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Cis-transgenesis: Novel Approaches to Achieve Durable Resistance against Potato Late Blight (*Phytophtora infestans*)

Adane Gebeyehu*

Plant Biotechnology Program; National Agricultural Biotechnology Research Centre, Holeta, Ethiopia

Abstract

The present study was conducted to investigate combining ability of multiple resistance genes to achieve durable resistance against potato late blight (*Phytophtora infestans*) through cisgenesis and transgenesis approaches. In this study, both cisgenesis and transgenesis approaches were followed to introduce different combinations of potato late blight R genes. Six constructs containing combinations of late blight R genes were transformed to the potato variety Desiree using *Agrobacterium tumefaciens* mediated transformation and a bacterial kanamycin resistance selection marker (NPTII). The construct Rpi-blb2:R8 had the lowest transformation efficiency while R8: Rpi-edn2 had the highest transformation efficiency followed by R8: Rpi-sto1. Functionality tests in the previously transformed marker free and marker-assisted transformation events were also done using the agro infiltration assay and detached leaf assay as well as transfer-DNA (T-DNA) and vector back bone integration by using PCR. Most of the transgenic events were also evaluated in the field for their resistance against *P. infestans*.

Keywords

Cisgenesis, Late Blight, Marker-Free Transformation, Resistance Gene, Transgenesis

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1. Introduction

1.1. The Potato Plant

Potato (*Solanum tuberosum L.*) is originating from South America. It belongs to the family *Solanaceae* having 12 chromosomes and its ploidy level ranges from diploid (2n=2x=24) to hexaploid (2n=6x=72). It is the third largest crop in the world, after rice and wheat (http://cipotato.org/potato/facts). Potato is a cross-pollinating and vegetatively propagated crop, with a genome size around 840 Mb, which was recently sequenced [1]. This genome sequence helps for better understanding of the crop and paves the way for resistance breeding. Most potato cultivars are tetraploid (4n=48), with breeding programs taking minimally 10–15 before a new cultivar is developed. According to a report by [2], the current global potato production reaches 365 megatons. Even though this increase in production seems attractive, the late blight disease that is caused by the oomycete *Phytophthora infestans* (Mont.) de Bary, is devastating potato and tomato all over the world [3]. Late blight can result up to 16% global yield loss [2].

1.2. Potato Late Blight

Late blight is one of the most destructive diseases of potato and tomato worldwide, It is caused by an oomycete called *Phythophthora infestans* (Mont.) de Bary, originating either from South America or Mexico both of which are believed to be centres of origin of the host plant [3]. *P. infestans* is a hemi-biotrophic oomycete that first develops black and brown lesions on living tissues 3-5 days after infection, then causes necrosis, and finally forms spores on the abaxial side

* Corresponding author
E-mail address: adyamrot@gmail.com

of leaves, stems or tubers of the potato plant [4]. At the biotrophic phase, the pathogen attached with the host cells by making a specialized structure called haustorium that can be used either for nutrient uptake from the host plant and/or for effector delivery into the host plant [5]. *P. infestans* favors cool (15 - 25°C) and humid (RH~ 100%) environment with moderate temperature for its fast growth and development [4]. Depending on the season, *P. infestans* isolates have both sexual (Oospores made from A1 and A2 mating types) and asexual (zoospores) reproduction systems [6].

Due to its extremely rapid spread, it can completely colonize and defoliate the whole potato field within weeks [7]. *P. infestans* disease management was relying on the application of fungicides like metalaxyl, that are environmentally unfriendly and its frequent applications results in fungicide resistance because of evolutionary events taken place on the *P. infestans* strains [8] that was related with the emergence of the blue 13 clonal lineage [9]. Therefore, introgression of durable resistance from wild *Solanum* relatives is paramount important.

1.3. Potato Breeding for Durable Late Blight Resistance

Breeding for increased resistance to *P. infestans* in commercial cultivars has been one of the main goals in traditional potato breeding programs, but due to the amazing capability of *P. infestans* to break resistance, there has been no success yet in developing commercial potato varieties with durable resistance [10]. Using GM approaches in conjunction with conventional potato breeding is suggested as one possible strategy to develop varieties with more durable resistance [7].

Durability of disease resistance means that the resistance remains effective for a long time during wide cultivation. The achievement of durable disease resistance remains a major issue. It is very important to combine multiple resistance (*R*) genes to breed potato cultivars with durable resistance against *P. infestans*. The rapidly evolving *P. infestans* population can easily break single *R* gene based resistance, since many *R* genes could stay durable for longer time. The existing gene pool in the Solanum species suggests enough possibilities to investigate new R genes against *P. infestans* [11].

Several broad-spectrum *R* genes against *P. infestans* have been cloned to date [12]. For example *Rpi-sto1*, *Rpi-pta1* and *Rpi-blb1* having avirulence effector *Avrblb1* [13], *Rpi-blb2* [14] and *Rpi-vnt1.1* [15]. The *RB/Rpi-blb1* gene from *S. bulbocastanum* showed a broad-spectrum resistance against various known *P. infestans* strains [16]. RB/Rpi-blb1 has been cloned and transformed into Katahdin, a highly susceptible potato cultivar [17]. Katahdin plants transformed with the *RB/Rpi-blb1* gene showed broad-spectrum resistance against many *P. infestans* strains in the greenhouse and field tests. This result shows that it is possible to develop resistant varieties by introgression of the RB gene from *S. bulbocastanum* into cultivated potato, by GM breeding methods. Now, attention is mostly paid to the *RB/Rpi-blb1* gene that originates from *S. bulbocastanum* [18].

Stacking of multiple *R* genes in a single genotype is promising strategy to bring more durable resistance, especially with broad-spectrum *R* genes for effective HR reaction between *R* genes and their corresponding *Avr* effectors in planta [7]. This can be achieved through using the genetic transformation (GM) approach.

1.4. The Genetic Transformation (GM) Approach

This is a kind of approach by which a gene of interest having desired trait is transferred and integrated into a recipient genome. It has many advantages over traditional breeding. For example, it is possible to transform one or more *R* genes to a potato cultivar that is under wide cultivation for more optimal production through making it resistant to various pathogens and insects. In addition, only desirable traits can be transferred unlike the conventional breeding that often brings undesirable traits from the wild *Solanum* species together with the desirable *R* genes in short called linkage drag [19]. The time to breed a cultivar is also much shorter using GM approach than the conventional breeding strategy. Genetic transformation can be done either through transgenesis or through cisgenesis. Transgenesis is a kind of genetic modification approach to introduce genes of interest originated from any source either from non-crossable plant species or from non-plant origin collectively known as transgenes. For example, the bacterial nptII gene that codes for kanamycin resistance is a transgene. The *Bt* gene from the bacteria *Bacillus thuringiensis* is also a transgene transferred to the cotton genome for pest resistance. Whereas, cisgenesis is an approach that introduce genes of interest from crossable plant species of its own gene pool called cisgenes under the control of its own promoter and terminator [20]. For example, the *R1* gene from wild *Solanum demissum* is a cisgene transferred to cultivated potato *Solanum tuberosum* for late-blight resistance.

Cisgenic plants are more acceptable by the consumers than transgenic plants in the EU [21]. A survey in the USA also shows that, 55-77% of the respondents preferred to eat cisgenic vegetables [22]. Now a days, researchers focus towards cisgenesis [23] due to its safety to consumers and the environment [24]. Recently, three cisgenic *R* genes were stacked in a susceptible potato cultivar [25]. Cisgenic breeding enables fast transfer of multiple *R* genes and prevents linkage drag, which is a real issue when using classical breeding [10].

The success of this approach in the future is heavily dependent on the availability of *R* genes. For this reason, the urge to find novel late blight *R* genes is high and many wild *Solanum* relatives were characterized.

1.5. *Agrobacterium tumefaciens* Mediated Transformation

Transformation can be done using four different methods. These are electroporation, transformation via viral vectors, particle bombardment by coating the gene of interest in tungsten or gold bullets and fired using gene gun and using the gram-negative *Agrobacteria*. Among those, *Agrobacterium* mediated transformation is the commonest method of transformation because of its efficiency of transferring the gene of interest (Transfer DNA) and low copy number of genes as compared to gene gun [26]. *Agrobacterium* mediated transformation depend on the use of a binary vector system to integrate the right boarder of the T-DNA plasmid and the left boarder even though the vector back bones are also integrated [27]. Therefore, the aim of this research was,

i. To investigate *R*-genes combining ability through the *A. tumefaciens* mediated transformation.

ii. To find an Agroinfiltration assay to detect *Rpi-edn2* activity.

iii. To compare marker-free and marker-assisted transformation regarding efficiency of functional R gene expression.

1.6. Experimental Outline

Part1. The transformation of different *R* genes and combinations of *R* genes in to a potato by means of *A. tumefaciens* transformations for the purpose of obtaining resistance to *Phytophthora infestans.*

Part2. The development of an *Avr-edn2* assay for the identification of Avr-edn2 activity

Part3. Testing already transformed potato plants on their recognition specificity for the presence of the desired *R* gene in the plant genome using PCR and the study of the activity of these genes using detached leaf assay (DLA) and the Agro-infiltration.

2. Materials and Methods

2.1. Plant Materials

Four groups of events derived from the same Desiree marker assisted transformation event: five A55 events (transformed with vnt1:blb3), four A53 events (transformed with blb3:chc1), six A99 events (transformed with vnt1:edn2:chc1) and three A100 events (transformed with vnt1:edn2:sto1). Two groups of *Rpi-edn2* transformation events consisting of fifty JV19 marker free transformation events (V108) and seven JV19 marker assisted transformation events (V73.4). Eight V109 events derived from JV19 marker free transformation event (transformed with vnt1:blb3) and two groups of events derived from JV19 marker free co-transformation with edn2:vnt1:blb3 (one V102.1 event and six V102.2-2 events) were tested for late blight resistance (Table 1). The vector pBINAW was used for marker free transformation whereas the vector pBINPLUS PASSA was used for marker-assisted transformation. All populations were evaluated for late blight resistance using the *P. infestans* isolates, IPO-C, Ec-1, Dinteloord and 90128 (Supplementary Table 1).

Table 1. The nine potato populations and its corresponding *P. infestans* isolates.

Cv.	Trafo code	Plasmid number	R genes	Vector	*P. infestans* isolates	
V	108	211	edn2	pBINAW	IPO-C	90128
V	73.4		edn2	pBINPLUS PASSA	IPO-C	90128
A	55	145	vnt1:blb3	pBINPLUS PASSA	Ec-1	IPO-C
V	109	107	vnt1:blb3	pBINAW	Ec-1	IPO-C
V	102.1	107&211	edn2:vnt1:blb3	pBINAW	IPO-C	90128
V	102.2	107&256	edn2:vnt1:blb3	pBINAW	IPO-C	90128
A	53	156	blb3:chc1	pBINPLUS PASSA	IPO-C	Dinteloord
A	99	224	vnt1:edn2:chc1	pBINPLUS PPASSA	IPO-C	
A	100	227	vnt1:edn2:sto1	pBINPLUS PASSA	IPO-C	

Key: V=JV19, A=Desiree

2.2. *P. infestans* Isolates

All events were evaluated for late blight resistance using the *P. infestans* isolates IPO-C, 90128, Ec-1 and Dinteloord (Supp. Table 2). These isolates were grown at 18°C on rye sucrose agar (RSA) medium supplemented with 2% sucrose. To feed mycelium for nutrients, cultures were grown in solid rye medium with sucrose (RS) in petri dishes for 2 weeks.

Table 2. List of *P. infestans* isolates used with their corresponding Avr effectors.

Isolates	Avr effectors
IPO-C	Avrvnt1, AVRedn2, avrblb3, AVRchc1, AVRsto1
90128	AVRvnt1, avredn2, AVRblb3, AVRchc1, AVRsto1
Ec-1	avrvnt1, AVRedn2, AVRblb3, AVRchc1, AVRsto1
Dinteloord	Avrvnt1, AVRedn2, AVRblb3, avrchc1, AVRsto1

2.3. Detached Leaf Assay

Detached leaf assays (DLA) were done in duplicate for each event after plants have grown for five weeks (DLA1) and seven weeks (DLA2), respectively in the green house. One hundred four plants containing the *R* gene *Rpi-edn2,* the positive control (untransformed JV19) and the transformed Desiree events containing the *Rpi-edn2* gene (A73.1-11) were used as negative control. The transformed Desiree events containing the *Rpi-vnt1* gene (A13-13) and transformed Desiree containing *Rpi-blb3* gene *(*A03-142) were used as negative controls for the events containing the *R* gene *Rpi-vnt1:blb3*. The transformed Desiree events containing the *Rpi-chc1* gene (A17-27), the *Rpi-vnt1* gene (A13-13), the *Rpi-blb3* gene *(*A03-142), containing *Rpi-sto1* gene (A09-267) and the transformed Desiree containing both *Rpi-vnt1* and *Rpi-chc1* genes (A19-46) were used as negative controls for events containing the R genes; *Rpi-chc1:blb3, Rpi-vnt1:edn2:chc1* and *Rpi-vnt1:edn2:sto1***.** In all experiments, JV19 was used as positive control instead of Desiree. Two plants per event and two leaves per plant were collected and leaves were inserted into wet floral foam bricks placed on wet filter paper in plastic trays. To induce the release of zoospores, the rye agar plates, containing the *P. infestans* were diluted with 10 ml of cold tap water and the sporangia were transferred to 50 ml blue cap tubes and incubated at 4^0C for 2 hours. Then, the suspension was further diluted to a concentration of $5x10^4$ zoospores per ml. After this, detached potato leaves were inoculated on the abaxial side with 10µl drop containing zoospores of the corresponding *P. infestans* isolates per leaf and were labeled showing cultivar name, event number and *P. infestans* isolates used. The trays were sealed in plastic bags to ensure humidity reaches 100%. The trays were incubated in a climate-controlled room at 15°C and 16h of light in a 24h period. Six days post inoculation the disease severity was measured using the scale in Supp. Table 3. Finally, results were compared to a negative control containing the *R* gene(s) and the positive control that is untransformed Desiree (JV19) plants. Transformants with marker and marker free transformants were also compared in response to resistance to *P. infestans* isolates.

Table 3. DLA disease severity scale.

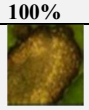
Symptoms	Score	Weighing factor	Group
No/hardly visible lesion	R9	0	R
lesion the size of the inoculated drop	R8	1	R
medium lesion	R7	2	R
large lesion	R6	3	MR
dry lesion, no sporulation	V5	4	MR
wet lesion, no sporulation	V6	6	S
sporulation on bottom side	V7	8	S
sporulation on both sides	V8	9	S

R=resistant; MR= moderately resistant; S=susceptible

2.4. Agro-infiltration of Avirulence (Avr) Genes

Agro-infiltration is a technique by which *A. tumefaciens* suspensions were injected into plant leaves of the selected events and control plants to show HR in response to their corresponding *Avr* genes. Transformation events were cultured in vitro on MS20 medium for two weeks in the growth chamber; they were transferred to the green house and grown for two weeks. Two days prior to the agroinfiltration assay, *A. tumefaciens* glycerol stocks containing the appropriate *Avr* gene(s) were inoculated on 3 ml LB medium with 50µg/ml of the appropriate antibiotic(s) like kanamycin and spectinomycin. These cultures were grown overnight at 30°C. The next day, the samples were transferred to YEB+++ medium and 20 ml of this medium was used and a calculated amount of overnight culture was transferred to this medium. The YEB+++ culture was incubated at 30°C for the calculated amount of time. On the day of the infiltration, the optical density (OD) of the samples was measured, followed by centrifuging them for eight minutes at 4000 rpm. The supernatant was then discarded and the pellet was re-suspended in a small amount of freshly made MMA+. After that, the OD was adjusted to 0.3. Then, the samples were ready to be infiltrated. A positive control consisting of two *A. tumefaciens* strains, one with *Avr3b* and one with *R3b*, were mixed together and a negative control consisting of an *A. tumefaciens strain* containing *R3B alone was* also infiltrated. Along with that, each plant was infiltrated with an *A. tumefaciens* strain containing the appropriate *Avr* genes. Plants that had JV19 were also infiltrated with RD28 and PITG_20303 to confirm the activity of the *Rpi-edn2* and *Avr2 & Avrvnt1* to check activity of the *Rpi*-blb3 and *Rpi-vnt1* genes respectively. A 1ml syringe was used to inject the *A. tumefaciens* strains in the leaves. Nine replicates were performed; three plants per event with infiltration of three leaves per plant were used. In all experiments, JV19 was used as positive control instead of Desiree. Responses were scored three days post inoculation using the HR severity scale in Supp. Table 4 and results were compared to the control plants.

Table 4. Images of spots with their corresponding scoring scales (adapted from Bijsterbosch Gerard, 2014).

% of necrosis	0%	25%	50%	75%	100%
Leaf display					
Numerical	0	0.5	1	1.5	2

N.B. Spots more than 0.5 but less than 1.0 gets a 0.75 and between 0 and 0.5 gets a 0.25

2.5. DNA Isolation

DNA extraction were performed from one unexpanded young leaf from green house plants and were put into non-sterile polypropylene 1.2 ml tubes (Collection Micro tubes (racked, 8 x 12) having two grinding balls inside. These tubes were stored at -80°C and cooled with liquid nitrogen prior to grinding the leaves in a Retch machine. Then, a mix of 400µl extraction buffer (Supp. Table 5), 500µl nuclei lysis buffer (Supp. Table 6) and fresh buffer working solution 50µl sarkosyl having 0.5% (w/v) sodium metabisulfite was added. Then the samples were shaken, and incubated in a water bath at 65^0C for 1 hour. The tubes were cooled on ice prior to the addition of 400µl chloroform and isoamyl alcohol in 24:1 ratio and mixed well. Then samples were centrifuged at 4600 rpm for 10 minutes and 350µl of the upper watery phase of each sample were pipetted into new deep well strips. Then 200µl isopropanol were added to each sample in order to precipitate the nucleic acids. The solutions were mixed by gently inverting the racks to allow precipitates to settle. Then the samples were centrifuged at 4600 rpm for 5 minutes. The supernatant were poured off. Then 300µl 70% ethanol was added; centrifuged at 4600 rpm for 5 minutes to wash the pellet and ethanol was poured off. The pellets were dried at 37^oC for 30 minutes and re suspend DNA in 100µl tris-EDTA (TE) buffer (10mMtris-HCl pH 8.0, 1mM EDTA pH 8.0 and 10µg/ml RNAse). The DNA samples were diluted 10 fold with MQ and stored at-20^oC. Finally, the quantity and quality of the DNA was checked using Gel electrophoresis.

Table 5. Extraction buffer preparation.

Extraction buffer stock	*for 500 ml:*
0.35 M Sorbitol	31.9 g Sorbitol
0.1 M Tris-HCl pH 8.0	50 ml 1M Tris-HCl pH 8.0
5 mM EDTA pH 8.0	5 ml 0.5M EDTA pH 8.0

Table 6. Lysis buffer preparation.

Lysis buffer stock	*for 500 ml:*
0.35 M Sorbitol	31.9 g Sorbitol
0.1 M Tris-HCl pH 8.0	50l 1M Tris-HCl pH 8.0
5mM EDTA pH 8.0	5ml 0.5M EDTA pH 8.0

2.6. PCR Assays

In order to investigate the absence of sequences outside left border (LB) and right border (RB) referred to as vector backbone sequences, as well as the presence of sequences in the T-DNA region, PCR analysis with four primer pairs (Two primer pairs for LB and two primer pairs for RB) were performed. The presence of vector backbone sequences were analyzed by PCR using primer pairs from eight vector backbone genes referred to as *tetR*, *oriV*, *traJ*, *oriv3+5*, *insB*, *nptIII*, *trfA*, and *tetA* as shown in figure 1 below.

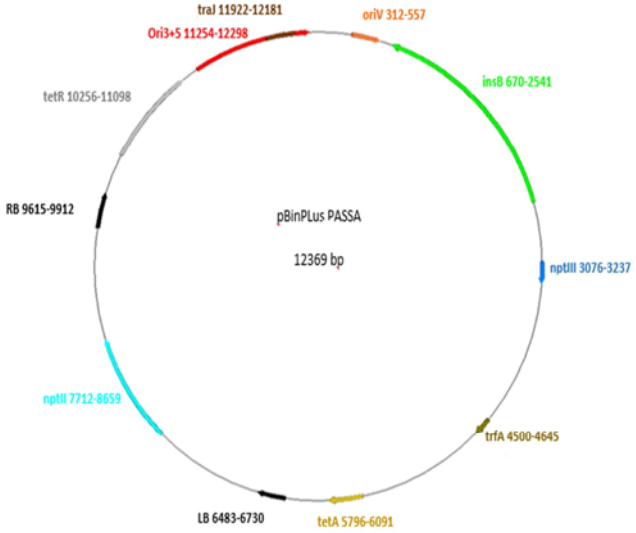


Figure 1. Location of markers on the pBinPLUS PASSA vector.

2.7. Plant Transformation

Stable insertion of the *Rpi* genes into susceptible potato cultivar Desiree was carried out using the binary vector pBINPLUS via *A. tumefaciens* mediated transformation as adapted by [25]. One month prior to transformation, the susceptible cultivar Desiree was grown in vitro on MS20 medium and placed at 25°C in the growth chamber. Two days before transformation, bacteria cultures containing *A. tumefaciens* with the appropriate *R* gene combinations were prepared and put on a shaker until those six *A. tumefaciens* constructs (Supp. Table 7) were grown.

Table 7. Constructs used for *A. tumefaciens* mediated transformation.

Trafo code	Insert	Plasmid number	Vector
114	R8:sto1	278	pBINPLUS-PASSA
115	R8:edn2	279	pBINPLUS-PASSA
116	blb2:R8	280	pBINPLUS-PPASSA
117	blb3:R8	281	pBINPLUS-ASSAP
118	blb3:edn2	282	pBINPLUS-ASSAP
119	vnt1:R8	283	pBINPLUS-PPASSA

One day before transformation, internodes of the in vitro grown plants were cut into 2-5 mm sized explants. Then, explants were placed on a plate containing R_3B medium, covered with two sterile filter papers and 1.5 ml liquid PACM medium.

Then, explants were submerged in *A. tumefaciens* suspension for 5-10 minutes, dried on sterile filter paper, placed back to

the R3B plates and sealed with parafilm. Two days after the transformation the explants were transferred to zcvk plates to ensure selective growth of transformants. Three controls were also used:

i. No bacteria, no selection (ZCV) to check failure to regenerate in to callus was due to *A. tumefaciens* or not.

ii. With bacteria, no selection (*A. tumefaciens* + ZCV) to check antibiotic functionality.

iii. No bacteria, with selection (ZCVK) to check regeneration efficiency of plants in the absence of *A. tumefaciens*. Explants were transferred to fresh ZCV plates every 2 weeks.

2.8. Late Blight Field Trials

Late blight resistance assays under field conditions were performed in the year 2014. *In vitro* potato plants were planted in the field on 22nd of May. Two replications, each with four plants per genotype, were planted. One month later, the first natural *P. infestans* infections were found in the susceptible varieties planted. The field was kept moist to sustain the late blight epidemic. Plants were examined every week from 27th of June until 18th of July. The percentage of the leaf area covered by late blight lesions was estimated by visual inspection.

3. Results

3.1. Transformation Efficiency Analysis of Constructs

The susceptible potato cultivar Desiree was transformed with six constructs (*R8*: *Rpi-sto1*, *R8*: *Rpi-edn2*, *Rpi-blb2: R8*, *Rpi-blb3: R8, Rpi-blb3: Rpi-edn2* and *Rpi-vnt1: R8*) containing double late blight resistance genes. During the experiment, 120 explants were used for each construct. In the first four weeks, none of the explants had shown any signs of regeneration into callus. After five weeks, transformants were regenerated into shoots and put into kanamycin containing (CVK) MS20 rooting medium to check for transformation efficiency. New shoots were harvested every two weeks (week 5, 7and 9) and put in to a CVK medium. Then kanamycin resistant events rooting on the medium were recorded each week after they were transferred in to the CVK medium (week 6,8 and10) and the transformation efficiency of each construct was calculated as the number of rooted shoots regenerated on kanamycin containing medium divided by the total number of explants used for each construct during the experiment (i.e.120 explants).

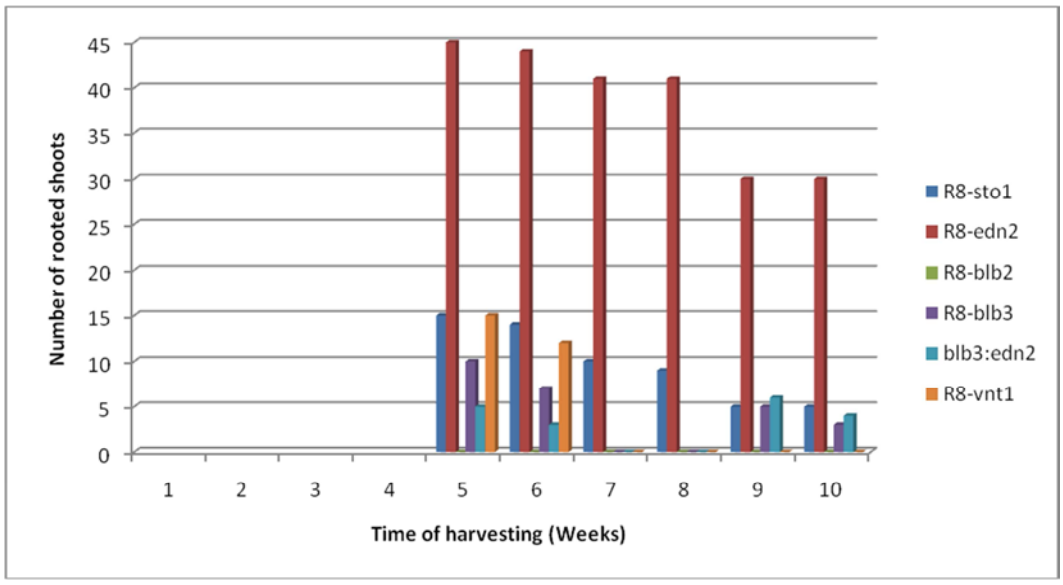


Figure 2. Transformation efficiency of different constructs.

When the transformants were compared to each other, differences in transformation efficiency were seen (Figure 2). The construct *Rpi-blb2:R8* does not have any transformants and considered as the lowest transformation efficiency (0%) while R8: *Rpi-edn2* had the highest transformation efficiency (95.8%) followed by *R8*: *Rpi-sto1* (23.3%). The construct *Rpi*-vnt1:R8 had 10% transformation efficiency and the construct *Rpi-blb3: R8* had only 8.33% transformation efficiency. The lowest transformation efficiency in *Rpi-blb2:R8* transformants was due to mutation in the pBINPLUS-PPASSA vector backbone (J.H. vossen personal communication). The transformation efficiency was also decreased over time. For example, the construct *Rpi-vnt1: R8* could not have any transformants 7 weeks after transformation as well as no transformants were seen for blb3: R8 and blb3:edn2 inserts in the 7th & 8th week.

3.2. Agroinfiltration Assay to Detect *Rpi-edn2* Activity

Functionality tests of the *Rpi-edn2* gene was carried out using agroinfiltration of the cognate effectors RD28 and PITG-20303. During the agroinfiltration assay, plants transformed with the resistance gene *Rpi-edn2* could not give specific hypersensitive response against both *Avr* genes used even though the OD was lowered from 0.3 to 0.2 assuming that lower ODs can reduce the degree of auto-necrosis and can give specific HR. The two *Avr* genes RD28 and PITG-20303 were not ideal for agroinfiltration assay in our potato plants. The *Avr* gene RD28 gave no response and the *Avr* gene PITG-20303 gave very light response in addition to auto-necrosis in JV19 plants that makes difficult in scoring *Rpi-edn2* activity in both marker free and marker assisted transformants.

3.3. Functional Tests of Transformants

a) Functional test of Rpi-chc1:blb3 transformants

Transformants were tested for presence or absence of the *Rpi-chc1:blb3* resistance genes using PCR and both of the transformants were containing both the *Rpi-blb3* and the *Rpi-chc1* genes. Functional expression of the introduced *Rpi-chc1* and *Rpi-blb3* genes were done using agroinfiltration of the matching *Avr* genes, *Avrchc1* and *Avr2* (Supp. Table 8; Figure 4). Those transgenic plants were also tested twice for resistance to the *P. infestans* isolates IPO-C and Dinteloord using a detached leaf assay (Supp. Table 8; Figure 3). Finally, the following results were obtained.

Table 8. Summary of *Rpi-chc1:blb3* transformants functionality test.

Genotype	chc1 PCR (+:-)	blb3 PCR (+:-)	No of events tested	IPO-C Dinteloord (DLA)R:S	Events expressing Chc1 (ATTA) (+:-)	Events expressing blb3 (ATTA) (+:-)
A53	2:0	2:0	2	2:0	2:0	2:0

Both of the transgenic events with marker (Figure 4) shown resistance to both *P. infestans* isolates IPO-C and Dinteloord. This indicates that the inserted late blight resistance genes *Rpi-chc1:blb3* were actively expressed, since the non-transformed control JV19 and the *Rpi-blb3* Desiree transformant A03-142 were susceptible to the isolate IPO-C and both JV19 and the *Rpi-chc1*transformant A17-27 were susceptible to the isolate Dinteloord.

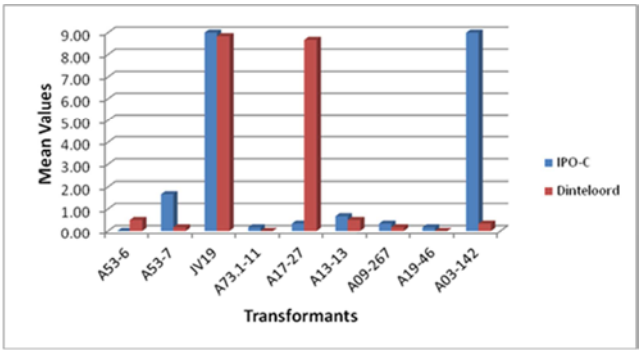


Figure 3. Detached leaf assay of transgenic potatoes transformed with *Rpi-chc1:blb3* gene constructs.

During the agroinfiltration assay, both plants transformed with the resistance gene *Rpi-chc1:blb3* gene constructs gave specific HR response against the *Avr* gene *Avr2* and only A53-6 gave HR for *Avrchc1* (Figure 4).

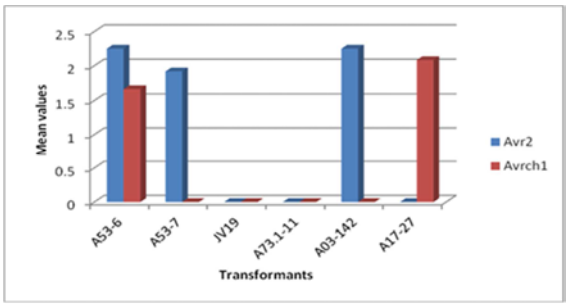


Figure 4. Agroinfiltration assay of transgenic potatoes transformed with *Rpi-chc1:blb3* gene constructs.

b) Functional test of Rpi-vnt1:blb3 transformants

The *Rpi-vnt1:blb3* transformants were tested for presence or absence of the *Rpi-vnt1:blb3* resistance genes using PCR and all of the transformants were containing the *Rpi-blb3* gene. The PCR result of the transgenic events was not reliable for the resistance gene *Rpi-vnt1* since the negative controls also show positive result. Functional expression of the introduced genes *Rpi-vnt1* and *Rpi-blb3* were tested using agroinfiltration of the matching *Avr* genes, *Avrvnt1* and *Avr2* (Supp. Table 9; Figure 6). Those transgenic plants were also tested twice for resistance to the *P. infestans* isolates IPO-C and Ec-1 using a detached leaf assay (Supp. Table 9; Figure 5). Field test was also done for their resistance to *P. infestans* and the following results were obtained.

Table 9. Summary of *Rpi-vnt1:blb3* transformants functionality tests.

Genotype	blb3 PCR (+:-)	No of events tested	IPO-C Ec-1 (DLA) R:S	Events expressing vnt1 (ATTA) (+:-)	Events expressing blb3 (ATTA) (+:-)
A55	5:0	5	5:0	5:0	5:0

All of the transgenic events with marker showed resistance to both *P. infestans* isolates IPO-C and Ec-1(Figure 5). This

indicates that the inserted late blight resistance gene *Rpi-blb3* was actively expressed, since the non-transformed control JV19 and the Desiree transformant containing only *Rpi-blb3* (A03-142) were susceptible to the IPO-C isolate. The control A13-13 was susceptible to Ec-1 whereas A03-142 was resistant to the isolate Ec-1 because of the presence of *Rpi-blb3* gene.

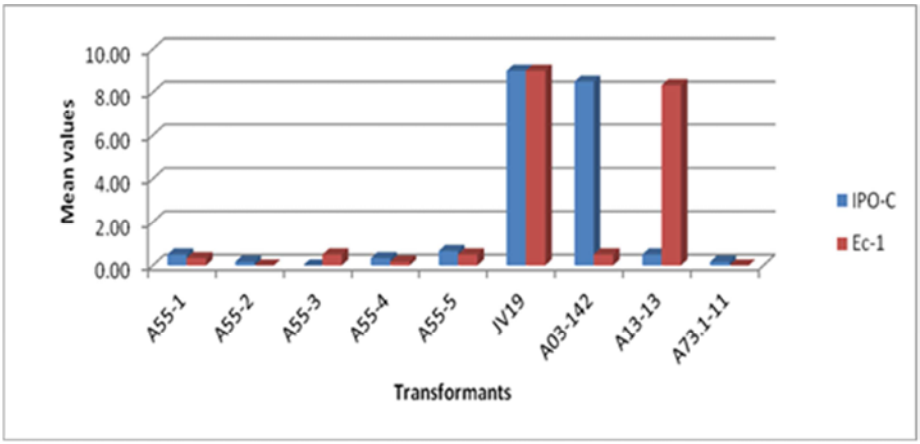


Figure 5. Detached leaf assay of transgenic potatoes transformed with *Rpi-vnt1:blb3*gene constructs.

During the agroinfiltration assay, plants transformed with the resistance gene *Rpi-vnt1:blb3* gene constructs gave specific HR response against the *Avr* genes used (Figure 6).

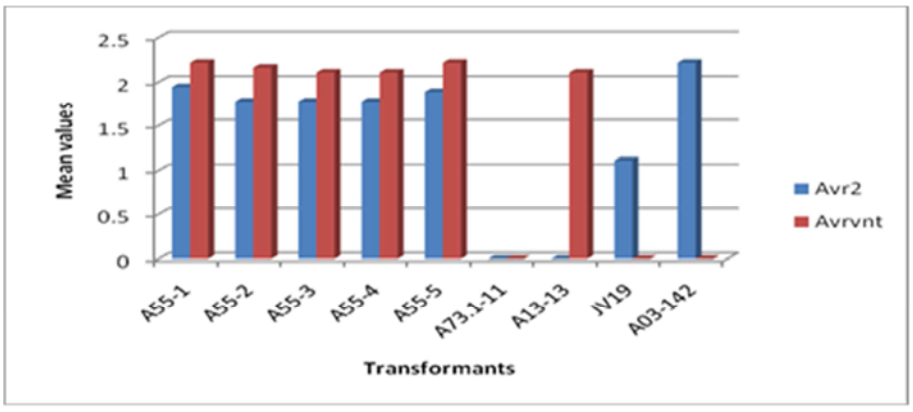


Figure 6. Agroinfiltration assay of transgenic potatoes transformed with *Rpi-vnt1:blb3* gene constructs.

c) Functional test of Rpi-vnt1:edn2:chc1 transformants

Transformants were tested for presence or absence of the introduced *Rpi-vnt1, Rpi-edn2 and Rpi-chc1* resistance genes using PCR and four of the transformants did not contain the *Rpi-edn2* gene and three of the transformants did not contain *Rpi-chc1* gene. The PCR result of the transgenic events was not reliable for the resistance gene *Rpi-vnt1* since the negative controls also show positive result.

Functional expression of the introduced *Rpi-vnt1*, *Rpi-edn2* and *Rpi-chc1* genes were done using agroinfiltration of the matching Avr genes (Supp. Table 10; Figure 7). Those transgenic plants were also tested twice for resistance to the *P. infestans* isolates IPO-C and Dinteloord using a detached leaf assay. The reason to repeat the DLA assay was because, the isolate Dinteloord was unable to infect transformants in the first experiment because of the low OD used (<5x10^4 zoospores/ml) and some mixing up of this isolate with the isolate IPO-C that was giving some false positive results during the DLA assay of the first experiment. In addition no clear spores were seen rather water soaked lesions were observed. Finally, the following results were obtained.

Table 10. Summary of *Rpi-vnt1:edn2:chc1* transformants functionality tests.

Geno type	PCR (+:-) edn2	PCR (+:-) chc1	No of events tested	IPO-C Dinteloord (DLA) R:S	Events expressing vnt1 (ATTA) (+:-)	Events expressing chc1(ATT) (+:-)	Field (natural infection) R:S
A99	2:4	3:3	6	0:6	0:6	6:0	0:6

All of the transgenic events with marker shown susceptibility to both *P. infestans* isolates IPO-C and Dinteloord. This indicates that the inserted late blight resistance genes *Rpi-vnt1:edn2:chc1* were not actively expressed, since the non-transformed control JV19 and the *Rpi-blb3* Desiree transformant A03-142 were susceptible to the isolate IPO-C.

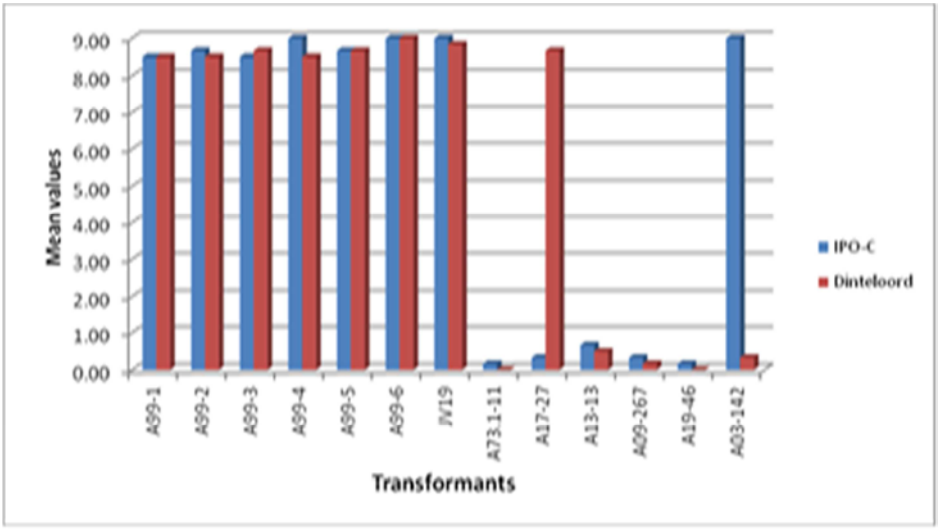


Figure 7. Detached leaf assay of transgenic potatoes transformed with *Rpi vnt1:edn2:chc1* gene constructs.

During the agroinfiltration assay, all plants transformed with the resistance gene *Rpi-vnt1:edn2:chc1* gene constructs gave specific HR response against the *Avr* gene *Avrchc* only (Figure 8).

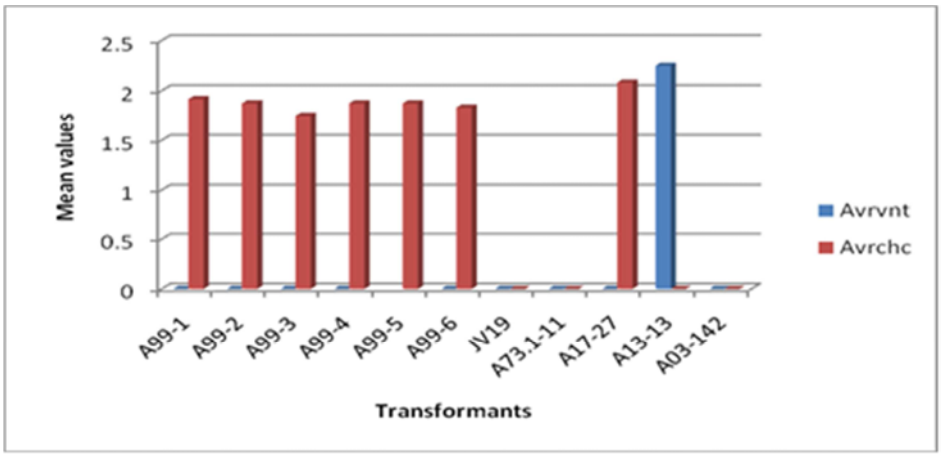


Figure 8. Agroinfiltration assay of transgenic potatoes transformed with *Rpi-vnt1:edn2:Chc1* gene constructs.

d) Functional test of Rpi-vnt1:edn2:sto1 transformant

The *Rpi-vnt1:edn2:*sto1 transformant, A100-1 was tested for presence or absence of the *Rpi-vnt1:edn2:*sto1resistance genes using PCR and the transformant was containing the both *Rpi-edn2* and *Rpi-sto1* genes. The PCR result of the transgenic events was not reliable for the resistance gene *Rpi-vnt1* since the negative controls also show positive result.

Functional expression of the introduced *Rpi-vnt1:edn2:*sto1 genes were tested using agroinfiltration of the matching *Avr* genes (Supp. Table 11; Figure 10). This transgenic plant was also tested twice since similar control plants were used per set, any conclusions cannot be drawn from the previous experiment for resistance to the *P. infestans* isolates IPO-C and Dinteloord using a detached leaf assay (Supp. Table 11; Figure 9), and the following results were obtained.

Table 11. Summary of *Rpi-vnt1:blb3* transformants functionality tests.

Genotype	PCR (+:-) edn2	PCR (+:-) sto1	No of events tested	IPO-C Dinteloord (DLA) R:S	Events expressing vnt1 (ATTA) (+:-)	Events expressingsto1 (ATTA) (+:-)
A100	1:0	1:0	1	1:0	1:0	1:0

The transgenic event A100-1 shown resistance to both *P. infestans* isolates IPO-C and Dinteloord (Figure 9). This indicates that the inserted late blight resistance genes *Rpi- vnt1, Rpi-edn2 and Rpi-sto1* were actively expressed, since the non-transformed control JV19 was susceptible to both isolates whereas, the transformed Desiree containing these *Rpi*-genes alone or in combination was resistant to both isolates (Figure 9).

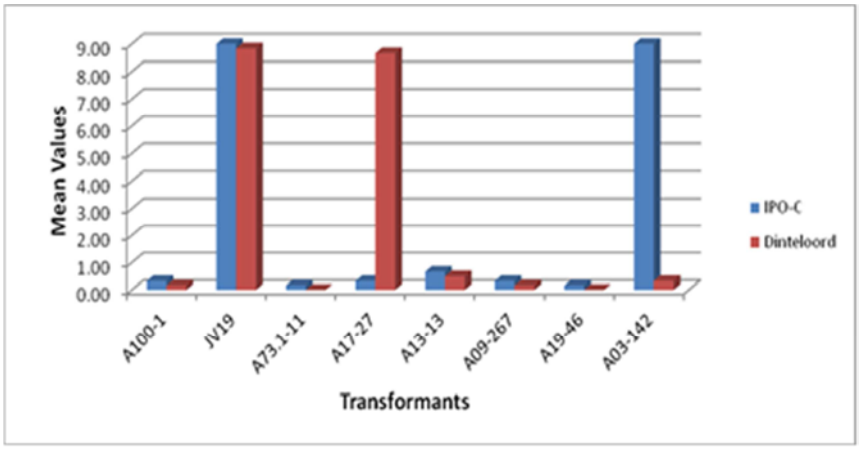


Figure 9. Detached leaf assay of transgenic potatoes transformed with *Rpi- vnt1:edn2:sto1* gene constructs.

During the agroinfiltration assay, the A100-1 plant that was transformed with the resistance gene *Rpi-vnt1:edn2:sto1* gene constructs gave specific HR response against both *Avrvnt* and *Avrsto* (Figure 10).

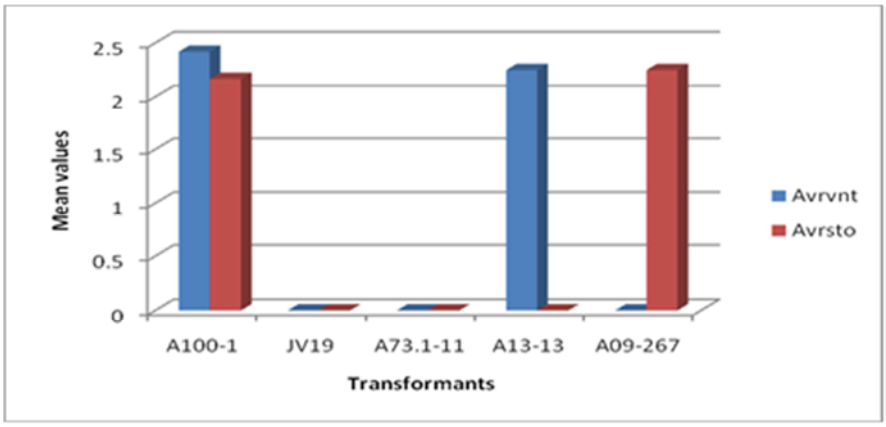


Figure 10. Agroinfiltration assay of transgenic potatoes transformed with Rpi-*vnt1:edn2:sto1* gene constructs.

e) Comparison of marker-assisted and marker-free Rpi-edn2 transformants

The *Rpi-edn2* transformants were tested for presence or absence of the *Rpi-edn2* resistance genes using the Ef1α-PCR and the inserted late blight resistance gene *Rpi-edn2* was lost in all transformants with marker and many marker free transformants except V108.10, 11, 18, 19, 21, 23, 25, 28, 32, 33, 35 and 42 (Supp. Table 12).

Table 12. Summary of *Rpi-edn2* transformants functionality tests.

Genotype	PCR (+:-)	No of events tested	Events expressing edn2 (DLA) R:S	Events expressing edn2 (field) R:S
V73.4 (MA)	0:7	7	0:7	0:7
V108 (MF)	4:39	43(30field)	11:32	4:26

These transformants were also inoculated with the *P. infestans* isolate IPO-C using a detached leaf assay and field evaluation was done to check resistance to *P. infestans*. Many of the transgenic events shown susceptibility to the *P. infestans* isolate IPO-C. Except for the marker free transformants; V108. 14, 23, 24, 28, 29, 35, 36, 40, 41, 42 and 43, IPO-C shown virulence towards *Rpi-edn2*.

f) Comparison of marker-assisted and marker-free Rpi-vnt1:blb3 transformants

The *Rpi-vnt1* and *Rpi-blb3* resistance genes containing transformants were tested for the functional expression of the introduced genes *Rpi-vnt1* and *Rpi-blb3* using agroinfiltration of the matching *Avr* genes, *Avrvnt1* and *Avr2* (Supp. Table 13). Those transgenic plants were also tested for resistance to the *P. infestans* isolates IPO-C and Ec-1 using a detached leaf assay (Supp. Table 13). Field test was also done for their resistance to *P. infestans*. Finally, PCR was done to check presence or absence of the *Rpi-:blb3* resistance gene and the following results were obtained.

Table 13. Summary of *Rpi-vnt1:blb3* transformants functionality tests.

Genotype	PCR (+:-)	No of events tested	Events expressing Vnt1:blb3 (DLA) R:S	Events expressing Vnt1:blb3 (ATTA) (+:-)	Events expressing Vnt1:blb3 (field)R:S
A55 (MA)	5:0	5	5:0	5:0	5:0
V109 (MF)	7:1	8	7:1	7:1	7:1

4. Discussion

4.1. Transformation Efficiency Analysis of Constructs

Transformation efficiency of constructs is determined by the Agrobacterium tumefaciens strain and classes of plasmids used [28]. Several experiments indicated that classes of Ti-plasmids are responsible for tumor induction and part of these plasmids known as T-DNA is transferred and incorporated into the plant genome. Therefore, Ti-plasmids are assumed to be used as vectors. If Ti-plasmids are very large and the T-DNA region does not have specific restriction endonuclease sites, it is difficult to clone a gene of interest into the T-DNA region [10]. Due to these reasons, plasmids have to be small to replicate both in *E. coli* and in *Agrobacterium* as well as contain specific restriction endonuclease sites. When the transformation experiments were compared to each other, differences in transformation efficiency were seen. The construct *R8*: *Rpi-blb2* does not have any transformants and considered as the lowest transformation efficiency (0%) whereas *R8*: *Rpi-edn2* had the highest transformation efficiency (95.8%) followed by *R8*: *Rpi-sto1* (23.3%). This difference in transformation efficiency could be due to difference in the vector used since the *Agrobacterium tumefaciens* strain AGL1, which was used during this experiment, was less efficient in transferring the T-DNA in to the potato genome than other strains [28]. This difference in transformation efficiency is most likely due to difference in the vector used since pBINPLUS-PASSA was used for both *R8*: *Rpi-edn2* and *R8*:*Rpi-sto1* whereas pBINPLUS-PPASSA was used for *R8*:*Rpi-blb2* transformation on which mutation was found in its vector backbone (Jack Vossen personal communication). It could also be because of mistakes during insertion of additional restriction sites or unstable gene integration due to partial digestion of the donor vector [29].

Transformation efficiency is highly dependent on cultivars used [30], age of explants, hormone combinations to the R3B pre-culture medium, pH (5.8), pre-culture of explants, concentration/number of *Agrobacterial* cultures (OD), acetosyringone concentration and period of co-cultivation with bacteria used for transformation [31]. According to a report by Rashid *et al.,* [32], acetosyringone with a concentration of 50-100µm is enough for effective transformation efficiency of wheat since higher concentration of acetosyringone kills the calli [33]. The concentration of *Agrobacterium* is the most critical factor of transformation efficiency. Excess number of *Agrobacterial* cells can cause Agrobacterium induced necrosis due to stress in the plants cells as a result of bacterial infection and too low concentration of *Agrobacterial* cells can affect the efficiency of transformation through delayed T-DNA transfer via few bacterial cells [34]. Jaiwal *et al.,* [35] reported that highest transformation efficiency was observed in *Vigna radiata* at the optical density (OD) of 1.0. Mannan *et al*., [34] observed similar result, since highest GUS expression was observed at an OD of 1.0 when the *Agrobacterial* culture was kept at 560nm as a result OD of 1.0 is optimum for efficient transformation of *Artemisia absinthium* because of a decrease in GUS expression as OD is further increased. However, the OD used in this experiment was 0.4 that might be the case for low transformation efficiency of the construct *R8*:*Rpi-blb2* since the recommended concentration of acetosyringone, hormone combination to the R3B pre-culture medium, pH (5.8), co-cultivation time to transfer T-DNA and similar cultivar and age of Desiree explants were used. According to [37], the larger T-DNA sizes of the construct due to stacked resistance genes, the lower the transformation frequency due to effect on the *Agrobacteria* to deliver them into plant cells. The *R8*: *Rpi-edn2* (95.8%) followed by had the highest transformation efficiency than other constructs such as; R8: *Rpi-sto1* (23.3%) and *Rpi-blb3: R8* (8.33%), shown that these two genes might be highly compatible as a result of correct insert size or due to the vector used since similar results were obtained from the controls used.

4.2. Agroinfiltration Assay to Detect *Rpi-edn2* Activity

Functionality tests of the *Rpi-edn2* gene was carried out using agroinfiltration of the cognate *Avr* genes RD28 and PITG_20303. The *Avr* gene RD28 was the *A. tumefaciens* transformant (GV3101) cultured in selective medium containing only Kanamycin whereas the *Avr* gene PITG_20303 was the *A. tumefaciens* transformant (*AGL1+pVirG)* cultured in selective medium containing Kanamycin and Chloramphenicol. During the agroinfiltration assay, plants transformed with the resistance gene *Rpi-edn2* could not give specific hypersensitive response against both *Avr* genes used. The two *Avr* genes RD28 and PITG_20303 were not ideal for agroinfiltration assay in our potato plants. The *Avr* gene RD28 gave no response and the *Avr* gene

PITG_20303 gave very light response in addition to auto-necrosis in JV19 plants that makes difficult in scoring *Rpi-edn2* activity in both marker free and marker assisted transformants. The reason for not to give specific hypersensitive response against the *Avr* gene RD28 might be due to the agrobacterial strain (GV3101) used or because the *Rpi-edn2* gene expressed more in the matured plants than the young ones, the hypersensitive response will be expressed late (J.H. Vossen personal communication). Most of these transformants were also shown to be susceptible to late blight during the DLA and field assays.

4.3. Functional Test of *Rpi-chc1:blb3* Marker Assisted Transformants

Transgenic plants harboring *Rpi-chc1:blb3* resistance genes were developed by transformation of the susceptible potato cultivar Desiree. The PCR result had shown the presence of both the *Rpi-blb3 and* the *Rpi-chc1* resistance genes in both transformants. During the agroinfiltration assay, A53-6 plants transformed with *Rpi-chc1:blb3* resistance genes constructs given specific HR response against the *Avr* genes *Avrchc* and *Avr2* whereas the transformant A53-7 did not gave HR for *Avrchc*. This indicates that the inserted late blight resistance genes *Rpi-chc1:blb3* was actively expressed in A53-6 plants and only *Rpi-blb3* is active in A53-7 plants. This result was in agreement with previous study of [29] done in *N. benthamiana* where *Rpi-chc1* recognizes its corresponding *Avr* effector, *A2-2*. Only one from the 19 plants responded to *A2-2* even though the experiment was repeated and the OD was changed from 0.1 to 0.2 [29]. During the detached leaf assay, both of the transgenic events shown resistance to both *P. infestans* isolates IPO-C and Dinteloord. This indicates that the inserted late blight resistance genes *Rpi- blb3* was actively expressed in both transformants, since the non-transformed control JV19 and the *Rpi-blb3* Desiree transformant A03-142 were susceptible to the isolate IPO-C and both JV19 and the *Rpi-chc1* Desiree transformant A17-27 was susceptible to the isolate Dinteloord. The DLA result was in agreement with previous studies of [36].

4.4. Functional Test of *Rpi-vnt1:blb3* Marker Assisted Transformants

The *Rpi-vnt1:blb3* transformants were tested for presence or absence of the introduced *Rpi-vnt1* and *Rpi-blb3* resistance genes using PCR. The result showed that all of the transformants were containing the *Rpi-blb3* gene even though the *Rpi-vnt1* PCR was not reliable. Functional expression of the introduced genes *Rpi-vnt1* and *Rpi-blb3* were also tested using agroinfiltration of the matching *Avr* genes, *Avrvnt1* and *Avr2*. All the *Rpi-vnt1:blb3* transformants gave specific HR response against both *Avr* genes used. All of the transgenic events with marker showed resistance to both *P. infestans* isolates IPO-C and Ec-1. This indicates that the inserted late blight resistance genes *Rpi-vnt1* and *Rpi-blb3* were both actively expressed, since the non-transformed control JV19 and the Desiree transformant only containing *Rpi-blb3* (A03-142) were susceptible to the *P. infestans* isolate IPO-C. The control A13-13 was susceptible to Ec-1 whereas A03-142 was resistant to the isolate Ec-1 because of the presence of *Rpi-blb3* gene. This result is in agreement with [6]; of which hypersensitive response (HR) was found in all transformants harboring the corresponding *R* genes; *Rpi-vnt1* and *Rpi-blb3*. It is expected that the whole T-DNA be transferred from LB to RB since 40% of the transformants were harboring the *trfA* and *nptIII* backbones that were closest to the LB than the RB. Both *Rpi-vnt1* and *Rpi-blb3* resistance genes were found to be always biologically active, leading to hypersensitive response (HR) in agroinfiltration assays and late blight resistance in DLA assays since, a close link was observed between the presence and biological functionality of stacked *Rpi* genes [6].

4.5. Functional Test of *Rpi- vnt1:edn2:chc1* Marker Assisted Transformants

The PCR result confirmed that three of the six transformants were only positive to *Rpi-chc1* gene alone. All of the transgenic events containing the triple resistance gene *Rpi-vnt1:edn2:chc1* shown susceptibility to both IPO-C and Dinteloord *P. infestans* isolates during the detached leaf assay since the non-transformed control JV19 and the *Rpi-blb3* Desiree transformant, A03-142 were susceptible to the IPO-C isolate. The transformed Desiree events containing the *Rpi-edn2* gene (A73.1-11), containing the *Rpi-chc1* gene (A17-27), containing the *Rpi-vnt1* gene (A13-13) and the transformed Desiree containing both *Rpi-vnt1* and *Rpi-chc1* genes (A19-46) were all resistant to the IPO-C isolate even though A17-27 was susceptible to Dinteloord because of lack of the *Rpi-blb3* gene unlike the A03-142 does. In the agroinfiltration assay, transformants of these construct given specific HR response against the *Avr* gene *Avrchc1* only. This shown that, the inserted late blight resistance genes *Rpi-vnt1, Rpi-edn2* and *Rpi-chc1* were not active. This might be because of mutations in the vector pBINPLUS-PPASSA. This might also be due to larger size of the insert that had an effect on the agrobacteria to transfer several resistance genes all at once into the plant genome [31]. It could also be because of mistakes during the insertion of an additional restriction sites. Generally, all of the triple resistance genes containing (*Rpi-vnt1:edn2:chc1)* transgenic events shown susceptibility to all functionality tests.

4.6. Functional Test of *Rpi-vnt1:edn2:sto1* Marker Assisted Transformant

The PCR result confirmed that this transformant was

positive to the *Rpi-sto1* gene even though the PCR result for the *Rpi-vnt1* gene was not reliable. During the agroinfiltration assay, the A100-1 transformant gave specific HR response against both *Avrvnt1* and *Avrsto1* avirulence genes. The transformant A100-1 containing the triple resistance gene *Rpi-vnt1:edn2:sto1* showed resistance to both *P. infestans* isolates IPO-C and Dinteloord whereas, the non-transformed control JV19 was susceptible during the DLA. The transformed Desiree cultivars containing the *Rpi-edn2* gene (A73.1-11), containing the *Rpi-chc1* gene (A17-27), containing the *Rpi-vnt1* gene (A13-13) and the transformed Desiree containing only the *Rpi-sto1* gene (A09-267) were all resistant to the IPO-C isolate even though A17-27 was susceptible to Dinteloord because of lack of the *Rpi-blb3* gene unlike the A03-142 does. This indicates that the inserted late blight resistance genes *Rpi-vnt1* and *Rpi-sto1* were actively expressed in the A100-1 transformant. This result is in agreement with [6]; of which hypersensitive response (HR) was found in all transformants harboring the corresponding *R* genes; *Rpi-vnt1* and *Rpi-sto1*. Both *Rpi-vnt1* and *Rpi-sto1* resistance genes were found to be always biologically active, leading to hypersensitive response (HR) in agroinfiltration assays and late blight resistance in DLA assays since, relation was observed between the presence and biological functionality of stacked *Rpi*-genes [6]. This might be due to either the pBINPLUS-PASSA strain was efficient for *Rpi-vnt1* and *Rpi-sto1* transformation or these two genes were highly compatible during the transformation process due to the correct insert size used.

4.7. Comparison of Marker-Assisted and Marker-Free *Rpi-edn2* Transformants

Because, those two candidate *Avr* genes, RD28 and 20303 were not ideal for agroinfiltration assay, the functional test for *Rpi-edn2* activity was based on the DLA, PCR and field tests. All marker-assisted and many of the marker free transgenic events except eleven marker free transformants shown susceptibility to the *P. infestans* isolate IPO-C. The field result also confirmed that all marker-assisted and many of the marker free transformants showed susceptibility to *P. infestans*. This might be because of youngness of the plants since one-month-old transformants were tested for the DLA assay. The *Rpi-edn2* gene expressed more in the matured plants than the young ones during both DLA and agroinfiltration assays (J.H. Vossen personal communication).

4.8. Comparison of Marker-Assisted and Marker-Free *Rpi-vnt1:blb3* Transformants

The PCR result shown that the resistance gene *Rpi-blb3* was absent in V102.2-6 marker free transformants. Transformants were also tested for the functional expression of the introduced *Rpi-vnt1* and *Rpi-blb3* genes using agroinfiltration of the matching *Avr* genes, *Avrvnt1* and *Avr2*. All the transformants tested in the agroinfiltration assay showed specific HR to both *Avr2* and *Avrvnt1* except V109-4 and V102.2-4, which did not respond to both *Avr* genes, and V102.2-6 that responds only to *Avrvnt1*. Those transformants were also tested for resistance to the *P. infestans* using a detached leaf assay and all the transgenic events with marker and many of the marker free transformants except V109-4, V102.2-3 and V102.2-4 shown resistance to both IPO-C and Ec-1 isolates. This indicates that the inserted late blight resistance genes *Rpi-vnt1 and Rpi-blb3* were both actively expressed, since the non-transformed control JV19 and the Desiree transformant only containing *Rpi-blb3* (A03-142) were susceptible to the IPO-C isolate. The difference in expression level between marker assisted and marker free transformants could be due to the difference in cultivars since marker assisted transformants were transformed in Desiree cultivar whereas marker free transformants were transformed in JV19 cultivars that might have different level of gene expression. This could be due to difference in the transformation efficiency of the vector used. The *Agrobacterium tumefaciens* strain used during this experiment (AGL1) was less efficient than the strains LBA4404 and AGL0 [37] in transferring the T-DNA in to the potato genome. The difference in expression level might be due to the *Rpi-edn2* gene in addition to the *Rpi-vnt1:blb3* gene that might result less frequency of gene expression due to large insert size in marker free transformants.

5. Conclusion

The construct *Rpi-blb2:R8* does not have any transformants whereas the *R8: Rpi-edn2* had the highest transformation efficiency (95.8%) followed by *R8: Rpi-sto1* (23.3%) thought that these two genes might be highly compatible. The *Avr* genes RD28 and PITG_20303 were not appropriate for the functional test of *Rpi-edn2 activity*. They both gave non-specific HR during the agroinfiltration assay.

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