57	+	-	R	R
A61-58	+	+	R	R
A61-59	+	+	R	R
A61-60	+	+	R	R
A61-61	+	+	R	R
A61-62	+	+	R	R
A61-63	+	+	R	R
A61-64				
A61-65	-	U	R	R

"+": presence of T-DNA gene

"-": absence of T-DNA gene

"u" : unclear about the integration of the T-DNA gene

"R": Resistance

"S": susceptible

Appendix B: List of plants tested for backbone genes

Transformant event	gene construct	Vector backbone genes					
		tetA	trfA	NPTIII	oriV	traJ	tetR
A115-2	R8:edn2	-	-	-	-	q	-
A115-3	R8:edn2	-	-	-	-	-	-
A115-12	R8:edn2	-	-	-	-	-	-
A115-13	R8:edn2	-	-	-	-	-	-
A115-16	R8:edn2	+	+	+	+	+	+
A115-17	R8:edn2	-	-	-	+	-	-
A115-18	R8:edn2	-	-	-	-	-	-
A115-22	R8:edn2	+	+	+	-	+	-
A115-24	R8:edn2	-	-	-	-	-	-
A116-1	blb2:R8	-	+	-	-	-	-
A116-4	blb2:R8	-	-	-	+	+	+
A116-5	blb2:R8	-	-	-	-	-	-
A116-10	blb2:R8	+	+	-	+	q	-
A116-19	blb2:R8	-	-	-	-	-	-
A116-22	blb2:R8	-	-	-	q	-	-
A116-26	blb2:R8	-	+	-	-	q	-
A116-28	blb2:R8	-	-	-	q	-	-
A118-2	blb3:edn2	+	+	+	+	+	-
A118-9	blb3:edn2	-	-	-	q	-	-
A118-11	blb3:edn2	-	-	-	-	-	-
A118-14	blb3:edn2	-	+	-	-	-	-
A118-18	blb3:edn2	-	+	-	-	q	-
A118-22	blb3:edn2	-	-	-	-	-	-
A118-23	blb3:edn2	-	-	-	-	-	-
A119-1	vnt1.1:R8	-	-	-	q	-	-
A119-3	vnt1.1:R8	+	+	+	+	+	+
A119-4	vnt1.1:R8	+	+	+	+	+	-
A119-7	vnt1.1:R8	+	+	+	+	-	+
A119-8	vnt1.1:R8	-	-	-	q	-	-
A119-9	vnt1.1:R8	+	+	+	-	+	+
A119-10	vnt1.1:R8	-	-	-	-	-	-
A119-11	vnt1.1:R8	-	-	-	-	q	-
A119-13	vnt1.1:R8	-	-	-	-	-	-
A119-15	vnt1.1:R8	+	+	+	+	+	-
A119-16	vnt1.1:R8	+	+	+	+	+	-
A119-17	vnt1.1:R8	-	-	-	-	-	-
A119-21	vnt1.1:R8	-	-	q	q	q	-
A119-22	vnt1.1:R8	+	+	+	+	+	-
A61-12	blb2:edn2	-	-	-	-	-	-

A61-22	blb2:edn2	+	+	+	q	-	-
A61-33	blb2:edn2	+	+	+	+	+	-
A61-34	blb2:edn2	-	-	+	+	q	+
A61-35	blb2:edn2	+	+	+	+	+	-
A61-44	blb2:edn2	-	-	-	-	-	-
A61-45	blb2:edn2	+	+	+	+	+	-
A61-46	blb2:edn2	+	-	+	q	q	-
A61-47	blb2:edn2	q	-	+	-	q	-
A61-49	blb2:edn2	-	-	-	-	-	-
A61-50	blb2:edn2	+	+	+	-	-	-
A61-55	blb2:edn2	+	+	-	+	+	+
A61-56	blb2:edn2	-	-	q	-	-	-
A61-58	blb2:edn2	+	+	+	+	+	+
A61-59	blb2:edn2	+	+	+	+	+	+
A61-60	blb2:edn2	+	+	+	+	+	+
A61-61	blb2:edn2	+	+	+	-	+	-
A61-62	blb2:edn2	+	-	-	-	q	-
A61-63	blb2:edn2	-	-	+	-	-	-

"+": presence of backbone genes (positive)

"-": absence of backbone genes (negative)

"q": invisible band (not clear)