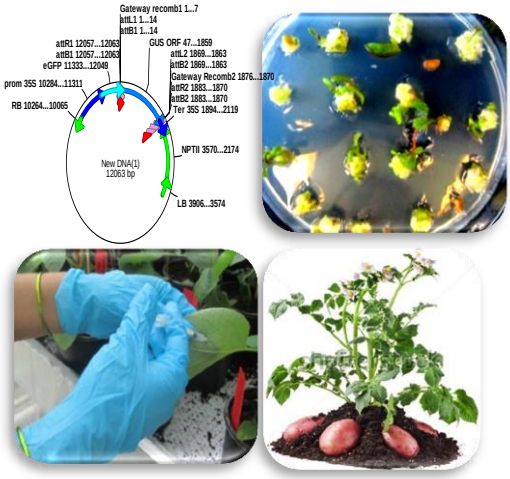
Marker Free Transformation of Potato

Master Thesis

Most. Shanaj Parvin

Reg. No. 810210585080



Supervisors

Jack Vossen
Marjan Bergervoet

Examiners

Evert Jacobsen
Henk Schouten

WAGENINGEN UNIVERSITEIT
WAGENINGEN UR

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Registration number. 810210585080

MSc thesis –Plant Breeding

(Thesis code: PBR 80436)

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Henk Schouten

Thesis submitted in partial fulfilment of the requirements for the degree of master of Sciences (MSc) Degree in Plant Biotechnology

(Specialization: Molecular Plant Breeding and Pathology)

August 2013
Wageningen, The Netherlands

ACKNOWLEDGEMENTS

It is my pleasure to take the opportunity to express my profound gratitude to my thesis supervisor Jack Vossen for his guidance which leaded me to finish the thesis in time. I would like to extend my heartfelt thanks to Marjan Bergervoet, Marjon Arens, Linda Kodde, Iris capel, Pereira Isolda and Gerard Bijster basch, lab technicians and Q, Weicong, PhD student, in the plant breeding and molecular biology laboratory for providing their technical support to me during my thesis work in lab. I would like to thank Dutch Government for awarding me the Netherlands Fellowship Programmes (NFP) to complete my thesis at Wageningen University.

Lastly, I wish to convey our deepest and sincere gratefulness to all of my friends, especially elder brother Tanjimul Islam, my husband Md. Ehsanul Haque, my mother-in-law Lutfun Nahar Begum and my beloved sons Shafin and Rhahin for their great support during this thesis tenure.

ABSTRACT

Potato has been accentuated for genetic improvement through gene transfer as its breeding process has shown some barriers due to self-incompatibility and heterozygosity. Although genetic modification was already possible since 1980, it continues to raise questions particularly, the safety issues for the people and the environment. To meet the public concern, several technologies have been developed for development of transgenic plant without having selectable markers (antibiotic resistance) such as site specific recombination, co-transformation and negative selection. Here we illustrated the use of a visible marker, i.e. *gfp,* as an alternative for antibiotic resistance. This research found that *gfp* based selection is not reliable as it has not been observed in the microscopic studies while a good percentage of shoots about 86% were found positive in PCR analyses. However, it is believed that *gfp* can be applied as an option to effectively sort for chimera and escapes in vivo. As expected, *gfp* and PCR can be used for the selection of transgenic tissues. Moreover, to establish a convincing and reliable protocol for the genetic transformation of potato several factors for instance; cultivars, phytohormones pre-treatment of the explant before transformation, age of the explants, period of co cultivation with bacteria, bacterial concentration for transformation, and use of acetosyringone as a virulence induction agent were investigated to enhance the regeneration performance and transformation efficiency. Among the studied four cultivars, Bintje showed highest regeneration efficiency (83%) in kanamycin selection media and 19% in marker free condition considering all the above mentioned factors. Notably, Atlantic and Russet Burbank showed moderately poor regeneration efficiency in both pre-cultured media with kanamycin and marker free condition. Furthermore, three different T-DNA length containing plasmids; 3kb, 13kb and 24kb were used to investigate the effect of larger insert on transformation process. Notably, the transformation frequency was found similar in 3kb and 13 kb T-DNA insert. After selection of marker free transformants, the functional expression of the inserted resistance genes (*Rpi-vnt1* and *Rpi – chc1* in V91 and P91 transformants) was studied. We noticed that *Rpi- vnt1* is active in P91 and V91 transformants through agro-infiltration and Detached Leaf Assay, while the activity of *Rpi-chc1* has to be tested in future.

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INTRODUCTION

Potato (*Solanum tuberosum* L.) is a starchy tuberous crop grown all over the world, notably due to its high productivity as well as high starch, vitamin and protein content (Beaujean et al. 1998). It is the worlds' fourth largest food crop and critical alternative to the major cereal crops for feeding the world population (Reader 2009). Potato provides approximately half of the world's annual production of all root and tuber based foods, making it the leading non-cereal crop. The world potato production approximately 324 million tons, from which two third were used for human consumption and rest for feed and starch production (FAO 2012). Initially, most potato were grown and consumed in Europe, North America, and former Soviet Union. Per capita Europe has still the highest production but the world potato sector is undergoing record changes. There has been a historic increase in potato production and demand in Asia, Africa and Latin America, where output rose from less than 30 million tons in the early 1960s to more than 165 million tons in 2007 (FAO 2012). At this moment, China is the world largest potato producing country whereas India is ranking second (FAO 2012). Potatoes are considered to play an important role in developing economies because they can be grown in a wide variety of climates and local conditions. About half a billion people in the developing countries consume potato as their diet (Ghislain M. 1999). Around 33 kg (73 lb) of potato are included in the annual diet of an average global citizen (FAO 2012).

Like other crops, potato production is hampered by different types of biotic and abiotic factors. Environmental stresses reduce the unconditional marketable yield of potato. Regarding to the biotic stress, the production or yield of a crop can fall dramatically, due to pathogen attack. Among the biotic stresses, the well-known disease late blight, caused by *Phytophthora infestans* causes serious yield loss on potato. The first epidemic of this disease that started in 1840 caused the Irish potato famine (Fry 2008). From that time *P. infestans* caused a tremendous effect on world agriculture. Today, late blight is the number one disease of potato. Cost of losses and protection, are estimated US$ 3.25 per annum (Latijnhouwers M 2003). Other estimates show global yield losses of 16% representing an annual financial loss of € 5.2 billion worldwide (Haverkort et al. 2008). It is now widely accepted that the remarkable genetic flexibility and rapid adaptation capacity of *P. infestans* to the natural selection pressure is escalating the global epidemic of late blight. Considerable breeding efforts have failed to produce durably resistant cultivars demanded by potato producer and consumer. Till now, twenty one *R* genes from various *Solanum spp* have been shown to provide resistance to specific *P. infestans* isolates (Vleeshouwers et al. 2011). From them, twelve resistance genes (*R1, R2, R3a, R3b, R5–R11)* have been introgressed from the wild species *Solanum demissum* (Vleeshouwers et al. 2011). Unfortunately, the *P. infestans* population quickly adapted, presumably by mutating or deleting the corresponding avirulence gene. As a consequence, to control the late blight, fungicides must be applied frequently, which causes harmful effect on environment and leads to develop fungicide-resistant *P.infestans* population (Deahl et al. 1993); (Goodwin et al. 1994); (Grünwald NJ 2001).

However, potato breeding is prospective due to many resistance sources are available in wild species. Approximately five thousand potato varieties prevail worldwide. Besides, there are about 200 wild species and subspecies reported, many of which can be crossbred with existing varieties. Despite the availability of resistance sources, the conventional ways of improving cultivar take considerable time, due to the possibility of linkage drag. In addition, high level of heterozygosity in

potato makes the breeding process more difficult. In these circumstances, transgenic technology could overcome the problem in linkage drag, and speed up introgression of the resistance to *P. infestans (Rpi*) gene by 10 fold (Jacobsen and Schouten 2007). Therefore, breeder are engaged to introduce multiple *Rpi* gene into cultivated potato from wild species via genetic engineering (Lamour and Kamoun 2009; Song et al. 2003); (Vander Vossen EAG 2003); (Vander Vossen EAG 2005); (Vossen et al. 2012). Recently, gene pyramiding (Halpin 2005); (Douglas and Halpin 2009) and functional stacking of *R* genes (Zhu et al. 2012) have been achieved to obtain broader resistance. Moreover, a number of new traits, such as PVX virus resistance (Hemenway C 1988); (Hoekema A 1989; Lawson C 1990), soft rot and wilt resistance (Düring K 1993); (Jaynes JM 1993), potato tuber moth and Colorado potato beetle resistance (Adang MJ 1993; Peferoen M 1990); (Perlak FJ 1993) but also quality related traits like increased starch content have been introduced into potato via transgenic technology. Hence, Agrobacterium-mediated genetic transformation offers great opportunities for further improvement of potato.

Although transgenic technology has shown considerable genetic improvement of different crops, the technology is still controversial. However, genetically improved crops in combination with advanced crop management have shown remarkable increases of production. Therefore, GM crops are one of the fastest adopted crop technology in the history of modern agriculture. The cultivation area of GM crop increased rapidly, almost 94 fold, since the first commercialization started in 1996 (James 2011). *Agrobacterium tumefaciens* mediated plant transformation is frequently used to introduce foreign DNA into plant cells, however, microinjection, particle gun, or protoplast transformation, are also used but seem to be relatively inefficient. For distinguishing the transformed cells that have integrated the DNA into their genome, in most cases, a selectable marker gene is co-introduced with the gene of interest. Such a selection step is essential since success of plant transformation is never 100%. To date, roughly 50 different selection systems have been developed, however, the antibiotic kanamycin (*nptII*), hygromycin (*hpt*) or the herbicide phosphinothricin (*bar*) resistance genes have been used most frequently in plant transformation. Noteworthy, mature plants do not require the antibiotic and herbicide resistance marker genes when they are cultivated in fields. Even they might act detrimental, especially when they can inhibit organelle protein synthesis system (Bevan 1983). Moreover, the use of antibiotic or herbicide resistance genes during transformation has raised public concern. Through crossing or horizontal gene transfer to bacteria, the genes might enter the environment in an uncontrolled way and create ineffective antibiotics (ISAAA 2012).

Therefore, commercialization of GM crops is a major problem for the plant breeder. Rather, it is better to remove this selectable gene from transformed plants when their job has been done. For instance site-specific recombination, transposition and homologous recombination methods were developed where markers are eliminated after transgene insertion (Darbani et al. 2007). As an alternative, some visible selection markers were used in plant transformation that has little or no endogenous activity in the plant to be transformed. At present, a few reporter genes are used widely, these being *β-glucuronidase* (*gus*) from *Escherichica coli* (Jefferson et al. 1987), green florescent protein gene (*gfp*) from jellyfish (Cubitt et al. 1995), firefly protein luciferase (Ow et al. 1986), plants red-purple anthocyanin's have also been used as visible markers for selecting transformed cell (Kortstee et al. 2011).

To increase the acceptability of GM crop a new biotechnological approach, cis genesis, has been introduced to reduce the limitation of GM crop (Schaart 2004); (Schaart et al. 2004); (Krens et al. 2003). In cisgenesis, the genetic makeup of existing cultivars is fully maintained in contrast to traditional breeding. In traditional potato breeding, the genetic makeup of existing cultivar can never be fully restored in the progeny, as these crops are heterozygous in nature. However, in case of cisgenesis only one or a few desired genes from same or closely related species along with their native promoter are added to an existing cultivar without using foreign selection markers (Schouten 2006). Therefore, deliberate release of cisgenic plants into the environment might be equally or more safe than traditionally bred plants (Schouten 2008). However, the limitation of this technology is that regulatory bodies have not discriminated cisgenic from transgenic plants.

Against the backdrop, an effective regeneration protocol is a prerequisite for transgenic plant development via genetic transformation (Cardoza 2008). Several studies reported that successful recovery of transgenic plant without selective media during regeneration such as citrus (Domínguez et al. 2004) wheat and triticale. The regeneration ability is attributed to the totipotency characteristics of plant cells and the use of specifically designed growth media and hormones (Cardoza 2008). Thus, the knowledge of each plant species and the explants type is very essential to the development of an efficient regeneration system. Nowadays in vitro regeneration protocols are available for most of the major crops. However, such protocols need to be optimized for the variety of interest, in order to get good result and avoiding any inefficiency that could arise from varietal difference. Heterozygosity, self-incompatibility has extremely reduced the efficiency of traditional methods for potato breeding. Therefore, an alternative approach has been practiced for further improvement of commercial potato cultivar, such as in vitro techniques, somatic hybridization, mutagenesis and genetic transformation. Potato was the first crop to be genetically modified and so far different potato cultivars were used for genetic transformation (Heeres P 2002). Till now, several transformation regeneration protocols have been developed, but most of these have long regeneration period and low frequencies (Romano et al. 2001). What has been more; the transformation protocols in potato are genotype dependent which restricted their universal usage for all genotypes.

Discussing all the innovation to facilitate the acceptability and commercialization of GM crops to the consumers, growers, and regulatory bodies, the present project is giving focus to develop marker free transformation of economic important crop potato. Since, aforementioned potato is heterozygous, self-incompatible and breeding process has taken considerable time to develop a new cultivar. Improvement of these crops would therefore benefit tremendously from GM approaches.

Broad view of this project

The project intends to develop marker free transformation in potato by considering the pros and cons of using antibiotic, visible and PCR markers, along with the importance of development of resistant cultivars of potato to the oomycete pathogen *P. infestans*. In a broad sense, this research might eventually allow to enrich existing or new cultivars with resistance to various diseases, pest and storage problem arising in major production areas. Furthermore, marker free transformed crop could reduce the cost and time required for safety evaluations that could speed up the deregulation process required to introduce a new variety to the market.

Specific objectives in present study

Marker-free transformation might help to stacking multiple transgene in one-step or by sequential transformation cycles. Therefore, I aim to study the process of marker free transformation of potato in order to understand the consequences of different selection methods to ultimately optimize the protocol and to efficiently select for plants with optimal performance of the introduced genes.

Research questions and Approaches

Improving factors influencing *Agrobacterium* mediated transformation by increase regeneration capacity of potato transformants

Recent research has found that, by changing physical conditions of the explants or media composition during or just prior to the transformation, the regeneration efficiency (93%) of stable transformants increases (Chakravarty and Wang-Pruski 2010). This study the effect of age of explants, cultivars, and hormone combinations, pre-culture of explants, period of co-cultivation with bacteria and concentration of bacterial cultures used for transformation on efficiency of transformation were analysed. A major improvement was obtained by the addition of IAA and trans Zeatin to the R3B pre-culture medium (Shu Zhang personal communication). With this in mind, the present study tested the reproducibility of this method and aimed to further test this protocol in other cultivars.

Usefulness of *gfp* reporter gene for selection of transgenic tissue

The reporter gene *gfp* is widely used as a selectable marker in many transformation studies. Compared to other selectable markers, such as gus and luc that have been used to visualize transformed cell, *gfp* is more convenient (Molinier et al. 2000); (Ghorbel et al. 1999); (Zhang et al. 2001). *gfp* can be used as an alternative selection markers in replace of antibiotic resistant marker that arise public concern (Halford 2004). Moreover, *gfp* selection is a non-destructive method and does not require any exogenous sophisticated substrate in contrast to gus and luc selection gene. Therefore, using *gfp* can be exploited for in vivo analysis of transgene expression by fluorescence microscopy or even laboratory fluorescent illumination (Molinier et al. 2000). The report for using *gfp* in potato is a very few and has been used combined with *nptII*, to monitor the expression of transformed genotypes (Rakosy-Tican et al. 2007)**.** Previous study also indicated that *gfp* is an excellent visible marker for visual screening of transgenic cell and in vivo discrimination of escapes and chimeras (Rakosy-Tican et al. 2007). Chimeric transformants can arise during tissue culture and its frequency may be higher during marker free transformation because selection by PCR lacks selection against non-transgenic tissue. Therefore, visual marker *gfp* can be effectively used to identify chimeric tissue which can reduce the cost and time associated with PCR based selection. Therefore, I aim to use *gfp* as a selection marker instead of antibiotic selection marker gene to optimize the marker free transformation protocol. In order to use *gfp* as a transformation marker present study may have considered PCR based identification for all the regenerants.

Activities

The *Agrobacterium* vector containing *gfp* and *nptII* gene was introduced into potato genotypes. Subsequently, regenerants were selected under maker assisted (kanamycin) and marker free transformation.

In case of marker assisted selection, the regenerants were first selected with antibiotic selection. Afterwards, they were identified phenotypically (for *gfp*) under microscope and at the DNA level by PCR amplification.

Whereas in marker free selection, the regenerants were selected for *gfp* and DNA level selection under microscope and by PCR respectively. This experiment may have revealed the effects of antibiotic selection on organogenesis and transformation. Previous research found higher frequencies of transgene silencing occurred in the lines having the presence of integrated marker gene compared to PCR based identification (Francis and Spiker 2005). In kanamycin selection, when marker gene *nptII* was silenced, even non-expressed, then few numbers of transformants were obtained for counting silenced insert. As a result, a lot of transformants may have cancelled because of marker gene silenced. Therefore, in marker free transformation, without kanamycin selection might reveal the exact percentage of silencing insert.

To investigate the effect of larger insert on transformation process, more than two genes with *gfp* was introduced into potato

Recently, several researches have been applied to introduce more than two resistance gene in one cultivar to achieve durable resistance. For instance in potato three resistance genes were introduced against *P. infestans* (Zhu et al. 2012). Introducing several resistance genes simultaneously, making the insert larger, could have effect for the *Agrobacteria* to deliver them into plant cells. Therefore, this research included three plasmids that contain three different T-DNA length for example *AGL0* (*35S+gfp+gus*) containing 3kb length of T-DNA, *AGL0 (pMF1-gfp-pMdRbcS-gus)* containing 13 kb and *AGL1*+virG (*pRIABI.2MFchc-short-vnt1-blb3-stol1*) having 24 kb length of T-DNA for investigating this question.

The overall planning of this experiment was attached in Appendix-1 (Figure 1)

Testing Resistance gene activity in marker free transformants

The ultimate aim of this project is to improve the protocol for marker free transformation of potato. In previous research with this marker free technology, the susceptible cultivar V (JV-19) and P(JV18) were transformed with two resistance genes, *Rpi-chc1* and *Rpi-vnt1* (construct 91), resulting in V91 and P91 plants. Here, I aim to test these transformants by Detached Leaf Assay (DLA) and agro-infiltration method in order to test the functionality of V91 and P91 plants containing *Rpi-chc1* and *Rpi-vnt1*.

Approach

When V91 plants are inoculated with the isolate *Katshaar* (containing effectors genes *Avrvnt1, AVR2* and *avrchc1*), they are expected to be resistant because the *Avrvnt1* is recognized by the resistance gene *Rpi-vnt1*. Similar results were also expected with another isolate, *Ec-1* (containing the effector genes *avrvnt1, Avrchc1* and *AVR2*) because *Avrchc1* will be recognised by *Rpi-chc1*. However, V91 transformants showed resistance only to *Katshaar*, but not to the isolate *Ec-1* (personal communication, Jack Vossen). Similar results were found by inoculating the cultivar Desiree (A) and A91 transformants with these isolates (personal communication, Jack Vossen). These results suggested that the resistance gene *Rpi-chc1* in V91 and A91 plants is inactive. It could be the reason *Rpi-chc1* gene is not active or the isolate mutated the *Avrchc1*.

The cultivar P (JV=18) already has a resistance gene namely *R2*. Therefore, P91 transformants have three resistance genes, *Rpi-chc1*, *Rpi-vnt1* and *R2*. P and P91 plants were inoculated by the same isolates, *Katshaar* and *Ec-1*. In previous research they found both control and transformed plants were shown resistant to these isolates. The manifestation of resistance in control plant P implies that the presence of avirulent gene *AVR2* in both isolates (*Katshaar* and *Ec-1*) responsible for that resistance. Interestingly, we used another isolate *IPO-C* (having virulent *avr2*) to inoculate these plants for further confirmation. Because if resistance occurred due to the presence of *AVR2* on those isolates then *IPO-C* inoculation may have showed susceptibility on P91 transformants.

I want to know the activity of the resistance genes *chc1*&*vnt1* in V91 and P91, therefore decided to perform effectors response tests in V91 and P91 plant by the *Avrchc1* and *Avrvnt1* gene constructs.

Therefore, I set an experiment according to our research questions.

Research questions

i) Is *Rpi-chc1* active or not in V91 and P91 plant?
ii) Do plants resistant to isolates respond to the corresponding *Avr*?
iii) Is the corresponding *Avr* mutated in the isolate, or not?
iv) Which R gene causes *IPO-C* resistant in P91?

MATERIAL AND METHODS

Plant material

Potato cultivars, Desiree, Atlantic, Bintjeand Russet Burbank were *in vitro* clonally propagated on MS20 medium (MS medium incl. vitamins 4,4 g/l, saccharose 20g/l,micro-agar 8 g/l was dissolved in a 1 liter of MQ water with pH 5.8) to test their regeneration ability after transformation. Stock plants were provided by Marjan Bergervoet. The plants were grown in a climate chamber at 24^{o}C and 16/8 hrs day and night regime. Four weeks later, internodal stem (2-6 mm) of these plants were used as a source of an explants.

Agrobacterium strains and culture

The *Agrobacterium tumefaciens* strains *AGL0* and *AGL1* harbouring the plasmids that contains three different length of T-DNA, were used in this experiment. The *AGL0* harbouring the plasmid *pMF1-gfp-pMdRbcS-gusi*, having 13 kb base of T-DNA that was controlled by apple derived promoter and terminator *MdRbS* (*Malusxdomestica* ribulose biphosphate carboxylase small subunit) was used [Figure 1(a)] and was provided by Iris Capel. The apple Rubisco promoter combination with same terminator has shown high level of expression in tobacco (Schaart et al. 2011). Another strain of *AGL0* contains the plasmid PK7WGF2 (*35S+gfp+nptII +gus*), having 3 kb T-DNA [Figure 1(b)] and was provided by Ahmed Abdel Haliem. Third, an *AGL1*+virG strain harbouring a plasmid having 24 kb base T-DNA [Figure 1(c)] was provided by Marjon Arens.

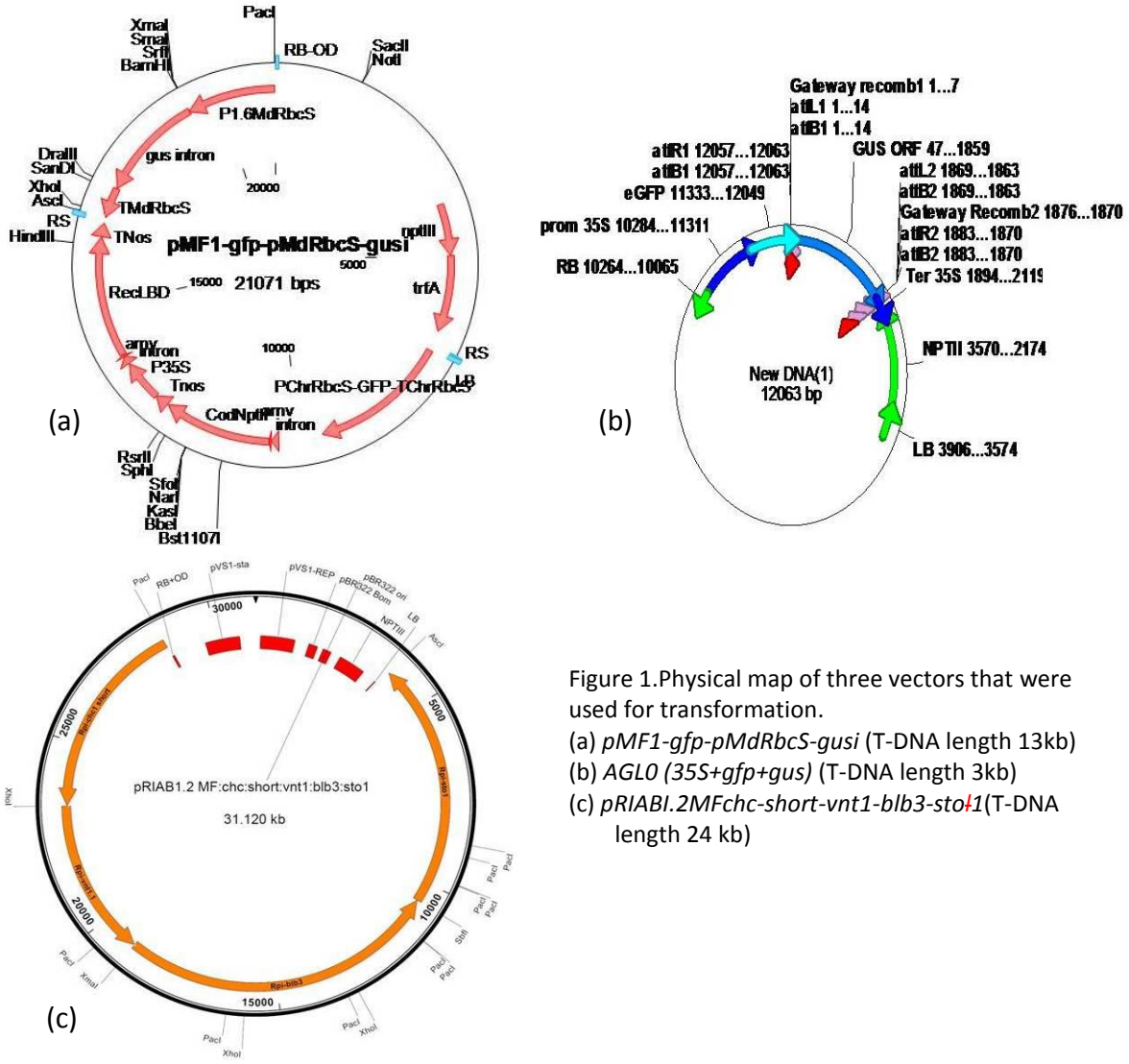

Figure 1.Physical map of three vectors that were used for transformation.
(a) *pMF1-gfp-pMdRbcS-gusi* (T-DNA length 13kb)
(b) *AGL0 (35S+gfp+gus)* (T-DNA length 3kb)
(c) *pRIABI.2MFchc-short-vnt1-blb3-sto~~l~~1*(T-DNA length 24 kb)

Preparation of the bacteria

The glycerol stock of *AGL0* (*pMF1-gfp-pMdRbcS-gusi*) and *AGL1*+virG (*pRIABI.2MFchc-short-vnt1-blb3-stol1*) strains were directly available. On the other hand, plasmid PK7WGF2 containing *35S+gfp+gus* gene were isolated from *E. coli* following QIAGEN Plasmid mini prep kit and transformed to *AGL0* strain. Subsequently glycerol stocks of this strain were made for further proceeding.

From the glycerol stock, each strain of bacteria were inoculated to LB medium (Pepton select Gibco: 10 g, yeast extract select Gibco: 5 g, NaCl: 5 g, agar: 8 g rest 1L water) containing appropriate antibiotics:Kanamycin (50 mg/L) and Rifampicin 25 mg /L are required for *AGL0* (*pMF1-gfp-pMdRbcS-gusi*) construct, Kanamycin (50mg/L), chloramphenicol (50mg/L) for AGL1 +virG (*pRIABI.2MFchc*-short-*vnt1-blb3-stol1*). The *AGL0* (*35S+gfp+gus*) construct needsSpectinomycin (100 mg/ml). Thereafter, the bacteria were grown overnight (16 hr) on a shaker 180 rpm at 28^{o}C. The optical density (OD) of bacteria was measured at 600 nm (OD600). The final OD600 was 0.4-0.6 during transformation. However, in factors influencing *Agrobacterium* mediated transformation experiment only AGL0 (*35S+gfp+gus*) construct were considered and used at OD=0.2

Pre-culture inoculation and co-cultivation

The internodes of potato were cut into pieces of 2-5 mm, then transfer them to petri dish containing R3B medium (MS+3% Sucrose + 0.8% Agar + 4mg/ml NAA + 1mg/ml BAP, pH5.8). Before transferring the explants, two sterile filter paper was placed on R3B medium then 1.5 ml PACM media (MS+3%Sucrose + 0.2% Caseine hydrolysate+1mg/ml 2,4-D + 1mg/ml Kinetine, pH6.5) was poured on the filter paper. After two days of pre-culturing, the explants were inoculated with the *Agrobacterium* by incubating iin a petridish containing bacteria suspension for 5-10 min. Subsequently, the explants were blotted on sterile filter paper to remove the bacterial suspension and placed back on R3B medium. Finally, they were placed in climate cell (24°C temp, 16h light& 8h in dark).

For improved regeneration experiment, half of the explants were transferred to R3B and rest half R3B+IAA+Zeatin medium (MS+3% Sucrose + 0.8% Agar + 4mg/ml NAA + 1mg/ml BAP+0.1 mg/LIAA+0.1mg/L trans-Zeatin, pH5.8) and followed the procedure as in R3B. The improving factors followed in this experiment are presented in table 1.

Table 1. Physical factors and media composition of R3B and R3B+IAA+Zeatin pre-cultured media

Factors	R3B	R3B+IAA+Zeatin
Age of the explants	4 weeks old	5 week old
Media composition	MS+3% Sucrose + 0.8% Agar + 4mg/ml NAA + 1mg/ml BAP, pH 5.8	MS+3% Sucrose + 0.8% Agar + 4mg/ml NAA + 1mg/ml BAP+0.1 mg/LIAA+0.1mg/L t-Zeatin, pH5.8
PACM	1.5ml	2ml
Pre-culture time	2 days	4 days
Used OD	0.4-0.6	0.2
Transformation (IM)	No induction medium used	Acetosyringon (19mg/l)
Co-cultivation	2 days(16 hrs light and 8 hrs dark	2 days in dark condition
Selection and regeneration	Zeatin (0.1mg.l-1) cefotaxime (200 mg/l) vancomycine(200mg/l)	Zeatin (0.1mg.l-1), cefotaxime (200 mg/l), vancomycine (200mg/l IAA ($0.1mg.l^{-1}$), t-zeatin ($0.1mg.l^{-1}$)

Regeneration & Selection

For regeneration and selection, two days later, the transformed explants were equally transferred on fresh ZCV (MS + 2% Sucrose + 0.8% Agar + 1mg/ml Zeatine + 200mg/ml Cefotaxime 200mg/ml Vancomycine, pH5.8) and ZCVK selection medium for regeneration of transgenic shoots. However, in updated protocol for regeneration, t-Zeatin (0.1mg/l) and IAA (0.1mg/l) were added on first regeneration medium, either with or without kanamycin. Every two weeks, the explants were transferred on fresh ZCVK or ZCV medium medium for marker assisted and marker free transformation, respectively.

gfp selection

Three weeks later putative transgenic callus and shoots were selected on kanamycin and without kanamycin containing media checked by visualizing *gfp* expression by fluorescence microscopy. In the explants stage both control and treated explants displayed green auto-fluorescence, so transgenic callus selection was not reliable at this stage. On the other hand, in shoot stage, transformed shoots showed auto-fluorescence and non-transformed shoots were visible as black appearance within the GFP filter.

Molecular analysis

DNA Isolation

For genomic DNA isolation leaves were harvested into 2 ml eppendorf tubes pre-placed on ice. Each tube contains four carbon steel balls for crushing the leaves. Meanwhile, the eppendorf tubes were frozen in liquid nitrogen before grinding the leaf materials in Retsch tissue lyser machine. Afterward, the tubes were placed back on liquid nitrogen and stored at -80^{0}C. Subsequently DNA was isolated following the CTAB protocol (Appendix-1). The DNA quality was assessed by gel electrophoresis and concentration was measured in nanodrop.

PCR amplification and screening of transformed regenerants by analyzing the presence of nptII, *gfp* and *virG* gene

There generants were analyzed for the integration of *nptII* and *gfp* gene by PCR amplification. For reliable screening, bacterial contamination was also checked by amplification of *VirG* gene in PCR by using *virG* gene specific primers LK37 and LK38. The presence of *nptII* and *gfp* genes were checked by using their specific primers LK160 and LK161 and GFP F and GFP R respectively. Primer sequences and annealing temperature are represented in Appendix 1 table 1. As a positive control A73.1-54 genotype (provided by Linda Kodde) that contain both *nptII* and *virG* gene was used to check whether PCR reaction is correct or not. The PCR program and protocol were described in Appendix part (table 2 and 3). After amplification, the *virG* gene containing shoots were considered as false positive transgenic selection while comparing with *nptII* and *gfp* gene containing shoot.

Screening of regenerants for the presence of the *Rpi-chc1* gene in AGL1 (pRIABI.2MF:chc-short:vnt1:blb3:stol1) treated explants

The *R* gene containing larger constructs that contain about 24 kb long T-DNA were used to see the effect of T-DNA length on transformation. Four *R* genes *chc1, vnt1, blb3* and *stol1* are present in this construct, where *Rpi-chc1* is present near the right border of the plasmid [Figure (1c)]. Gene present near the right border can easily enter into the plant cell, that's why chc1 was selected to check the transformed shoot. The *Rpi-chc1* specific primers LK57a and LK65 were used for amplification. The positive control A91-1 used to check whether PCR reaction is right or not. The PCR program and protocol was attached on appendix 1 (Table 2 & 3).

Testing resistance gene activity in marker free transformants

Detached Leaf Assay (DLA)

Detached leaf method was used to evaluate the response of potato genotype reaction *to* different *P. infestans* isolates. The mycelium of *P. infestans* isolates were cut intocube (1x1 cm) and placed on rye medium containing 20 g/l glucose two weeks prior of inoculation. After two weeks, the mycelium had grown all over the plate, and then sporangia were extracted with cold tap water and collected in blue cap tube. Before inoculation the sporangia were incubated for two hours at 4^{o}C.Subsequently, infection unit (Zoospores +non germinated sporangia) were counted in a microscope and adjusted the concentration of sporangia suspension to 50X10^{4} zoospores per millilitre.

The fully extended primary leaves with 2 cm petiole were collected from greenhouse and stored together with the corresponding label. Afterward, the leaves were put in 4X4 cm water soaked floral foam blocks for 20 minutes. Two leaves from the same individual were placed in opposite direction of each other in each foam block with the abaxial side up. Then they were placed in a plastic tray on water soaked filter paper. The level for each individual was fixed with toothpick. Each leaf was inoculated with five 10 µl droplets of zoospore suspension (50X10^{4} spores/ml) of two different isolates an the left and right abaxial sides respectively. Seven days after inoculation the leaves were then evaluated by eye and scored based on infection and resistance response (Table 2 and 3).

Table 2. The virulence spectrum of *Pi* isolates used in present experiment

***P. infestans* isolate**	***Rpi***	
	vnt1	***chc1***
EC1	virulence	avirulence
Katshaar	avirulence	virulence
IPO-C	avirulence	avirulence

Table 3. Scoring detached leaf assay symptoms

Symptoms	**Score**
No infection at all	R9
HR size of the inoculum drop	R8
HR size somewhat bigger than the drop	R7
Large HR lession	R6
Large dry lesion, no sporulation	V5
Large water soaked lesion, no obvious sporulation	V6
Large lesion, sporulating on the dark side of the leaf	V7
Sporulation on both sides of the leaf	V8

Agro-infiltration

To test the activity of resistance genes *Rpi-vnt1* and *Rpi-chc1* in marker free transformants V91 and P91, agro-infiltrations were performed by using cognate effectors *Avrvnt1* and *Avrchc1*. Simultaneously, as a positive control *R3b-Avr3b* and negative control *Avr3b* construct were infiltrated in V91 and P91 plants. The construct *AVR2* also used in cultivar P background plant. Moreover, positive control plant A13-13 for *Avrvnt1*, A27-17 plant for *Avrchc1* and A03-104 plants for *AVR2* were also included in this experiment for confirmation of the right construct that used in agro-infiltration. All constructs and control plants were provided by Marjon Arens and Marjan Bergervoet.

All the plants were multiplied 4 weeks before agro-infiltration. Two media were used to grow the effectors LB and YEB media. The composition of LB (10g/l Bactopeptone, 10g/l NaCl, 5g/l yeast extract) and YEB (5g/l beef extract, 5g/l bactopeptone, 5g/l sucrose, 1 g/l yeast extract, 2ml 1M MgSO4 (246g/l) media. Three days before infiltration, all the construct were inoculated from glycerol stock in 3 ml LB medium with appropriate antibiotic and grown two nights at 28-30C and 200 rpm. Next day, to determine the growth of the bacterial suspension, 100 µl cultures was diluted with 900µl LB medium and OD600 was measured. Successively, 15 ml of YEB medium was inoculated with X l culture (according to the following calculation).

X=V*Z/OD of the pre-culture
Where $Z=800/2^{(\Delta t/td)}$
X=volume in micro-litre to be inoculated
V= culture volume in millilitre
Δt= desired culture time,
td = doubling time

On the following day, MMA medium was prepared with 20g/l sucrose, 5g/l MS salts (no vitamins), 10ml 1M MES pH=5.5, 1ml acetosyringone (200 mM).The YEB bacteria cultures OD600 were measured and centrifuged for 10 mins at 4000rpm. The cell yield was calculated in OD units (multiplied volume of YEB and measured OD_{600}). Subsequently, the required OD for agro-infiltration was adjusted by adding MMA. After 1 hours of incubation, they were infiltrated by 1 ml syringe on fully expanded young leaves of plants. In each plant 4 leaves were used for agro-infiltration. After 2 days, the leaves were scored in terms of percentage of infiltrated area showing hypersensitive response (HR). The following table (Table 4) indicates the expected HR in transgenic plant infiltrated by several constructs.

Table 4. Expected HR in transgenic plant with corresponding HR

Plants used in Agro-infiltration	***Avrchc1***	***Avrvnt1***	***Avr3b+R3b***	***Avr3b* (negative control)**	***AVR2***
V91 (*chc1&vnt1*)	HR	HR	HR	No HR	No HR
P91 (*chc1,vnt1 &Rpiabpt1)*	HR	HR	HR	No HR	HR
P (*Rpiabpt1*)	No HR	No HR	HR	No HR	HR
A13-13 (positive control for *Avrvnt1*)	No HR	HR	HR	No HR	No HR
A17-27 (positive control for *Avrchc1*)	HR	No HR	HR	No HR	No HR
A03-142 (positive control for *AVR2*)	No HR	No HR	HR	No HR	HR

RESULTS

Influencing hormone during pre-culture and selection

Two pre-culture media R3B and R3B+IAA+Zeatin were used to evaluate the callusing and regeneration potential of four different cultivars; Bintje, Atlantic, Desiree, and Russet Burbank after inclining *Agrobacterium* mediated transformation, with or without kanamycin condition. In R3B media the explants were pre-cultured two days before bacteria inoculation while in R3B with Zeatin and IAA they were cultured 4 days prior to inoculation. After transformation R3B pre-cultured explants were placed in 16hrs light and 8 hrs in dark whereas R3B including with IAA and zeatin treated explant were kept in dark condition at two days than they were placed in normal 16 hrs light and 8 hrs in dark in climate cell. In these respect, each treatment was considered three times. The range of explants varied 40-160 for each experiment. The percentage of callusing and regeneration response was counted on the basis of number of shoot regenerated from the initial explants that were accommodated in each petri dish as 20 explants (Appendix-1, Table 1 & 2).

The callusing response was considered by callus grown ability of explant. Among the four cultivars, it has been noticed that callus response was considerably higher in R3B+IAA+Zeatin pre-cultured explants than in R3B pre-cultured explants in kanamycin selection [Figure 2(a)].The respective cultivars Bintje, Desiree, and Russet Burbank were found to be a pronounced percentage of callus induction that was just above 90% in R3B+IAA+Zeatin pre-cultured in Kanamycin selection media. In R3B pre-cultured media, Atlantic and Bintje were found just above 40% callusing while Desiree and Russet Burbank were observed well above 70% callusing in kanamycin assist selection as mentioned in table 5.

While in marker free transformation, the callusing response in explantsshowed a marginal difference in R3B+IAA+Zeatin and in R3B pre-cultured media [Figure 2(b)]. For instance, the callus induction in Bintje and Russet Burbank explants varied by 8% in R3B+IAA+Zeatin and 5% in R3B pre-cultured media for marker free (Table 6).

Table 5. Callus grown ability (%) of explant of 4 different cultivars which were pre-cultured with 2 different media under kanamycin selection (Data 6 weeks post transformation)

Cultivar	(R3B)					(R3B+IAA+Zeatin)				
	Initial number of explants	No of explant having callus grown ability	No of shoot regenerated from initial number of explants	% Callusing	% Regeneration	Initial number of explants	No of explant having callus grown ability	No of shoot regenerated from initial number of explants	% Callusing	% Regeneration
Bintje	100	41	7	41	7	120	115	100	95	83
Atlantic	64	30	2	46	3	60	50	1	83	2
Desiree	140	100	27	71	19	160	150	45	93	28
Russet Burbank	100	72	1	72	1	40	38	1	95	3

Table 6. Callus grown ability (%) of explant of 4 different cultivars which were pre-cultured with 2 different media under kanamycin free selection (Data 6 weeks post transformation)

Cultivar	(R3B)					(R3B+IAA+Zeatin)				
	Initial number of explants	No of explant having callus grown ability	No of shoot regenerated from initial number of explants	% Callusing	%Regeneration	Initial number of explants	No of explant having callus grown ability	No of shoot regenerated from initial number of explants	%Callusing	%Regeneration
Bintje	105	93	28	88	26	100	100	19	100	19
Atlantic	100	95	7	95	7	40	40	7	100	18
Desiree	100	100	37	100	37	100	100	28	100	28
Russet Burbank	60	50	1	83	2	40	37	1	92	3

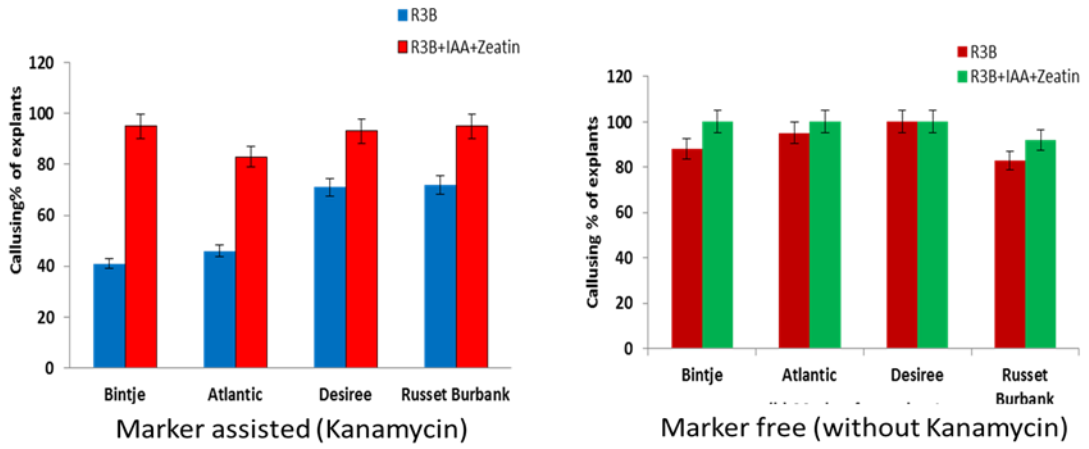

Figure 2. Callus formation in stem explants of 4 different cultivars that were pre-cultured in two different media separately, in R3B for two days and R3B+IAA+zeatin for four days prior transformation process. Data were obtained from both kanamycin and marker free transformations at 6 weeks post transformation. The range of explants varied from 40 to 160 for each experiment**.**

Interestingly, the regeneration capacity of Bintje was markedly higher in R3B+IAA+Zeatin pre-cultured explants than in R3B pre-cultured explants under Kanamycin selection [Figure 3(a)]. It was well above 80% in R3B+IAA+Zeatin pre-cultured explants in Kanamycin while in R3B pre-cultured explants showed approximately 10% (Table 5). Conversely, in Atlantic and Russet Burbank showed very poor regeneration efficiency just below 5% in both pre-treatments media of the explants with kanamycin [Figure 3(a)]. The cultivar Desiree showed 25% regeneration capacity in R3B+IAA+Zeatin pre-cultured explants with Kanamycin selection while in R3B pre-cultured explants showed approximately 19% regeneration (Table 5). By contrast, in marker free condition, Bintje & Desiree cultivars showed a higher regeneration response in R3B pre-cultured explants than in R3B+IAA+Zeatin pre-cultured explants [Figure 3 (b)].

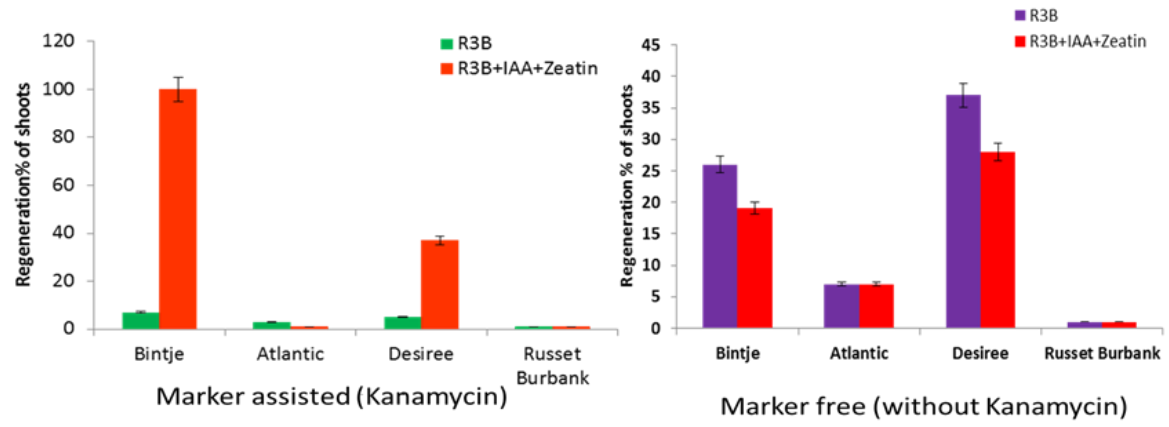

Figure 3. Shoot regeneration capacity of 4 different cultivars that were pre-cultured with two different media, in R3B for two days and R3B+IAA for four days prior transformation process. Data were obtained from both kanamycin and marker free transformations at 6 weeks post transformation .The range of explants varied from 40 to 160 for each experiment**.**

Molecular analysis

The four different cultivars were subjected to histological (*gfp*) and molecular analysis (PCR) that were pre-cultured on R3B and R3B+IAA+Zeatin media before transformation both kanamycin and without kanamycin media. In marker free selection, cultivar Bintje regenerated 91 shoot from R3B+IAA+Zeatin pre-cultured explant, 30 of these was found PCR positive for *gfp* and 3 for *nptII* , 7 shoots were visually positive. Whereas in kanamycin selection, out of 6 shoots, 6 were *gfp* and 2 were *nptII* positive in PCR. The performances of other cultivar were presented in table 7. Among the all four cultivars Bintje were performed better after R3B+IAA+zeatin pre-cultured explant.

Table 7. Performance of four different cultivar that are pre-cultured in two different media R3B and R3B+IAA+Zeatin

Cultivars	R3B								R3B+IAA							
	K-				K+				K-				K+			
	Total shoots	***gfp* + visually**	***gfp* + in PCR**	***nptII* + in PCR**	**Total shoots**	***gfp* + visually**	**gfp+ in PCR**	***nptII* + in PCR**	**Total shoots**	***gfp* + visually**	***gfp* + in PCR**	***nptII* + in PCR**	**Total shoots**	***gfp* + visually**	***gfp*+ in PCR**	***nptII*+ in PCR**
Bintje	27	x	x	x	4	2	2	2	91	7	30	3	6	1	6	2
Desiree	18	x	7	x	2	1	1	2	21	x	9	5	1	1	1	2
Atlantic	x	x	x	x		x	x	x	11	1	1		13	3	10	4
Russet Burbank	x	x	x	x	x	x	x	x	x	x	x	x	4	2	2	2
Total	45	x	7	x	6	3	3	4	123	8	40	8	24	7	19	10

'x' = not tested

Table 8. Performance of four different cultivars that are pre-cultured in two different media R3B and R3B+IAA+Zeatin in percentage

Cultivars	R3B								R3B+IAA							
	K-				K+				K-				K+			
	Total shoots	*gfp* + visually	*gfp*+ in PCR (%)	*nptII* + in PCR	Total shoots	*gfp* + visually (%)	gfp+ in PCR (%)	*nptII* + in PCR (%)	Total shoots	*gfp* + visually (%)	gfp+ in PCR (%)	*nptII*+ in PCR (%)	Total shoots	*gfp* + visually (%)	*gfp* + in PCR (%)	*nptII*+ in PCR (%)
Bintje	27	x	x	x	4	50	50	50	91	7.6	33	3.2	6	16	100	33
Desiree	18	x	38	x	2	50	50	100	21	x	42	23	2	25	25	100
Atlantic	x	x	x	x	x	x	x	x	11	9	9	x	13	23	76	30
Russet Burbank	x	x	x	x	x	x	x	x	x	x	x	x	4	50	50	50

'x' = not tested

Physical difference of explants between two media

The growth performances of four different cultivars were evaluated in kanamycin media and marker free media after pre-treatment of explants with R3B+IAA+Zeatin and R3B media. In kanamycin selection, the explants of Bintje which was pre- treated with R3B+IAA+Zeatin media were found to be green in colour and vigorous in state, expanded callus region (Figure 4b).

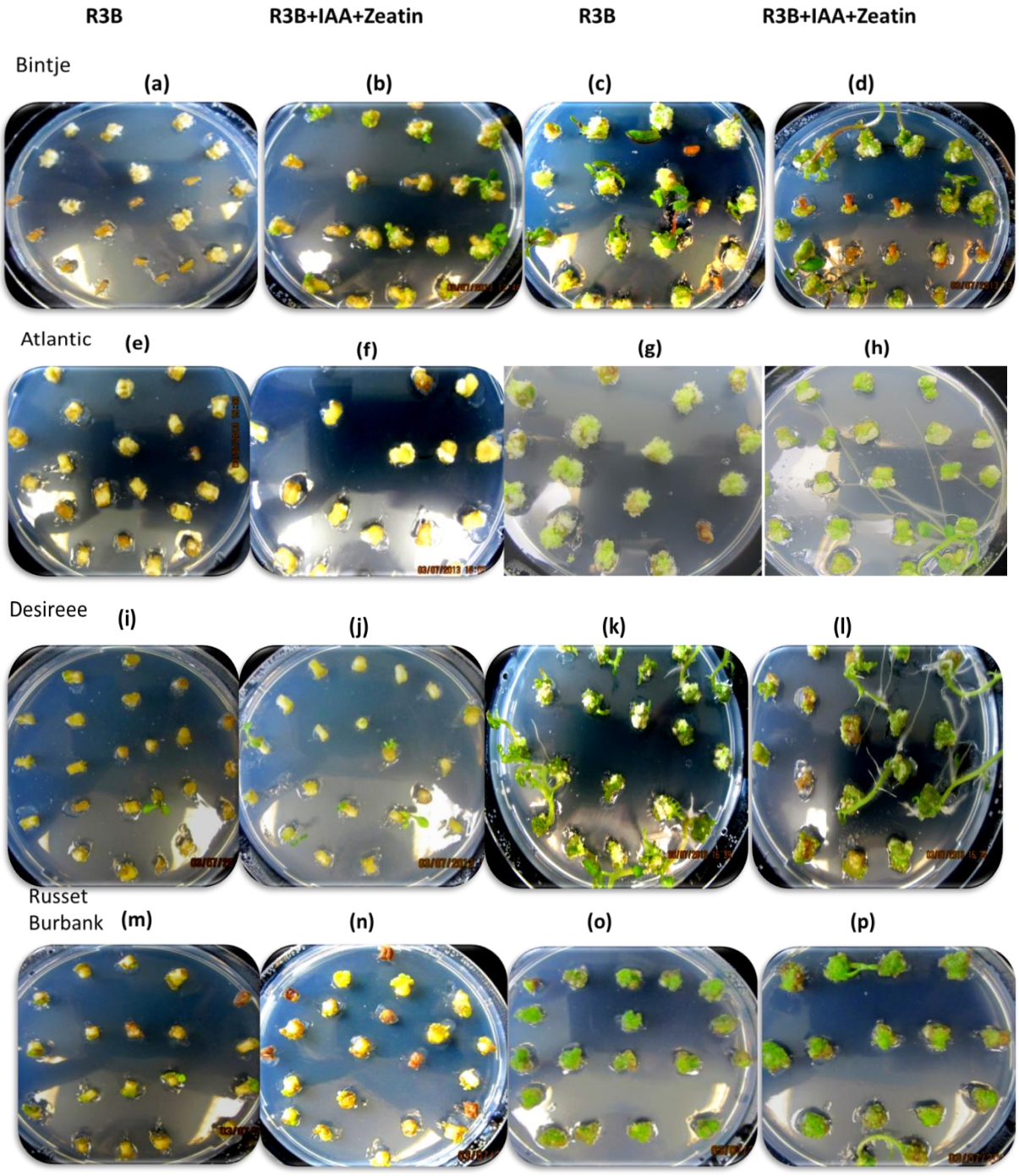

Figure 4. Callus formation and regeneration of Bintje (a,b,c,d),Atlantic (e,f,g,h), Desiree (I,j,k,l) and Russet Burbank (m,n,o,p) after transformation. The explants were pre-cultured on R3B (a,c,e,g,I,k,m,o) or R3B+IAA+Zeatin media (b,d,f,h.j.l,n,p) for two and four days respectively. Left two petridishes (a,b,e,f,I,j,m,n) contain explants under kanamycin selection and right two petridishes (c,d,g,h,k,l,o,p) without kanamycin selection.

By contrast, the R3B pre- treated explants were observed lean and thin callus region, and stressed cottony white (Figure 4a).

On the other hand, the explants of Bintje that were grown in kanamycin free media were found greenish, expanded callus in the pre-cultured explants of R3B+IAA+Zeatin media and in the R3B pre-cultured media [Figure 4(c) and (d)].

In the Atlantic cultivars, major differences were found among the explants of the two pre- treated media under kanamycin selection and a very slight variation was noticed in the two pre- treated media under marker free selection .For instance, in kanamycin selection the explants were found to be pale yellow in colour and very short callus region in the R3B pre-cultured explants (Figure 4e). By contrast, in the R3B+IAA+Zeatin pre-cultured explants under kanamycin selection showed yellow in colour and distinct callus region (Figure 4f). Moreover, the colour of the callus region was found whitish in both pre-cultured media. In kanamycin free media, R3B+IAA+Zeatin pre-cultured explants were found greener in colour and wider knot in the callus region than in R3B pre-cultured explants. On the other hand, R3B pre-cultured explants were found cottony white under marker free selection media (Figure 4, g and h).

In Desiree cultivars a narrow callus region were found among the explants of the two pre-cultured media under kanamycin selection whereas under marker free selection a very wide region of callus was found in both pre-cultured media (Figure 4, i and j). In kanamycin free media, R3B pre-cultured explants showed light greenish callus part while in R3B+IAA+Zeatin pre-cultured explants found greenish in colour and more vigorous growth (Figure 4, k and l).

There was a marginal difference among the explants of Russet Burbank in two different media under kanamycin selection, where explants were found pale yellow in colour and very narrow callus region (Figure 4, m and n). While in the marker free selection the explants were found to be green and yielded a pronounced callus region with big size and shape in the pre-cultured R3B+IAA+Zeatin media (Figure 4, o and p).

Usefulness of *gfp* as a selection marker in marker free transformation

The visual marker *gfp* was used to monitor the transformation of four different potato cultivars Bintje, Atantic, Russet Burbank, and Desiree in present research.An expression of *gfp* was observed under fluorescent microscope and finally DNA level section was done by PCR.
The auto-fluorescence was checked by including non-transformed explant and shoot with bacterial treated explant, which reduces the probability to screen false *gfp* expressing shoots. Moreover, for reliable screening, it was also checked whether *Agrobacterium* showed -fluorescence or not. It was noticed *Agrobacterium* showed fluorescence under microscope (Figure 6). Shoots emerging from kanamycin selection and marker free shoots were considered for *gfp* screening. In addition, chimeric and tissue specific expression was counted after screening (Table 9). A higher percentage of shoots also found as an escape.

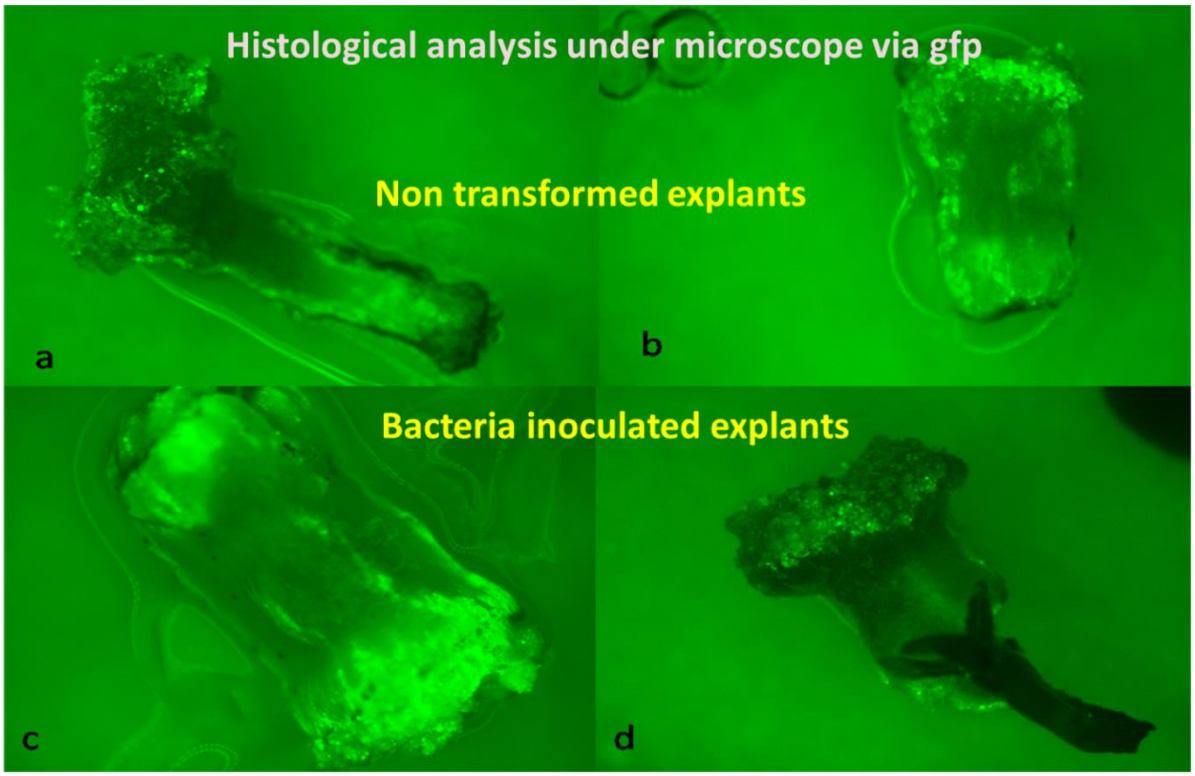

Figure 5. Expression of *gfp* during stages of callus development of potato explants. Both Control (a and b) and transformed (c and d) explants were showing auto-fluorescence in fluorescence microscope.

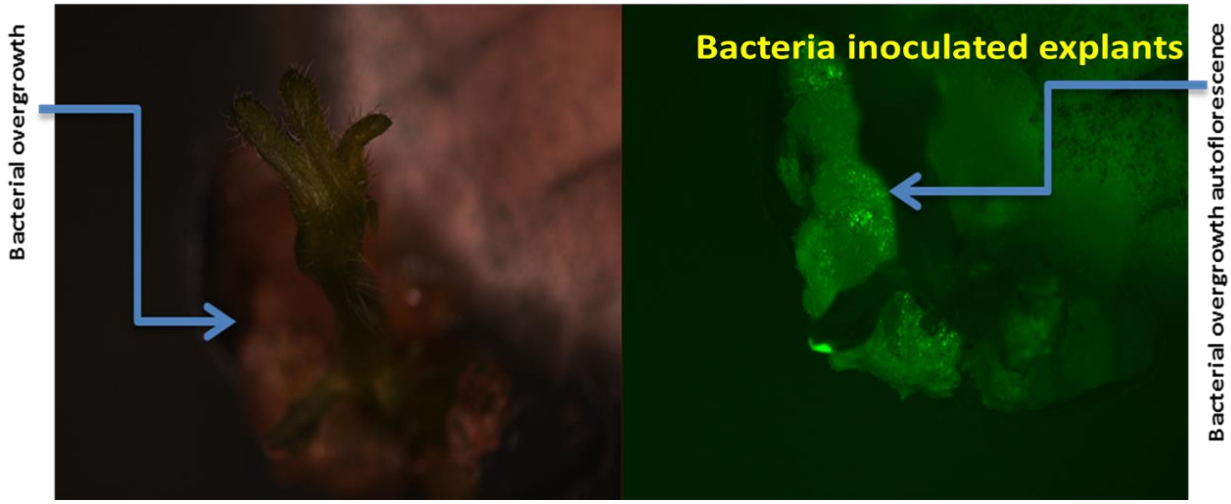

Figure 6. Agrobacterial overgrowth displaying fluorescence under fluorescence microscope

Visual screening

Identification of transgenic explant by visual screening of *gfp* at explant stage was not reliable, since both bacterial treated and control explants was noticed auto-fluorescence (Figure 5). Besides, variation in *gfp* expression was found in kanamycin resistance and marker free regenerated shoot. In kanamycin selection, a higher percentage of shoots was found that express tissue specific *gfp* expression (Figure 7). Moreover, the intensity of *gfp* expression was also higher comparing with marker free transformants (Figure 8). Whereas, shoots in marker free selection, expressing patchy small part of tissue green fluorescence, they are classified as potentially chimeric because in the available time frame it was not possible to distinguish tissue specific expression from chimeric transfromants.

Table 9. Visual screening of *gfp* under fluorescent microscope in kanamycin and marker free selection

Constructs	T-DNA length	Total explants		Total shoots-tested in Microscope		Visually gfp+ shoots	
		MF	Kan	MF	Kan	MF	Kan
(35S : *gfp* :gus +NPTII*)*	3kb	1468	1685	386	157	16	111
pMF1-pMdRbcS *gfp*: gusi+ NPTII	13kb	834	950	265	13	15	9
Total		2302	2635	651	170	31	120
per-cent						5 %	70 %

'MF'= Marker Free; 'Kan'= Kanamycin

Total 821 shoots were observed under microscope, among them 651 shoots obtained from marker free transformants and 170 were from kanamycin selection. The percentage was counted on the basis of total shoot number observed under microscope. In marker free situation, 5% transformants were found *gfp* positive, whereas in kanamycin selection 70% shoots was observed as *gfp* positive. A relatively higher percentage (74%) of potentially chimeric shoot was found in marker free selection. By contrast, in kanamycin selection the percentage was reduced, 16% shoots were potentially chimeric and approximate 50% shoots found that express *gfp* throughout the whole shoot (Table 10). In marker free, only 28% shoot was found gfp positive in PCR analysis, that means 72% shoots had not integrated *gfp* gene considered as escape. In kanamycin selection 47% shoots were found *gfp* positive in PCR and the rest 53% had not integrated *gfp* gene. This 72% shoots in marker free and 53% in kanamycin considered as escape (Table 12)

Table 10. Identification of shoots that expressing different kind of *gfp* under fluorescent microscope in kanamycin and marker free selection

Expression type	*gfp* positive shoots in MF condition	*gfp* positive shoots in Kanamycin selection	% in MF	% in Kan
Total shoots	31	120		
Tissue specific	3	40	10	33
chimeric	23	20	74	16
Homologous expression	1	60	3	50

'MF'= Marker Free; 'Kan'= Kanamycin

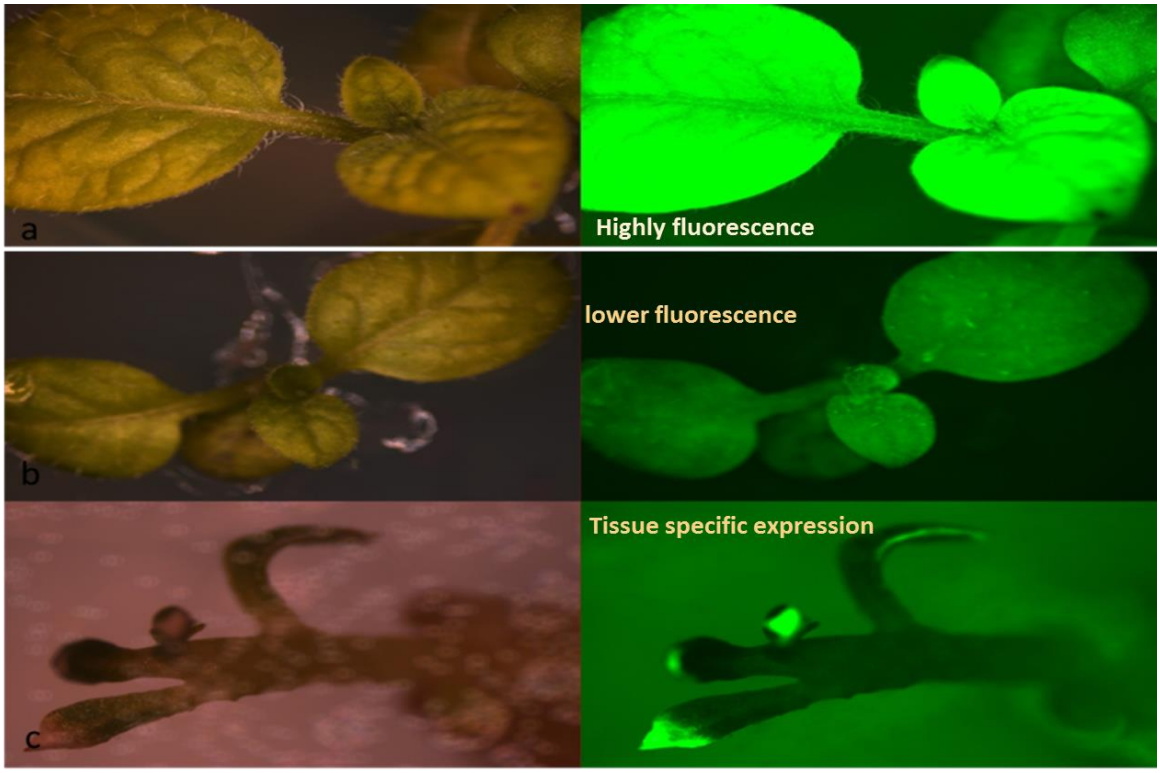

Figure 7. Expression of *gfp* in kanamycin resistant shoots of potato. The intensity of expression varies even in same cultivar and using same construct. Picture depicted the transformed shoots of Atlantic by using AGL0 (pMF1-gfp-pMdRbcS-gusi) construct, (a) Highly fluorescence (b) lower fluorescence (c) tissue specific expression. Pictures were taken in same exposure time; indicate that left image derives from exposure in visible light. Right image derives from UV exposure.

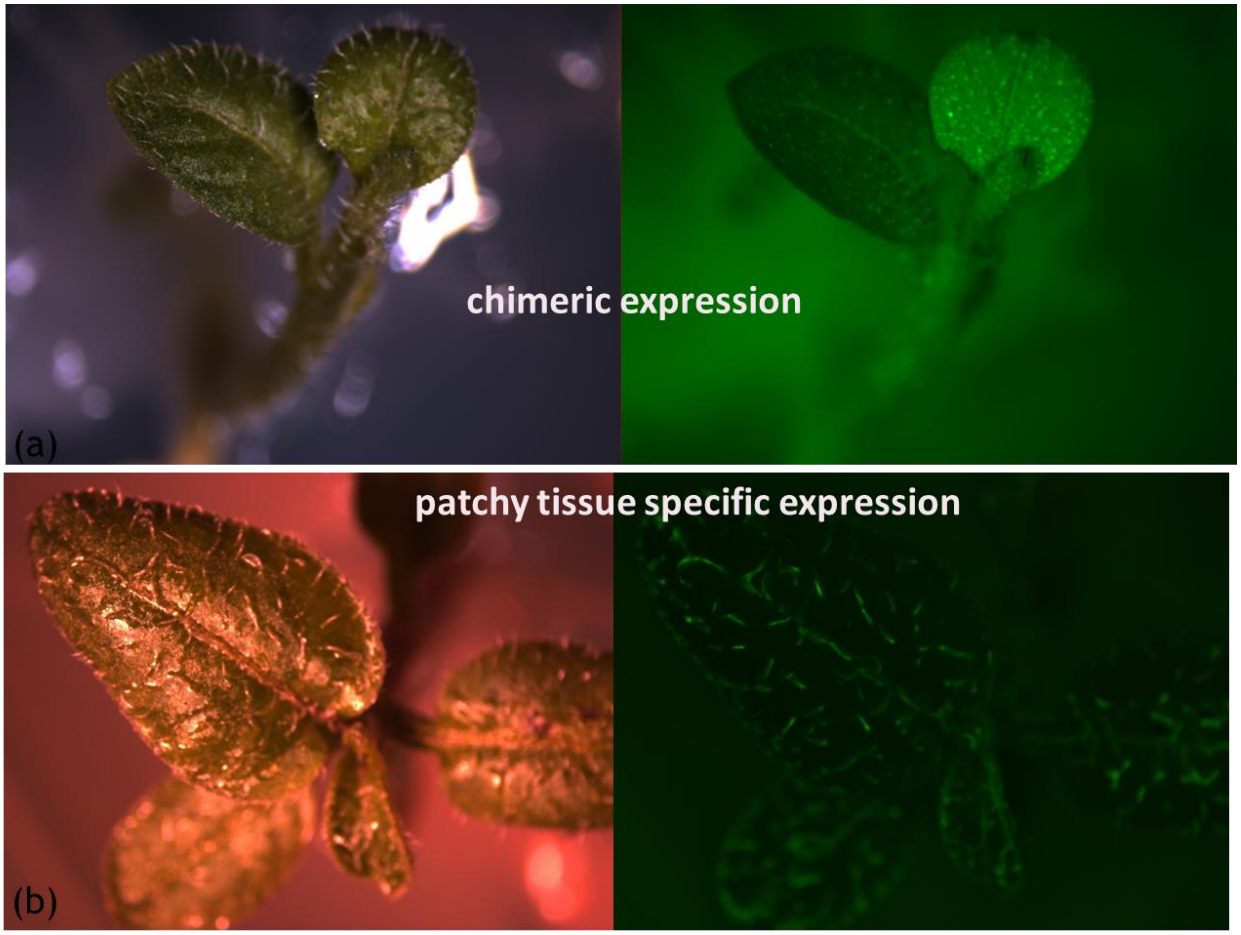

Figure 8. Expression of *gfp* in marker free shoot, (a) potential chimeric expression and (b) patchy tissue specific expression throughout the tissue.

It has been speculated that transformation efficiency is dependent on plant genotype. Here four potato cultivars were included to see their regeneration efficiency after transformation. Due to the poor regeneration it was not possible to check sufficient number shoot for *gfp* for all cultivars. Very few number of shoot of Atlantic and RB were found. On the other hand, more than 100 shoots of Bintje and Desiree were checked for *gfp,* where 44% shoots (including both kanamycin and marker free shoot) of Bintje and 17% shoots of Desiree were noticed for *gfp* positive.

Table 11. Shoots of different cultivars that pre-cultured in R3B and R3B+IAA+Zeatin media expressing *gfp* under fluorescent microscope

Cultivars	Total *gfp* tested shoot		Kan		MF		Total *gfp* positive shoot		*gfp* positive in Kan		*gfp* positive in MF	
	R3B	R3B+IAA+Zeatin	R3B	R3B+IAA+Zeatin	R3B	R3B+IAA+Zeatin	R3B	R3B+IAA+Zeatin	R3B	R3B+IAA+Zeatin	R3B	R3B+IAA+Zeatin
Bintje	33	120	8	94	26	26	10	58	7	50	3	8
Atlantic	14	4	x	2	14	2	5	2	x	2	5	x
Desiree	83	70	24	x	59	70	20	7	17	x	3	7
Russet Burbank	28	15	13	7	15	8	12	7	10	7	2	X
Total	158	209	45	103	114	108	47	74	34	59	13	8

'MF'= Marker Free; 'Kan'= Kanamycin; 'x' = not tested

Screening of transformants on the basis of presence of *nptII, gfp* and *VirG* gene

PCR analysis confirmed the presence of 722 bp *nptII* and 300 bp *gfp* products, indicating the presence of the plasmid in the regenerants [Figure 9, (a) and (b)]. A total 157 regenerants obtained from marker free condition and 40 from kanamycin selection were subjected to PCR analysis. It was found that 6 shoots in marker free and 15 shoots in kanamycin selection were PCR positive for *gfp* gene. This result indicated that over 47% of the regenerants in kanamycin were transgenic with successful integration of the plasmid in plant genome (percentage calculated on the basis of number of shoot subjected to PCR) (Table 12). By contrast, in marker free selection only 28% regenerants were found as transgenic. Moreover, the amplification of 692 bp of *virG* gene in the plasmid showed complete absence of this gene in most of the transformants [Figure 9, (c)]

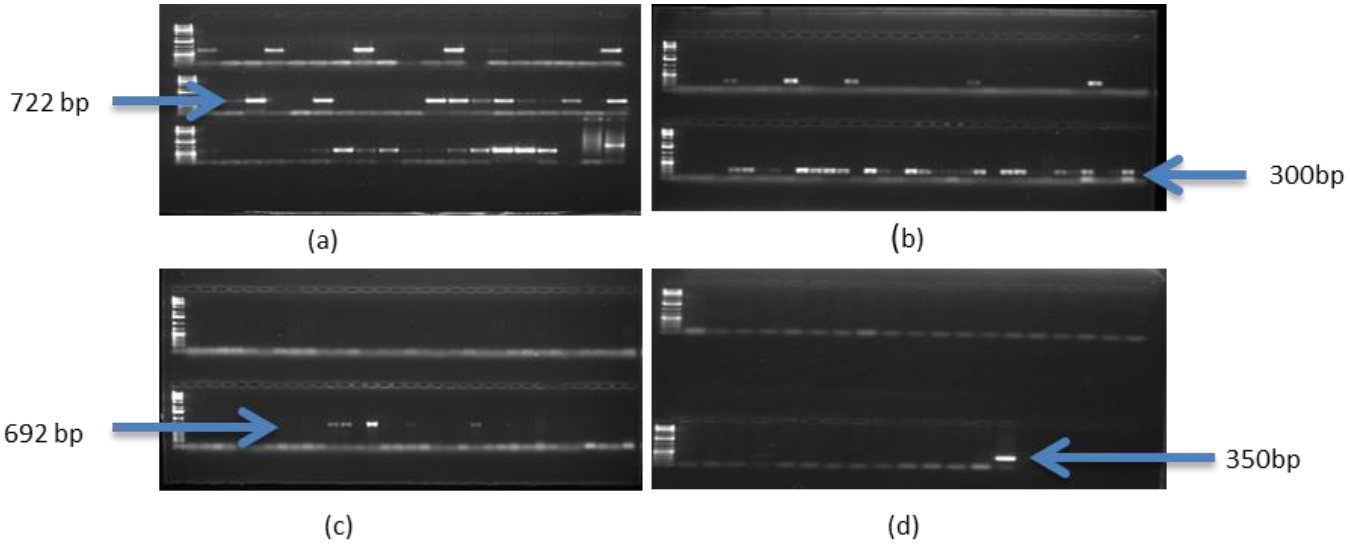

Figure 9. Amplification of (a) *nptII* (b) *gfp* and (c) *virG* (d) *chc1* product for screening of positive transformants

Table 12: Screening of shoots obtained from kanamycin and marker free selection by PCR amplification of *nptII* , *gfp*, and *virG* genes

Condition	Total shoot tested in PCR	*gfp* positive (%) under microscope	gfp positive in PCR (%)	nptII positive in PCR (%)	*gfp*+npt II+microscope gfp	*VirG* contaminated shoots (%)	Non-transformed (%)
Marker free	157	4	28	7	0	3	72
Kanamycin	40	37	47	30	15	2	53

Table 13: Screening of shoots obtained from kanamycin and marker free selection by PCR amplification of *nptII*, *gfp*, and *virG* genes

Constructs	T-DNA length	Total shoots tested in PCR		Visually *gfp* +ve		*gfp* +ve in PCR		*nptII* +ve in PCR		*VirG* contaminated shoot		chc1+ve shoots in PCR
		MF	Kan	MF	Kan	MF	Kan	MF	Kan	MF	Kan	
(35S : *gfp* :gus +NPTII*)*	3kb	135	27	5	12	40	15	10	9	3	x	
pMF1-pMdRbcS *gfp*: gusi+ NPTII	13kb	22	13	1	3	4	4	1	3	1	1	
pRIAB1.2MFchc:-short: -vnt1:- blb3:- sto1	24kb	66										x

'MF'= Marker Free; 'Kan'= Kanamycin; 'x'= Absence of the gene

Among 157 regenerants, 135 shoots were obtained from the explants treated with 35S:+ gfp: +gus) construct. Those explants were pre-cultured in two pre-culture media R3B or R3B in combination with IAA and Zeatin. In marker free selection, 123 regenerants were obtained after pre-treating explants in R3B+Zeatin+IAA media, of these 40 were found PCR positive for *gfp* and 8 for *nptII*. In kanamycin selection, 24 regenerants were obtained from the explants that pre-cultured in R3B+Zeatin+IAA media; of these 19 shoots were found positive in PCR for *gfp* and 10 were *nptII* positive.

Table 14. Performance of four different cultivars that are pre-cultured in two different media R3B and R3B+IAA+Zeatin

Cultivars	R3B								R3B+IAA+Zeatin							
	K-				K+				K-				K+			
	Total shoots	*gfp* + visually	*gfp*+ in PCR	*nptII*+ in PCR	Total shoots	*gfp* + visually	gfp+ in PCR	*nptII*+ in PCR	Total shoots	*gfp* + visually	gfp+ in PCR	*nptII*+ in PCR	Total shoots	*gfp* + visually	*gfp*+ in PCR	*nptII*+ in PCR
Bintje	27	x	x	x	4	2	2	2	91	7	30	3	6	1	6	2
Desiree	18	x	7	x	2	1	1	2	21	x	9	5	1	1	1	2
Atlantic	-	-	-	-	-	-	-	-	11	1	1		13	3	10	4
Russset burbank	-	-	-	-	-	-	-	-	-	-	-	-	4	2	2	2
Total	45	x	7	x	6	3	3	4	123	8	40	8	24	7	19	10

'x' = absence of gene; '-' = not tested

Whereas of 45 plants regenerated from R3B explants in marker free situation, of these 7 shoots were found *gfp* positive in PCR, but none of the shoots were found *nptII* positive or visually expressing GFP. In kanamycin selection, 6 were regenerated from pre-cultured explants in R3B media and 3 were found positive in PCR for *gfp* and 4 for *nptII* positive.

Effect of larger insert on transformation process

Three different lengths of T-DNA construct were used to see the effect of T-DNA length during transformation. Total 3000 explants were transformed by using a 3 kb construct (*35S*: *gus: gfp*). A total of 161 shoots were regenerated and subjected to PCR and 55 shoots were found *gfp* positive that indicated 1.8% shoots were transformed from the initial number of explants, but when the efficiency is expressed as the number of PCR positive shoots among the total number of shoots, a frequency of 33 % for (*35S*:*gus*:*gfp)and 22% for pMF1-gfp-pMdRbcS-gusi* was found.

Table 15. Number of transgenic shoot after transformation with three different T-DNA length containing plasmid

Constructs	T-DNA length	Total no. explants (MF + Kan)	Total shoots tested in PCR (MF + Kan)	gfp positive shoots in PCR (MF + Kan)	chc1 + shoots in PCR	Insert length percentage (%)
(35S : *gfp* :gus +NPTII*)*	3kb	3000	162	55	NA	33
pMF1-pMdRbcS *gfp*: gusi+ NPTII	13kb	1785	35	8	NA	22
pRIAB1.2MFchc:-short: -vnt1:-blb3:- sto1	24kb	1130	66	NA	-ve (not found)	NA

'MF'= Marker Free; 'Kan'= Kanamycin; 'NA'= Not applicable

A total 1780 explants were inoculated by the construct *pMF1-gfp-pMdRbcS-gusi* having 13 kb T-DNA on the plasmid. A total 35 shoots were regenerated and tested via PCR, of these 8 shoots for *gfp* positive which indicated 0.4% shoots were transgenic from initial number of explants, but when the efficiency is expressed as the number of PCR+ shoots among the total number of shoots, a frequency of *22%* % was found.

A third construct *pRIAB+2MFshortchvnt+blb* containing four resistance genes(T-DNA 24 kb) were used to inoculated 1130 explants and total 66 shoots were regenerated and PCR tested for having *chc1* . Notably, none of the shoots were shown *chc1* positive. By comparing the first and the second construct we noticed the transformation frequency was same in both cases by adjusting the initial number of explants.

Testing Rpi-vnt1 and Rpi-chc1 in cultivar P and V transformants

The *vnt1*:*chc1* construct (91) was previously transformed into cultivar P and V and it yielded P91 and V91 events. Ten P91 events were tested for the functional expression of *Rpi* genes by DLA and agro-infiltration method. For instance, in detached leaf assay, seven events of P91 were tested and they were found resistant to isolates *Katshaar* and *Ec1* (Table 16). The control plant P was susceptible while in previous experiment they found resistance to the isolate *Ec1* and *Katshaar*. While, in agro-infiltration system two different OD of each construct *Avrvnt1* (OD 0.4 and 0.2) and *Avrchc1* (OD 0.6 and 0.3) were used. However, agro-infiltration of *Avrvnt1* in seven transformants except P91-10, most of the transformants showed HR response to *Avrvnt1* without P91-4 (Table17). The positive control plant A13-13 was shown profoundly positive response in agro-infiltration. The details picture was presented in Appendix-2, Figure 3, 4, 5 and 6.

Table 16. Response of *vnt1:chc1* transformants to *Avrvnt1* and *Avrchc1* in DLA

Genotypes	*Ec-1*	*Katshaar*	*IPO-C*
P91-1	R8	R8	R9
P91-2	R8	R8	R9,V5
P91-3	R8	R8	R9,V5
P91-4	R8	R8	R9
P91-5	R8	R8	R9
P91-6	R8	R8	R9
P91-7	R8	R8	R9
P91-8	x	x	R9
P91-9	x	x	R9
P91-10	x	x	R9, V5
P	R8	R8	V8
V91-1	V7	V7	x
V91-2	V7	R8	x
V91-3	V6	R7	x
V91-4	V6	R7	x
V91-5	V6	V6	x
V91-6	V5, V6	R8	x
A17-27	R8	V8	x
A03-142	R7,R6	R6,V6	x
A13-13	V7	R8	x

'x' = not tested

On the flip-side, agro-infiltration through *Avrchc1* construct in P91 transformants (repeated three times), did not reflect HR (Figure 10 and data in table 17). The stability of the *Avrchc1* construct was confirmed by using the positive control plant A17-27, that showed HR (Figure 10). The *R3b-Avr3b* and *Avr3b* were included as positive and negative control, where all the experimental conditions were optimally controlled.

Table 17. Testing of *Avrchc1 and Avrvnt1* constructs in V91 and P91 transformants by agro Infiltration

Genotype	*Avrchc1*	*Avrvnt1*	*Avr3b*	*Avr3b+R3b*	*AVR2*
V91-1	1	1	0	1	x
V91-2	0.72	1	0	0.7	x
V91-3	1	0.5	0	1.3	x
V91-4	0.5	1	0	1.3	x
V91-5	0.5	0.5	0	1.6	x
V91-6	0	0	0	0	x
V91-7	0.7	1	0	0.72	x
A13-13	0	2	0	0	x
A17-27	0.8	x	0	2	x
P91-1	0	0.5	0	0.5	0.5
P91-2	0	0.5	0	0.5	0.5
P91-3	0	5	0	1	0.75
P91-4	0	0	0	0.5	1.6
P91-5	0	0.5	0	1	1
P91-6	0	0.5	0	0.3	0.3
P91-7	0	0.5	0	0	0.8
P91-8	0	0.5	0	0.1	0.3
P91-9	0	0.5	0	0.3	0.6
P91-10	0	x	0	1	1
P	0	0	0	0.1	0.5
A03-143	0	x	0	1	2

Response intensity was scored on a 0-2 scale, 2 days after infiltration. Three plant replicate and four leaves per plant were used for infiltrations. Each constructs two types of OD was used, *Avrvnt1* (OD=0.1 and 0.2), *Avrchc1* (OD=0.2 and 0.4) for V91 transformant and *Avrvnt1* (OD=0.2 and 0.4), *Avrchc1* (OD=0.3 and 0.6) for P91 trans formants. 'x' = not tested

In another stance, V91 transformants were inoculated with the isolates of *Ec1* and *Katshaar* in DLA method, was noticed that all the transformants were susceptible to the isolates *Ec1* (except V91-2 and V91-6 showed resistance to *Katshaar*) (Table 17). The *Ec1* interacted in a compatible manner with the V91 transgenic plants. On the other hand, four event of V91 (V91-2, 91-3.V91-4, V91-6) was observed resistance to the *Katshaar* and two event of V91 (V91-1 & V91-7) were susceptible to the *Katshaar*. Hence, it implied that *Rpi-vnt1* was active in most of the V91 transformed plants. However, positive control plant of A17-27 for *Avrchc1* and A13-13 for *Avrvnt1* showed resistance response to *Ec1* and *Katshaar*, it showed the evidence that both genes were not mutated by their corresponding isolates (Table 16 and 17).

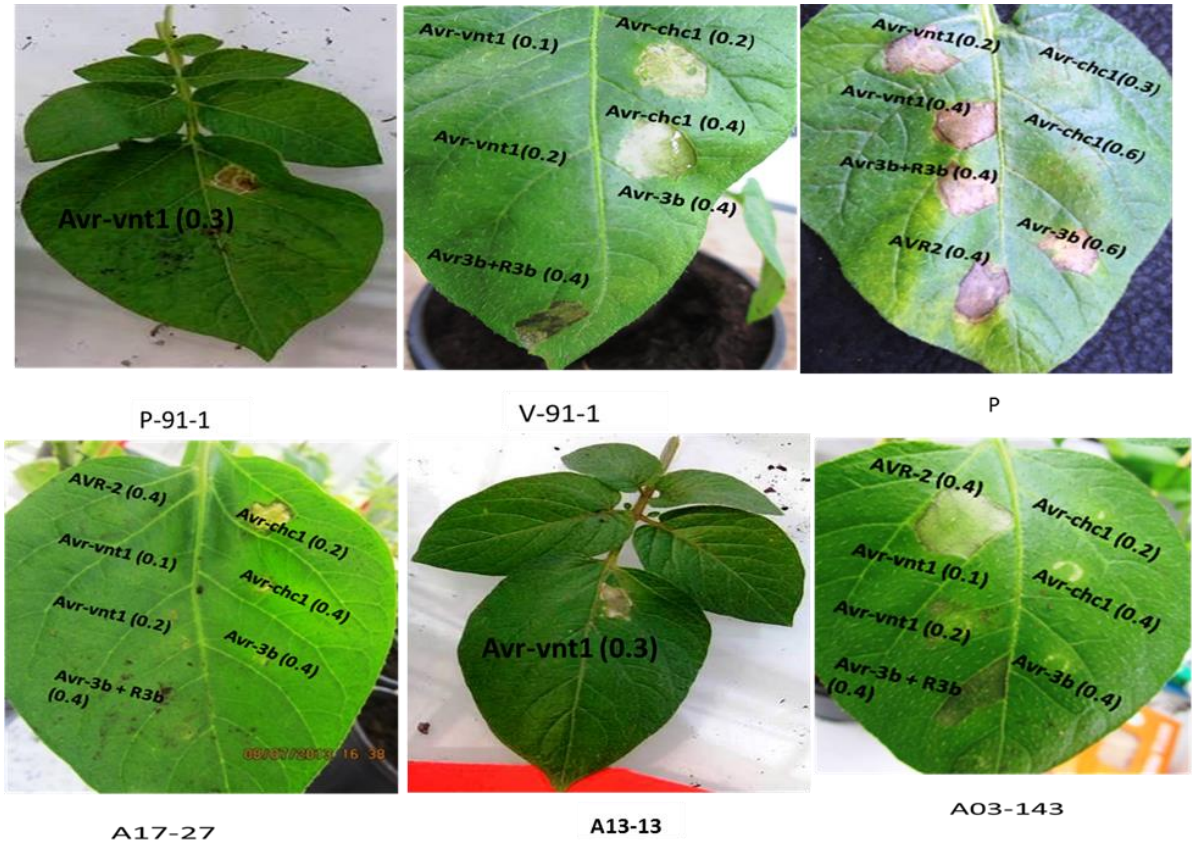

Figure 10. The response of *Rpi-vnt1* and *Rpi-chc1* to *Avrvnt1* and *Avrchc1* in P91 and V91 is shown. Co-infiltration of *Rpi-R3b* with *Avr3b* and *AVR2* (P plant) served as a positive control. The positive control plant A17-27 for *Avrchc1*, A13-13 for *Avrvnt1* and A03-143 for *AVR2* were used to confirm the stability of the constructs. Infiltration of *Avr3b* served as a negative control. Each construct two type of OD was used, *Avrvnt1* (OD=0.3), *Avrchc1* (OD=0.2 and 0.4) for V91 and for P plant *Avrvnt1* (OD=0.3), *Avrchc1* (OD=0.3 and 0.6). The positive construct *R3b*+*Avr3b*=0.4 and *AVR2*=0.4 and *Avr3b*=0.4, OD was used.

In agro-infiltration assay, all V91 events showed HR to *Avrchc1* except V91-6, since aberrant phenotypic growth of this plant was found (Table 17). The positive control plant A17-27 has shown strong resistance response (Figure 10). Similarly, the construct Avrvnt1 was shown matching R gene response in all V91 events including positive control A13-13 (Figure 10 and Table 17).

Furthermore, the isolate *IPO-C* (having virulent *avr2*, *Avrvnt1* and *Avrchc1*) was used to inoculate P91 transformants for further confirmation of the functionality of the *Rpi- vnt1*:*Rpi-chc1*.Three leaves per transformant, and four spot at each leaf were inoculated in a DLA assay. Out of ten, three transformants P91-2, P91-3 and P91-10, were shown to be moderately susceptible to this isolate (Figure 12). Seven other transformants were shown extreme resistance. The non-transformed P plants were fully susceptible to this isolates. In previous research, this P plants were shown resistance to *Ec1* and *Katshaar* and it was suspected that the resistance is due to the presence *AVR2* in *Ec1* and in *Katshaar* which was recognized by P plant having *Rpi-abpt1*. However, in this study both DLA and agro-infiltration, the transformed plants P91 were shown resistance to *IPO-C*, but the control plants P were susceptible. Therefore, it can be concluded that the resistance in P91 to the *Katshaar* and *Ec-1* was not due to the presence of *AVR2* gene. It was due to the presence of either *Rpi-chc1* or *Rpi-vnt1* or both.

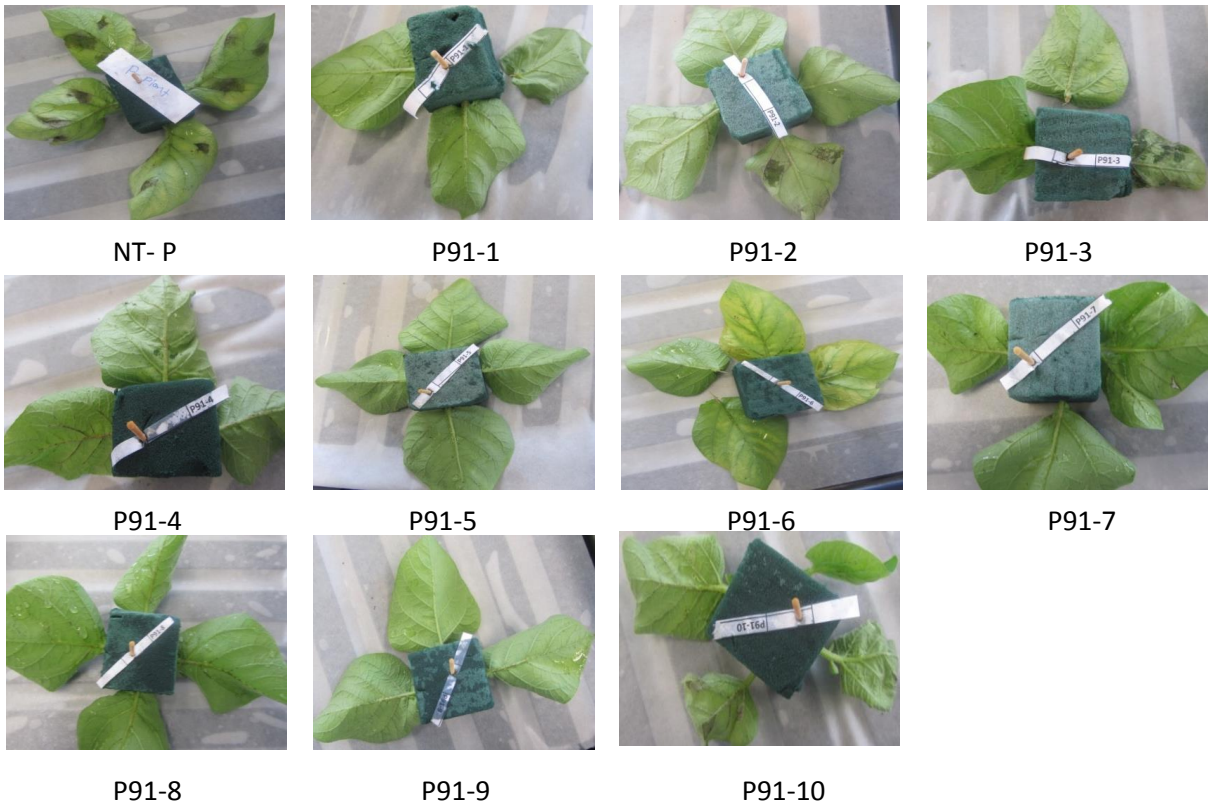

Figure 11. Detached leaf assay in P91 transformants by using IPO-C isolates in which six 10 µl droplets of a zoospore suspension (50,000 spores/ml).

DISCUSSION

Factors influencing *Agrobacterium* mediated transformation by increasing regeneration capacity of potato transformants

To study the effect of pre-culture media with phytohormone, the explants (stem: inter-nodal) were pre-cultured on R3B for two days and R3B in combination with IAA and t-Zeatin for 4 days before transformation both in kanamycin and marker free condition. In marker free situation, these two pre-cultured media grown explants did not show any difference. The conspicuous differences were observed among the pre-cultured explants of these two media in kanamycin selection. Probably, the phyto-hormone pre-treatment combination with several physical factors which influence *Agrobacterium* mediated transformation, assist to recover transgenic shoot from the effect of kanamycin. The explants on R3B combination with hormone treatment IAA and t-Zeatin for 4 days showed increase in regeneration response in comparison with the explant in R3B pre-cultured at 2 days [Figure 2(a)], that has also been found in previous report of potato (Chakravarty and Wang-Pruski 2010) and citrus species (Domínguez et al. 2004). The highest callusing and regeneration response was noticed after 4 days pre-treatment on R3B combination with IAA, and t-Zeatin pre-cultured explants under kanamycin selection. Moreover, they were also found to be less sensitive to overgrowth of *Agrobacterium*. It may be the reason of lower 0.2 OD for R3B+IAAA+Zeatin, as compared to higher 0.4 to 0.8 OD in the R3B. Phytohormones pre-treatment of the explants before co-cultivation is important for transformation as it activate cell division, involves formation of new and thin cell walls probably influences specific attachment capacity to *Agrobacterium (Chen et al. 2007)*.Another factor was using the phenolic compound acetosyringone in the inoculation media as a virulence stimulating agent, triggering in an efficient T-DNA delivery and transformation rate (Li et al. 2003). Moreover, bacterial concentration for inoculation and period of co-cultivation also greatly influenced the transformation efficiency as shown in the figure 2 Appendix-1. As expected, increased inoculation and co-cultivation time usually yields more efficient T-DNA delivery but often incurs higher cell-damage and necrosis sign and leads to death of tissues. Therefore, 2 days of co-cultivation and diluting the bacterial suspension to OD600 = 0.2 profoundly increased the transformation rate which has also been shown in the previous findings (Chakravarty and Wang-Pruski 2010), (Chen et al. 2007), (Zaragoza et al. 2004). It was also found that diluting bacterial concentration also reduced the number of explants with overgrowth of bacteria. In this study, 4 week old explants were pre-cultured on R3B media and 5 weeks old explants were pre-culture on R3B+IAA+Zeatin media. The age of explants is an important factors that influencing the T-DNA delivery into plant cell. However, in this comparative study, age factor was not critically analyzed because there was only one week difference between these two pre-cultured media grown explants. Besides, previous findings mentioned, 5 week old explants showed optimum results as compared to the explants from the older explants bearing more hard tissues that decreased regeneration response and reduced the transformation efficiency (Chakravarty and Wang-Pruski 2010). The results suggested that physiological state of the starting material is also crucial to ensure successful transformation.

The combination of IAA and t- Zeatin in first regeneration media with kanamycin selection, influence higher number of shoot regeneration. Subsequently, transferring of the regenerating explants to shooting media without auxin could promote further elongation and growth of shoots. By contrast, without IAA and t-Zeatin in kanamycin selection medium contain few number of shoots (after 6

weeks of transformation). Although numerous shoots were produced from each explant, only one shoot was cut and transferred into container in order to avoid duplicate transgenic for further analysis. Four different cultivars were considered to investigate their regeneration performance after pre-treatment with those media. However, cultivar Bintje was found the most responsive genotype in kanamycin selection that supported the result of previous research (Chakravarty and Wang-Pruski 2010). It was also observed that cultivar Desiree performed better regeneration response having minimum callus phase compared to Atlantic, and Russet Burbank.

Usefulness of *gfp* reporter gene for selection in marker free transformants

gfp and the selectable marker gene *npt*II, were used to select the transformed shoot both visually and at DNA level, both in kanamycin and marker free situation. It was noticed that *gfp* based selection at explant stage was not reliable, as the callus part of explants both transformed and non-transformed showed autofluorescence, therefore distinguishing *gfp* expression from autofluroscence is difficult at this stage (Figure 5). The reason of autofluorescence at callus part could be explained by stress due to damaging of cells during cutting of the explants. By contrast, *gfp* selection at shoot stage was reliable because shoots were not displaying autoflurosecence. The transgenic shoots displayed clear green fluorescence compared to the non-transgenic that appeared as black under fluorescence microscope. In this experiment, *gfp* expression on kanamycin selection showed less patchy fluorescence suggesting that tissue specific expression and/or chimeric shoots were less frequently obtained compared to the marker free selection where most of the shoots appeared as chimeric. The higher level of *gfp* expression and entire transformed plant was found in kanamycin resistant shoot compared to the shoot regenerated from marker free condition that often show patchy or chimeric expression of *gfp*. Variation in *gfp* expression during development could be interpreted by integration site-specific gene activation. A similar expression of *gfp* in tobacco also noticed by (Bastar et al. 2004). Alternatively, the frequency of finding chimeric shoots could be higher in marker free transformation due to the absence of selection against non - transgenic tissue in the same plant that contains the transgenic tissue.

Current research showed in marker free situation only 4 percent shoots were *gfp* positive visually in microscope (Table 12), whereas in PCR analysis found 28 percent shoots were *gfp* positive and 7 % shoots *nptII* positive. Notably, 24% plants were found to be PCR positive but no fluorescence was observed under microscope (Table 12), indicated large percentage (86%) of gene was not expressed which intended to the previous assumption as in potato(Rakosy-Tican et al. 2007). While in PCR analysis only 7% of the shoots found to be integrated with *gfp* and *nptII* gene . The absence of *nptII* gene in *gfp* integrated shoot could be explained that presence of *gfp* gene near the right border of the plasmid [Figure 1(a)] easily transfer delivered to the plant cells.

By contrast, in kanamycin selection 37% shoots were noticed *gfp* positive in microscope, where as 47% shoots found that had integrated *gfp* gene and 30% shoots for *nptII* positive. We have noticed a large percentage of regenerated shoots approximately 53% were not expressing *gfp* visually even not integrated *gfp* and *nptII* gene under kanamycin selection in all potato genotypes tested (Table 12), these are possibly escapes from kanamycin selection. In routine kanamycin transformation, these escapes will be lost in rooting medium containing kanamycin. It was noticed that *gfp* is very efficient to distinguish escapes and chimeras both in kanamycin and marker free condition.

In marker free situation, 75% shoots were found as non-transformed after PCR analysis. Such a high percentage of these shoots may be possible because auxin and zeatin pretreatment (0.1 mg L−1IAA and 0.1 mg L−1 zeatin for 4 days), as an initial step of the protocol (Kumar 1995), could act as a signal stimulating the regeneration.

Effect of larger insert in transformation process

Three different length of T-DNA such as 3kb, 13kb, and 24kb were used to investigate the effect of larger insert during transformation process. There was no difference found between 3 kb and 13 kb T-DNA length containing construct although the number of shoots was much lower with the13 kb TDNA. The larger insert which contain 24 kb T-DNA length was included in this experiment but the number of PCR tested shoots was too low to draw any conclusion about transformation efficiency. Due to lack of *gfp* and *nptII* gene in this plasmid, shoots were not subjected to test for *gfp* and kanamycin selection.

Testing resistant genes activity in marker free transformants

This study was conducted to test the activity of *Rpi-vnt1* and *Rpi-chc1* in V91 and P91 transformants through detached leaf and agro-infiltration assay. Detached leaf assays can reveal the effectiveness of the stack of resistance genes to selected isolates. While agro-infiltration with specific *Avr* genes can allow to verify the functionality of individual *Rpi* genes.

In this study, ten P91 and seven V91 events were tested. In detached leaf assay all P91 events were shown resistant to *EC1*, *Katshaar* and *IPO-C*, while non-transformed P plant (having R2 gene) were also found resistance to Ec1 and Katshaar while susceptible to *IPO-C* (Table 16). This result was corroborated the findings of previous research, where non-transformed P plant showed resistance to the isolates *EC1* and *Katshaar* (Vossen J., personal communication).Therefore, It was suspected that resistance manifestation on P background plant might be due the presence of R2 gene, which could recognized the *AVR2* gene containing by the isolates *EC1* and *Katshaar*. Therefore, for further confirmation present study was included the third isolates *IPO-C* (containing avr2 gene), expected that if resistance occurred due to the interaction of *R2-AVR2* then P background plant may have shown susceptibility. However, we found that resistance in P background plant was not due to the interaction of *R2-AVR2*. In DLA seven P91 events and non-transformed P was tested and found resistanse to these isolates (*Ec1, Katshaar* and *IPO-C*) (Table 16). While 10 events of P91 and P plants were tested by *IPO-C* inoculation. It was also noticed; all the P91 events showed extreme resistance (three events one out of three leaves has shown susceptibility) (Table 16, Figure 12). Interestingly, non -transformed P plant was found highly susceptible to *IPO-C* (Figure 12).Then we confirmed resistance had occurred due to the presence of *Rpi-vnt1* or *Rpi-chc1* or both.

Subsequently, we did agro-infiltration to unveil which *Rpi* genes (either Rpi-*vnt* and *Rpi-chc1* or both) are active in P91 and V91 transformants. It was found that all P91 transformants were responded to *Avrvnt1* but not the construct *Avrchc1* (Table 17). However, the agro infiltration response for *Avrchc1* was not correspond to these DLA, where P91 transformant were found resistant to *Ec1* (containing *Avrchc1*). It could be the *Rpi-chc1* gene is present but not expressed or copy number is too low that's not able to produce HR response immediately.

On the other hand, V91 transformant were found susceptible to both isolates *Ec1* and *Katshaar* in detached leaf assay, except V91-2 and V91-6 that showed resistance to *Katshaar* (Table 12). The agro-infiltration assay was repeated three times. The first and second set of V91 transformants were responded to *Avrchc1*, however third set of plant were not responded except V91-1 (Table-16). On the flip side, all the events of V91 were showed resistance response during agro-infiltration with *Avrvnt1* and *Avrchc1* indicated both *Rpi-vnt1* and *Rpi-vnt1* were active on V91 transgenic plant.

Discussing the entire event in DLA and Agro-infiltration, it was confirmed that *Rpi-vnt1* was active in both P91 and V91 transformants. In this study *Rpi-chc1* is not active in P91 transformants, it might be the expression of *Rpi-vnt1* suppress the expression of *Rpi-chc1* in a double construct which was also mentioned previous thesis of Jeron Stellingwerf using Desiree transformants. Although in agro-infiltration, most of the events of V91 showed resistance responses to *Avrchc1*, but in DLA, they showed susceptibility to *Ec1*. Variation of result in this two s experiment has suggested for further study about the functionality of *Avrchc1*.

CONCLUSION AND RECOMMENDATION

According to the first research question, phytohormones in pre-culture media combined with some pre-scheduled physical factors (age of explants, days of pre-culture, co-cultivation period, bacterial concentration, adding acetosyringone in inoculation media) increased the regeneration efficiency of the transformants. Among the four studied cultivars, Bintje showed the highest and regeneration efficiency about 83% in R3B+IAA+Zeatin pre-cultured media with marker assisted (kanamycin) selection and followed by Desiree about 28% regeneration efficiency in the same condition. Interestingly, Bintje & Desiree cultivars showed about 19% and 28% regeneration efficiency in R3B+IAA+Zeatin pre-cultured media with marker free condition. While in R3B pre-cultured condition they showed 26% and 37% regeneration efficiency. Notably, Atlantic and Russet Burbank showed very poor regeneration efficiency in both pre-cultured media with kanamycin and marker free condition. This research suggested it should not be applicable to practice this protocol in marker free condition. However, only Bintje with kanamycin selection protocol can be recommended.

The second research question was if visual marker *gfp* can be used as selection marker instead of antiobiotic? In histological study, well above 4% regenerants were observed *gfp* positive under microscope whereas, 28% regenerants were found *gfp* positive by molecular approach (PCR analysis). It indicated a higher percentage (around 86%) of shoots not expressed in marker free condition. Therefore, selection based on *gfp* expression is not reliable because higher percentage of transgenic shoots will be discarded because of non -expression. It could be possible that the gene is expressed in later developmental stages. However, *gfp* can be used for in vivo discrimination of escapes and chimera because only PCR analyses could not distinguished chimeric part, it is very essential to identify such kind of transformed tissue for further molecular investigation. In marker free transformation, both *gfp* and PCR can be used for selection of transgenic shoot. It is also recommended to test large number of transformed shoot in PCR for further validation of this study. In this available time it was not possible to check all the transformants by PCR analysis. In kanamycin selection, 47% shoots were found *gfp* positive in PCR and 37% shoots were observed *gfp* positive in microscopic analysis that indicated 78% shoots positive in PCR also visually observed in microscope.

Three different length of T-DNA such as 3kb, 13kb, and 24kb were used to investigate the effect of larger insert during transformation process. There was no difference found between 3 kb and 13 kb T-DNA length containing construct although the number of shoots was much lower with the13 kb TDNA. The larger insert which contain 24 kb T-DNA length was included in this experiment but the number of PCR tested shoots was too low to draw any conclusion about transformation efficiency. Due to lack of *gfp* and *nptII* gene in this plasmid, shoots were not subjected to test for *gfp* and kanamycin selection.

The fourth research question is testing *Rpi-vnt1* and *Rpi-chc1* gene activity in P91 and V91 transgenic plant. It was found *Rpi-vnt1* was functional in both kinds of transgenic plant by DLA and agro-infiltration. On the other hand, *Rpi-chc1* was showed functionally active in V91 transformant but not in P91 transformants through agro-infiltration .While in DLA, P91 showed resistant to the both isolates *Ec1* and *Katshaar*. In another stance, V91 transgenic was showed susceptibility to the *Ec1, suggested* to re-test the functionality of *Rpi-chc1* on V91.

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Appendix-I

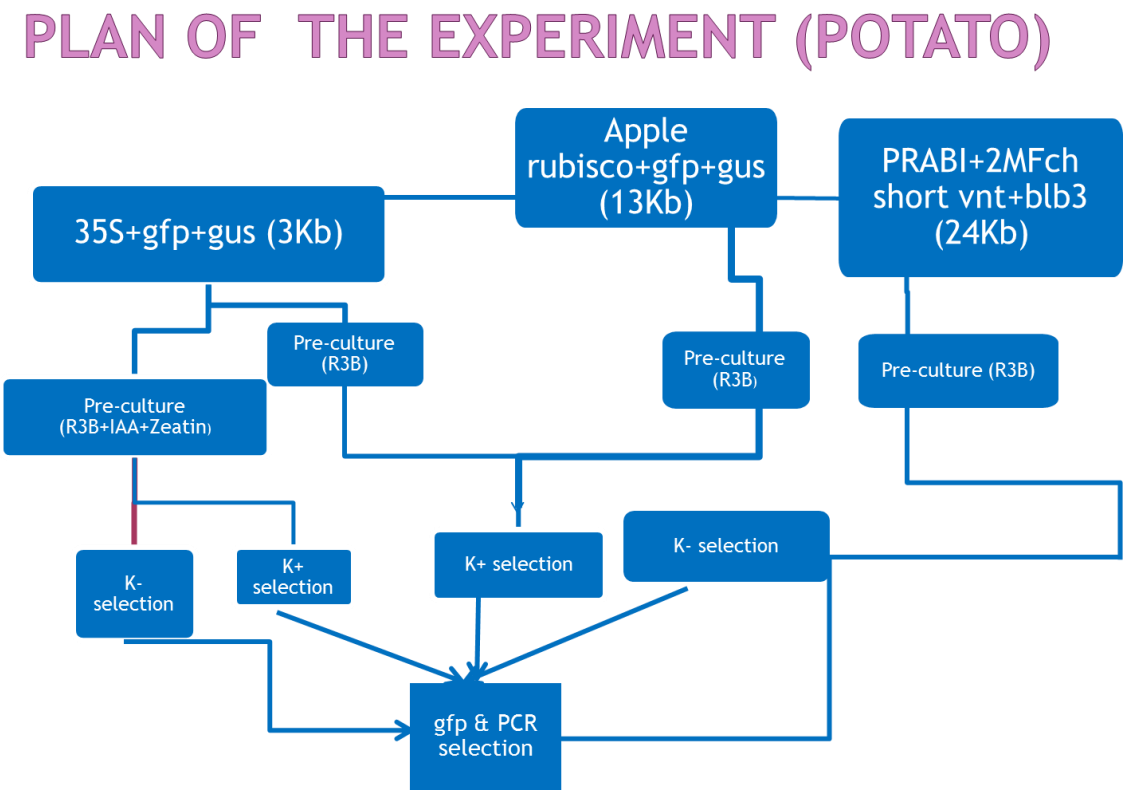

Figure 1. Overall planning of the Experiment

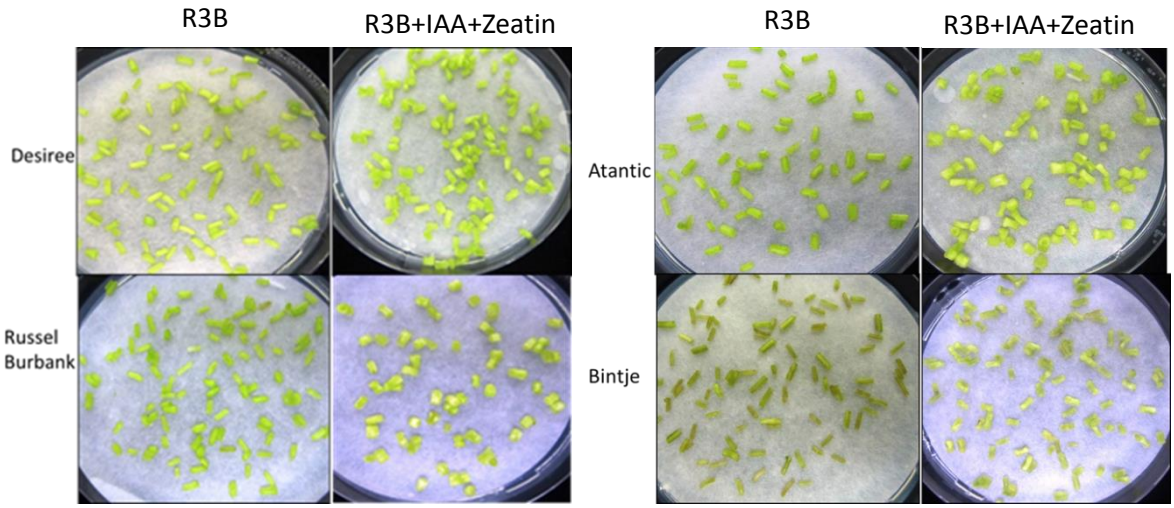

Figure 2. Picture represent the physical condition of the explants of Desiree, Russel Burbank, Atlantic and Bintje after pre-cultured in R3B and R3B+IAA+Zeatin media for two and four days respectively, before transformation.

Protocol for DNA isolation

Buffer stock solutions:

Extraction buffer stock
0.35 Sorbitol
0.1 M Tris-HCl pH 8.0
5 mM EDTA pH 8.0

Lysis buffer stock
0.2 M Tris-HCl pH 8.0
0.05 M EDTA pH 8.0
2M NaCl
2% CTAB

Method

- Harvest leaf material in a deep-well block containing two tungsten carbide beads(3 mm, Qiagen) in every well. Keep on ice during harvesting.
- Freeze in liquid Nitrogen, fit the deep-well block into the shake adapters and shake for 1 minute at 20 cycle/s in the RETSCH machine
- Put the deep well block back in liquid nitrogen immediately
- Store at -80^0 C until DNA isolation
- Take the block from the -80^{0}C an add 400 µl isolation buffer, mix manually
- Put at 65^0 C (minimum 1 hour), mix occasionally
- Cool down to room temperature
- Add 400 µl chloroform/isoamylalcohol (24:1) and mix vigorously
- Centrifuge for 20 minutes at 6000 rpm
- Remove the supernatant into a new deep-well block (2x 175 µl) and add 0.8 volume of isopropanol mix gently
- Centrifuge for 8 minutes at 6000 rpm
- Pour off the supernatant and centrifuge briefly
- Remove remaining ethanol by pipetting
- Dry pellet at room temperature

Dissolve in TE + RNase (10 µg/ml

Table1. Primer sequence and concentration used for screening of regenerants

Amplified gene	Annealing temperature (^{o}C)	Primer sequence
nptII	56	LK160:5'CTCCTGTCATCTCACCTTGC3' Lk161:5'TAATCATCGCAAGACCGGC3'
gfp	60	GFP F:5'GCACGACTTCTTCAAGCTCGCCATGCC3' GFP R:5'GCGGATCTTGAAGTTCACCTTGATGCC3'
chc1	55	LK57a:5'-ATTTGGGACATTCTGATATA3' LK65-:5'ACAGATAATAATTTTCAACAG3'
virG	55	LK37:5'CAATAGTAGCTGTAACCTCG3' LK38:5'ACCTGCCGTAAGTTTCACAC3'

Table 2. PCR reaction mixture (total volume 15 µl) used to screening regenerants

Components	Volume (µl)
10x PCR reaction buffer	1.5
DNA (120ng)	1.0
Primer F (10 µM)	0.6 (stock concentration of primer (10 pmol/ µl)
Primer R (10 µM)	0.6 (stock concentration of primer (10 pmol/ µl))
dNTPs (5mM)	0.8
Dream Taq (5U/µl)	0.03
MilliQ H_2O	Add to 15

Table 3. PCR program for screening of regenerants

Step	Cycles	Temperature(^oC)	Time
1	1	94	4 min
2	35	94	30 sec
		55 (*chc1,VirG*) 56 (*nptII*) 60 (*gfp*)	30 sec
		72	1 min
		72	10 min

Appendix 2

Table 1. Testing *Avr3b* and *Avr3b*+R3b constructs in V91 transformants by agro-infiltration

Genotype	1st set	2nd set	3rd set	1st set	2nd set	3rd set
	Avr3b	***Avr3b***	***Avr3b***	***Avr3b+R3b***	***Avr3b+R3b***	***Avr3b+R3b-***
V91-1	**0**	**0**	**0**	**1.4**	**0**	**1.4**
V91-2	**0**	**0**	**0**	**1**	**0.45**	**0.6**
V91-3	**0**	**0**	**0**	**2**	**1.4**	**0.6**
V91-4	**0**	**0**	**0**	**1.3**	**1.5**	**1.1**
V91-5	**0**	**0**	**0**	**2**	**1.5**	**0.4**
V91-6	**0**	**0**	**0**	**0**	**0**	**0**
V91-7	**0**	**0**	**0**	**1**	**0.8**	**0.37**

The positive construct *R3b*+*Avr3b*=0.4 and *AVR2*=0.4 and Avr3b=0.4, OD is used.

Table 2. Testing *Avr3b* and *Avr3b+R3b* constructs in P91 transformants by agro-infiltration

Genotype	1st set	2nd set	3rd set	1st set	2nd set	3rd set
	Avr3b	*Avr3b*	*Avr3b*	*Avr3b+R3b*	*Avr3b+R3b*	*Avr3b+R3b*
P91-1	0	0	0	0	0.5	0.6
P91-2	0	0	0	0	0.5	1
P91-3	0	0	0	0	1	1
P91-4	0	0	0	2	0.5	1
P91-5	0	0	0	0	1	1
P91-6	0	0	0	0	0.3	1
P91-7	0	0	0	0.5	0	1
P91-8	0	0	0	0	0.1	0.5
P91-9	0	0	0	0	0.3	0.5
P91-10	0	0	0	0	1	2
P	0	0	0	0	0.1	0.5
A13-13	0	0	0	0	0.1	0
A17-27	0	0	0	0	2	0
A03-143	0	0	0	0	1	0.5

Table 3. Testing of *Avrchc1 and Avrvnt1* constructs in V91 transformants by agro-infiltration

	1st set		2nd set		3rd set		1st set		2nd set		3rd set		4th set
Genotype	***Avrchc1* (low)**	***Avrchc1* (high)**	***Avrchc1* (low)**	***Avrchc1* (high)**	***Avrchc1* (low)**	***Avrchc1* (high)**	***Avrvnt1* (low)**	***Avrvnt1* (high)**	***Avrvnt1* (low)**	***Avrvnt1* (high)**	***Avrvnt1* (low)**	***Avrvnt1* (high)**	***Avrvnt1* (high)**
V91-1	2	2	0	0	0.69	1.5	0	0	0	0	0	0	1
V91-2	0.92	0.92	1	1.5	0	0	0	0	0	0	0	0	1
V91-3	1.4	1.4	1	1.6	0	0	0	0	0	0	0	0	0.5
V91-4	1.3	1.6	0	0	0	0	0	0	0	0	0	0	1
V91-5	1.1	1.1	0.32	0.7	0	0	0	0	0	0	0	0	0.5
V91-6	0	0	0	0	0	0	0	0	0	0	0	0	0
V91-7	1.4	1.4	0.7	0.7	0	0	0	0	0	0	0	0	1
A13-13	0	0	0	0	0	0	0	0	0	0	0	0	2
A17-27	1.5	1.5	0.5	1.5	0.1	0.1	0	0	0	0	0	0	x
A03-143	0	0	0	0	0	0	0	0	0	0	0	0	x

Response intensity was scored on a 0-2 scale, 2 days after infiltration. Three plant replicates and four leaves per plant were used for infiltrations. Each construct two type of OD is used, *Avrvnt1* (OD=0.1 and 0.2), *Avrchc1* (OD=0.2 and 0.4).

Table 4. Testing of *Avrchc1* and *Avrvnt1* constructs in P91 transformants by agro-infiltration

Genotype	1st set		2nd set		3rd set		1st set	2nd set	3rd set	1st set		2nd set		3rd set		4th set
	Avrchc1 (low)	*Avrchc1* (high)	*Avrchc1* (low)	*Avrchc1* (high)	*Avrchc1* (low)	*Avrchc1* (high)	*AVR2*	*AVR2*	AVR2	*Avrvnt1* (low)	*Avrvnt1* (high)	*Avrvnt1* (low)	*Avrvnt1* (high)	*Avrvnt1* (low)	*Avrvnt1* (high)	*Avrvnt1* (high)
P91-1	0	0	0	0	0	0	0	1	1	0	0	1.6	1.4	1	1	0.5
P91-2	0	0	0	0	0	0	0	1	1	0	0	1.2	1.4	1	1	0.5
P91-3	0	0	0	0	0	0	0	2	1	0	0	1.5	2	2	2	5
P91-4	0.5	0.5	0	0	0	0	2	1	2	0	0	0.78	0.85	2	2	0
P91-5	0	0	0	0	0	0	0	2	1.5	0	0	2	2	2	2	0.5
P91-6	0	0	0	0	0	0	0	0.1	1	0	0	1.4	1.4	2	2	0.5
P91-7	0	0	0	0	0	0	1.3	0.1	1	0	0	1	1	1	1	0.5
P91-8	0.8	0.8	0	0	0	0	0	0.5	0.5	0	0	1.6	1.6	1	1	0.5
P91-9	0	0	0	0	0	0	0	1	1	0	0	1.3	1.3	1	1	0.5
P91-10	0	0	0	0	0	0	0	1	2	0	0	2	2	2	2	x
P	0	0	0	0	0	0	0	0.5	0.5	0	0	2	2	2	2	0
A03-143	0	0	0	0	0	0	0	2	2	0.5	0.5	2	2	0.1	0.1	x

Each construct two type of OD is used, *Avrvnt1* (OD=0.2 and 0.4), *Avrchc1* (OD=0.3 and 0.6).

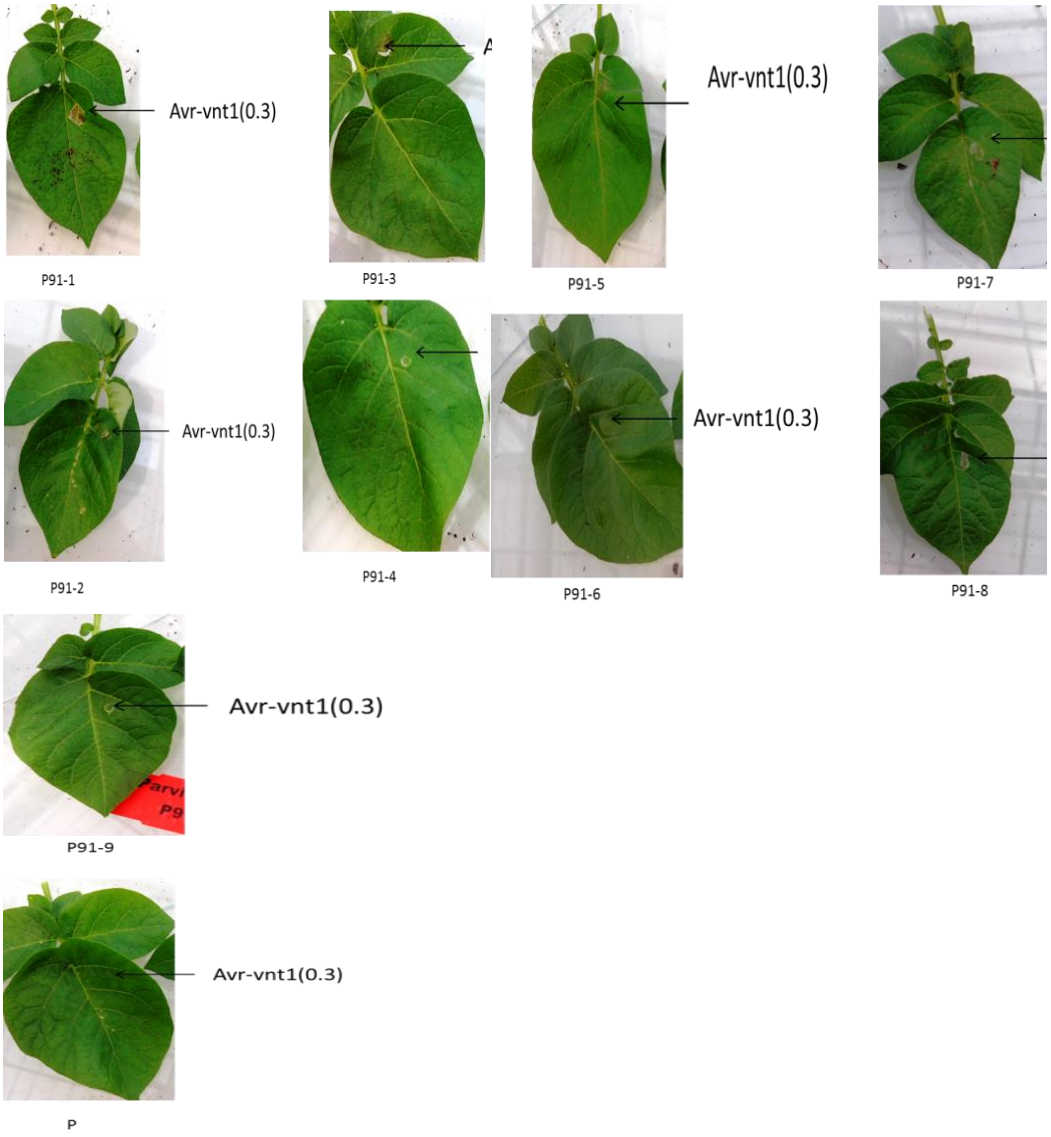

Figure 3. The response of *Avrvnt1* on P91 and P transformants were shown. The used OD for *Avrvnt1* is 0.3.

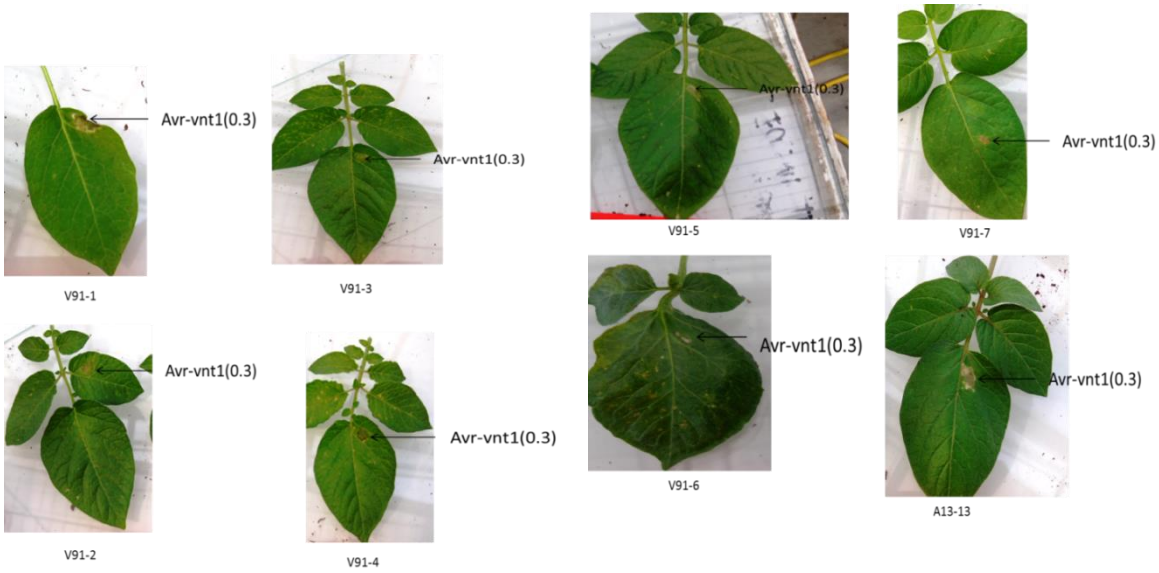

Figure 4.The response of *Avrvnt1* on V91 and P transformants were shown. The used OD for *Avrvnt1* is 0.3.

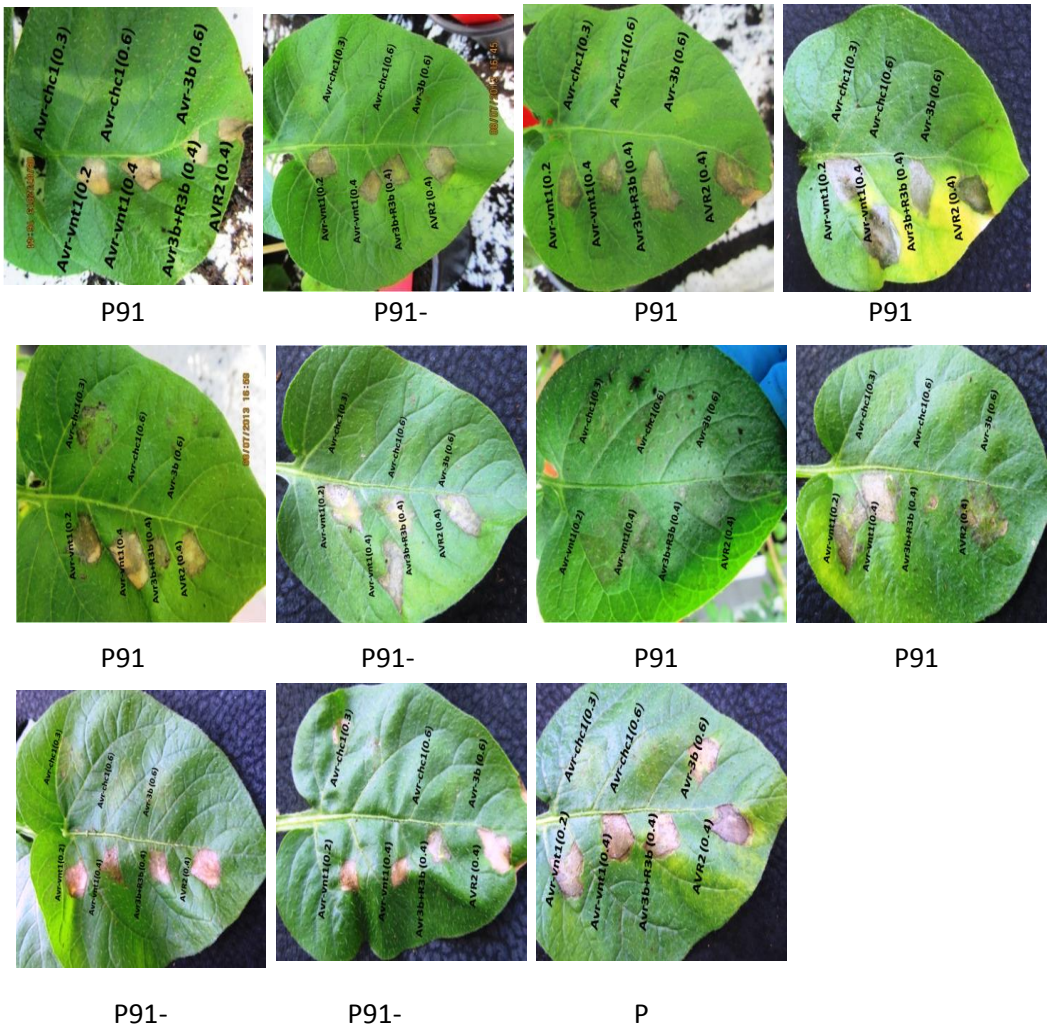

Figure 5. The response of *Rpi-vnt1* and *Rpi-chc1* to Figure 8. The response of *Rpi-vnt1* and *Rpi-chc1* to *Avrvnt1* and *Avrchc1* in P91 transformantsand P leaves is shown. Co-infiltration of *Rpi-3b* with *Avr-3b* and *AVR2* served as positive control. Infiltration of *Avr3b* served as a negative control. Each construct two type of OD is used, *Avrvnt1* (OD=0.2 and 0.4), *Avrchc1* (OD=0.3 and 0.6). The positive construct *R3b*+*Avr3b*=0.4 and *AVR2*=0.4 and *Avr3b*=0.6,OD is used.

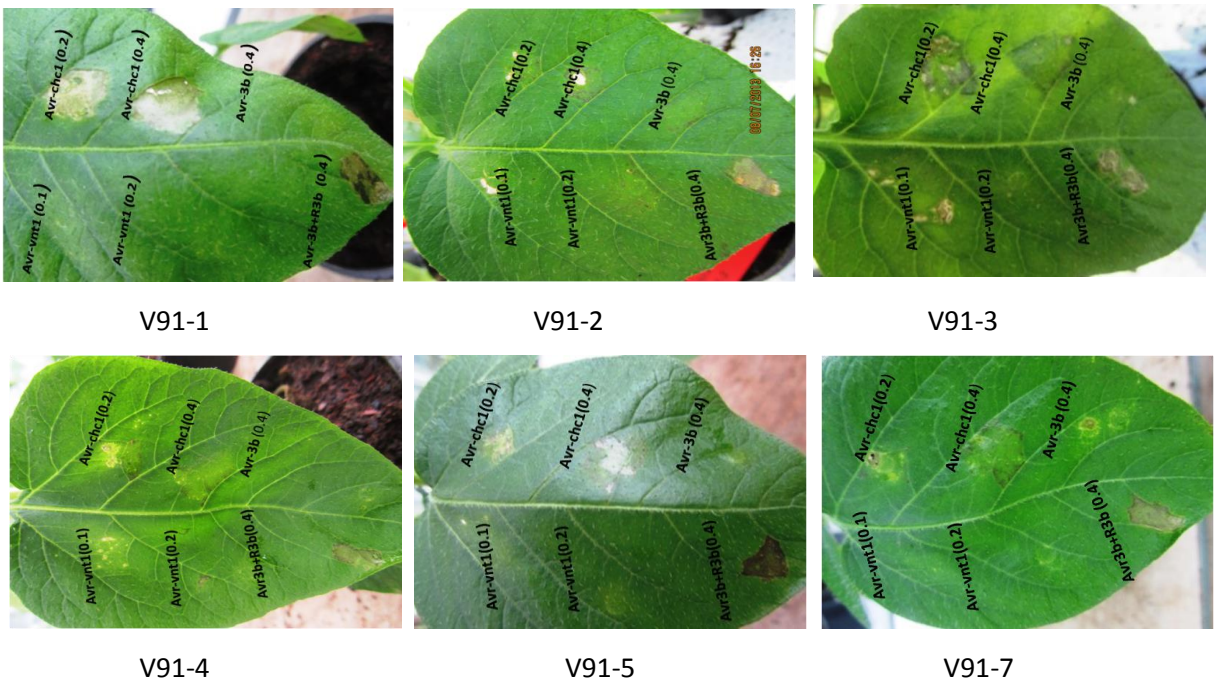

Figure 6. The response of *Rpi-vnt1* and *Rpi-chc1* to *Avrvnt1* and *Avrchc1* in V91 transformants leaves is shown. Co-infiltration of *Rpi-3b* with *Avr-3b* served as positive control. Infiltration of*Avr3b* served as a negative control. Each construct two type of OD is used, *Avrvnt1* (OD=0.1 and 0.2), *Avrchc1* (OD=0.3 and 0.6). The positive construct *R3b*+*Avr3b*=0.4 and *AVR2*=0.4 and *Avr3b*=0.4, OD is used.