IMPROVING GENE TRANSFER IN POTATO

MSc Thesis Report

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Abstract

Agrobacterium mediated plant genetic modification is an important and widely used indirect gene transfer method. However, *Agrobacterium* mediated gene transfer has not been exploited fully for all plant species or cultivars due to its low transformation efficiency. Several factors affect the success of *Agrobacterium* mediated gene transfer and improvement of these factors will enhance successful gene transfer. Mainly, improvement of tissue culture conditions increases the efficiency of *Agrobacterium*-mediated gene transfer. Here, I will report on the effects of light types and salicylic acid biosynthesis inhibitors (AIP and paclobutrazol) as well as on the regeneration capacity of two different potato genotypes in transformation. The effect of tissue culture conditions on transformation efficiency was different between potato genotypes. Two potato genotypes, Karnico and VIP099-19, were used to study the effect of light types. The two genotypes were grown both in white and blue LED lights and these plants were transformed with *Agrobacterium* strain AGL0(pCambia1301) carrying a binary Ti plasmid construct containing the GUS reporter gene. After transformation, the explants were distributed in both white TL and blue LED lights i.e., plants grown in white TL light were transferred either into white or into blue LED lights after transformation; and, the same is true for those plants grown in blue LED light. The transformation efficiency of these explants were evaluated at day-3 and day-14 in a way that at these days the explants were stained with GUS and evaluated for their GUS gene expression. Type of light had an effect on gene transfer efficiency. Its effect was genotype dependent. Karnico had better transformation efficiency at white light type whereas, VIP099-19 had better transformation efficiency at blue LED light. Comparison of genotypes indicated that Karnico had better overall transformation efficiency than VIP099-19 in many of the light types. Salicylic acid inhibitors also have significant effects on transformation efficiency. Three concentrations (0µM, 10µM and 50µM) of AIP and paclobutrazol were tested independently on potato genotype Desiree. Explants inoculated with *Agrobacterium* strain AGL0(pCambia1301) were cultured on MS medium containing the above indicated concentrations and evaluation of the explants was done at day-3 and day-14. AIP increases transformation efficiency at 10µM concentration. However, the effect of paclobutrazol was lower but there is an indication that 10µM of paclobutrazol enhanced transformation efficiency to some extent although not significantly. Regeneration capacity of transformed potato genotypes was also affected by genotype but not type of transformation vector. Two potato genotypes, VIP038-1 and VIP097-27 were transformed with two *Agrobacterium* constructs AGL0(pBin19-TT12) and AGL0(pBin19-TT19) and cultured in normal light and evaluated for their regeneration capacity after 60 days of culturing on selection medium. VIP038-1 had higher regeneration capacity than VIP097-27, irrespective of the construct used.

Acknowledgement

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1. Introduction

Plant genetic modification can be conducted in a direct and indirect ways. In the indirect DNA delivery methods, gene of interest is delivered to the plant cell via bacteria; for instance, *Agrobacterium tumefaciens or Agrobacterium rhizogenes* (Tzfira and Citovsky, 2006). The direct DNA delivery method does not use bacterial cells as a medium to transfer DNA to plant cells. Development of direct DNA delivery methods have been initiated due to the low success rate of *Agrobacterium* mediated DNA delivery method to monocots and recalcitrant plant species. There are several direct DNA delivery methods: microprojectile bombardment, electroporation-mediated transformation, polyethylene glycol (PEG)-mediated transformation and so on, which are used to transform different plant cells/ tissues (Breitler *et al*., 2002; Salmenkallio-Marttila *et al*., 1995; Davey *et al*., 2005). However, direct DNA delivery methods have several drawbacks such as integration of multiple copies of the desired transgene which can lead to silencing of gene of interest in the transformed plants, superfluous DNA sequences, limitation of some of the techniques (such as, electroporation-mediated transformation) to few plant species, lower transformation efficiency, and occurrence of fertility problems on regenerants (reviewed in Barampuram and Zhang, 2011). As compared to direct DNA delivery methods, *Agrobacterium* mediated DNA transfer method has several advantages. The ability of the *Agrobacterium* to deliver large sized DNA segment to the plant cell, low copy number of the transgene, the stable integration of the inserted gene to the plant cell and its consistent inheritance with continuous expression of the inserted gene in subsequent generations are some of the advantages of *Agrobacterium* mediated transformation method. And, the continuous success of improvement of *Agrobacterium* mediated gene transfer method through the improvement of plant tissue culturing methods made the *Agrobacterium*-mediated DNA delivery method more preferable than the direct DNA delivery methods (Barampuram and Zhang, 2011).

Agrobacterium tumefaciens has a large Ti (tumor-inducing) plasmid in its virulent strain which enable the *Agrobacterium* to induce tumor in plant cells. The Ti plasmid contains T-DNA (transferred DNA); and the T-DNA contains two types of genes: oncogenic genes and opine synthesis genes (figure 1). Oncogenic genes code for enzymes that are responsible for the synthesis of auxins and cytokinin, plant hormones involved in tumor induction, whereas the opine synthesis genes are involved in opine production which is used as a source of nitrogen and energy by *Agrobacterium*. The *virulence (vir)* genes, which found outside the T-DNA on the Ti plasmid and bacterial chromosome, are essential for the transfer of T-DNA to

the plant cell. The expression of *vir* genes is triggered by host derived phenolic compounds such as, acetosyringone, and sugar compounds which enhance the *vir* genes of the *Agrobacterium* to initiate processing of T-DNA from the Ti plasmid and subsequent transfer of the T-DNA from the *Agrobacterium* to the plant cell (Gelvin, 2000).

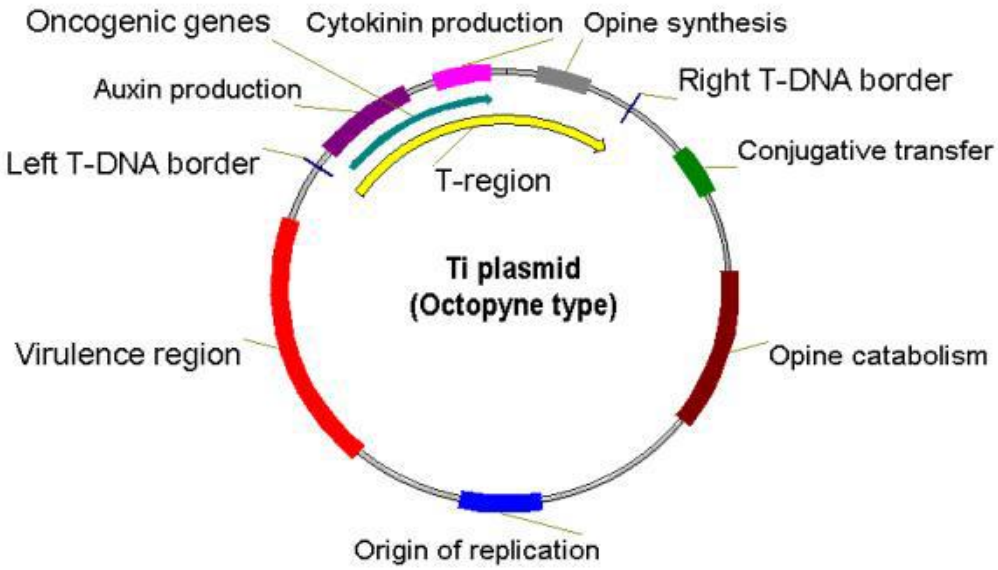

Figure 1. Tumer inducing (Ti) plasmid

Several steps are comprised in the *Agrobacterium* mediated transformation process: starting from recognition of plant phenolic compounds and attachment of the *Agrobacterium* to the plant cell to the integration of the T-DNA to the plant genome. In the T-DNA transfer processes, several *vir* genes are involved, which function either in the *Agrobacterium* (before T-DNA transferred to plant cell) or in the plant (after T-DNA transferred to the plant cell) (figure 2). Before the T-DNA is exported to the plant genome, the *vir* genes play roles in perceiving the plant phenolic compounds which, in turn, initiates T-DNA processing and transferring to plant genome. After transferring of the *Agrobacterium* T-DNA to plant cells, the bacterial *vir* genes interact with a number of plant genes in order to integrate the T-DNA into the plant genome (Gelvin, 2000; Tzfira and Citovsky, 2006). The T-DNA region is flanked by left border (LB) and right border (RB). Each of the left and right borders has a 25-bp direct repeat, which acts as a *cis* element signal for the T-DNA transfer. Any DNA between the two borders will be transferred into the plant cell (Zupan et al., 2000).

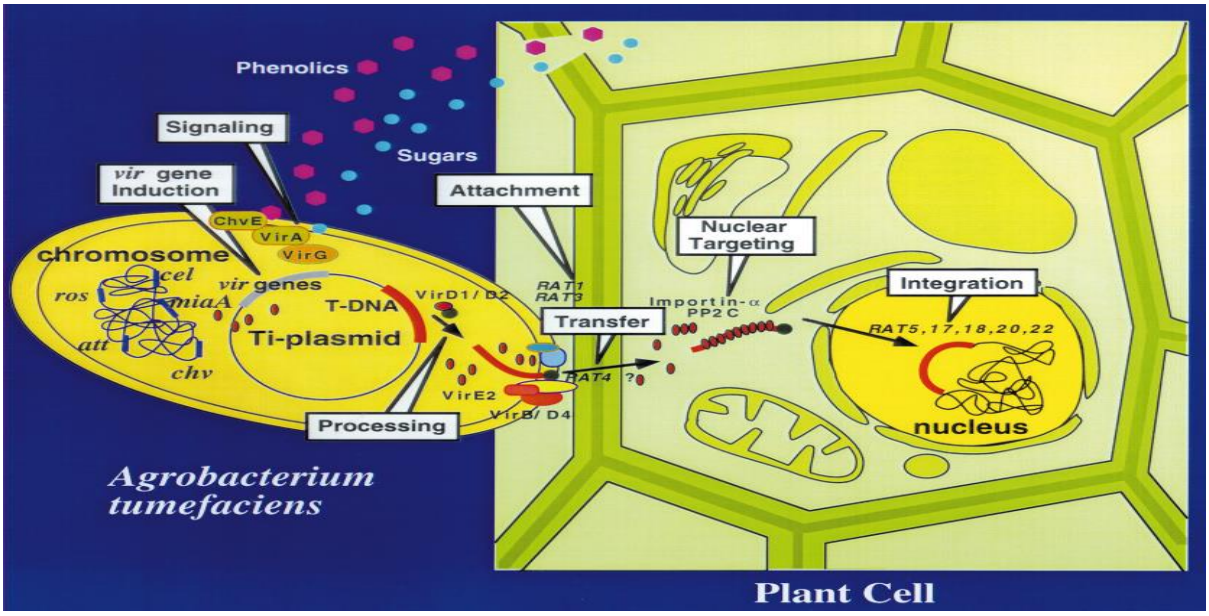

Figure 2. Schematic representation of *Agrobacterium* infection processes. The highlights indicate the critical steps that occur within *Agrobacterium* (phenolic signaling, *vir* genes induction, and T-DNA processing) and within the plant cell (attachment of the *Agrobacterium* to the plant cell, T-DNA transfer, nuclear targeting and T-DNA integration). Figure adapted from Gelvin, 2000.

There are essential requirements for successful gene transfer in plants. The ability to stably introduce the desired gene into the plant genome and the ability to regenerate plant from the transformed cell are the basic requirements (DeBondt *et al*., 1994). Efficiency of *Agrobacterium*-mediated transformation can be affected by a number of factors. Some of these factors are: *Agrobacterium* strain, construct, bacterial cell density, genotype of the plant, physiology of the plant and culturing conditions.

Transformation is a genotype dependent process so that different transformation procedures are needed for different genotypes. In a study conducted to evaluate the differences in transformation efficiency between different potato genotypes, it was observed that different genotypes showed different transformation efficiencies, even though, all genotypes were transformed with the same *Agrobacterium* strain and construct (Wenzler et al., 1989; Dale and Hampson, 1995; Heeres *et al*., 2002). This variation in transformation efficiency between genotypes could be resulted due to the physiological and other source of variations, such as type of explant, age of explant, contents of growing media and antibiotics used for selection (Dale and Hampson, 1995, Yepes and Aldwinckle, 1994).

The type and intensity of light is another factor which affects *Agrobacterium* mediated transformation efficiency of plants. For an efficient transfer of T-DNA into plant cells, exposure of explants to different light conditions during donor plant growing, cocultivation and/or cultivation of infected explants on selection medium is essential. For instance, the expression of *uidA* gene in transformed *Arabidopsis thaliana* and *Phaseolus acutifolius* was enhanced more when coculturing was done under continuous light than dark (Zambre, *et al*. 2003). The effect of light type on gene transfer efficiency was also observed on apple. Apple plants cultured under blue LED light showed a positive effect on both regeneration and transformation efficiency (Mariana, 2012). The difference in transformation efficiency of plants in relation to light conditions may be due to the fact that the different light conditions may affect the physiology of the plant differently, which, in turn, influence the competence of plants and *Agrobacterium* for T-DNA transfer. The physiological aspects within the plant which can be affected by light conditions include plant hormone production, cell proliferation and cell cycle stages (Seabrook, 2005; Lee *et al*., 2007). The effect of light on transformation efficiency of plants may also remain specific for *Agrobacterium* mediated transformation. The transient expression of the *CaMVp35S-uidA* gene, which was delivered to seedlings of *Picea* by particle bombardment, was not affected by light conditions. This elucidates the fact that the negative effect of light on the performance of plants to resist *Agrobacterium* attack contributes to the *Agrobacterium* to easily transfer its T-DNA (Ellis *et al*., 1991; Zambre, *et al*. 2003).

Plants can also suffer from both physiological and biochemical damage from exposure to different stresses such as pathogen attack, drought, air pollution and heat (Paliyath *et al*. 1997). The results of these injuries are reflected in most metabolic processes, which may reduce the growth capacity of the plant, resistance capacity of plants to pathogen attacks and other stresses. In response to pathogen attack, plants induce a systemic acquired resistance (SAR) against pathogens. Salicylic acid (SA) and systemic acquired resistance (SAR) -gene expression plays important roles in the induction and maintenance of SAR in plants (Lawton *et al*., 1995). Salicylic acid is required in signal transduction in systemic tissues to induce SAR (Vernooij *et al*., 1994). The involvement of salicylic acid in plants defense mechanism has been demonstrated in transgenic tobacco and *Arabidopsis* (Gaffney et al., 1993; Vernooij *et al*., 1994).

Salicylic acid, in plants, is produced through the shikimate pathway which leads to the primary metabolite chorismate; from this substrate two pathways can give salicylic acid: the isochorismate and the phenylalanine pathways. These pathways are activated by isochorismate synthase (ICS) and phenylalanine ammonia-lyase (PAL) enzymes (Figure 2). Usually, as a consequence of various biotic and abiotic stresses, an increase in phenylalanine ammonia-lyase (PAL) activity and accumulation of many phenolics are observed, which play a significant role for the plant to induce resistance (Solecka and Kacperska 2003; Sgarbi *et al*., 2003). Salicylic acid activated the accumulation of PAL in plants in response to stress (Wen *et al*., 2008). Salicylic acid (SA) belongs to a group of plant phenolics which regulate a large variety of physiological processes in plants. SA essentially plays significant roles in the expression of multiple modes of plant stress resistance (Clarke et al., 2004). However, inhibition of the SA could decrease the accumulation of phenolics and weaken the resistance of plants to stress, like pathogen attack (Janas *et al*., 2002; Solecka and Kacperska 2003). For instance, pre-treatment of Arabidopsis seedlings with 2-aminoindan-2-phosphonic acid (AIP), a highly specific PAL inhibitor, made the plants completely susceptible to *Peronospora parasitica* (Mauch-Mani and Slusarenko, 1996). It has also been reported by Xiang et al., 2011, that paclobutrazol, which is a plant hormone known to retard plant growth, also inhibits SA biosynthesis which is achieved by inhibiting benzoic acid 2-hydroxylase (BA2H). Benzoic acid 2-hydroxylase (BA2H) is an enzyme used to convert benzoic acid to salicylic acid. Although SA inhibition causes plant susceptibility to stress, it may also have significant positive implication on efficiency of plant transformation by *Agrobacterium*.

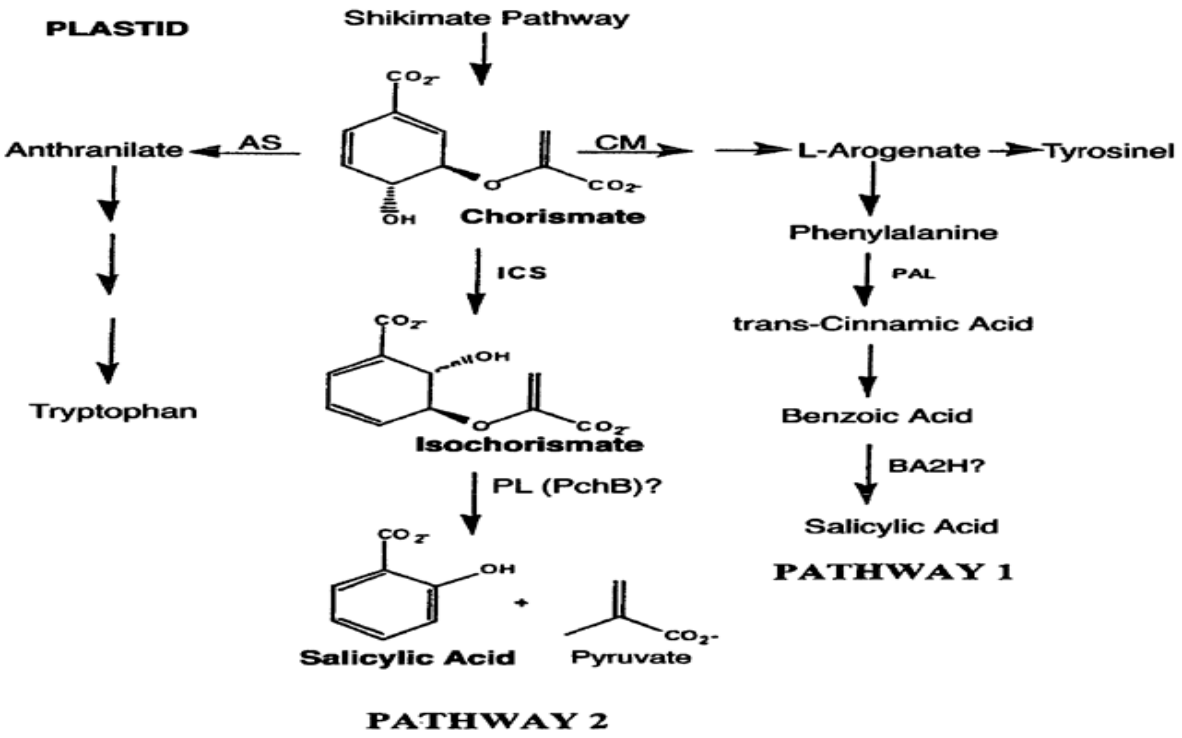

Figure 3. Salicylic acid biosynthetic pathways in plants. The enzymes involved in SA biosynthesis are indicated in abbreviations: anthranilate synthase (AS), chorismate mutase (CM), isochorismate synthase (ICS), pyruvate lyase (PL), phenylalanine ammonia-lyase (PAL) and benzoic acid 2-hydrozylase (BA2H) (Figure adapted from Wildermuth et al., 2001).

One of the agricultural crops which widely used for *Agrobacterium* mediated transformation studies is potato. Potato is the fourth most important crop in the world, next to maize, rice and wheat, with annual production approaching 365 million tons (http://faostat.fao.org/site/339/default.aspx) (FAO report, 2012). It is an excellent source of carbohydrates, proteins and vitamins. Considering its nutritional quality and ease of production, potato is the prime candidate crop for improvement through breeding and biotechnology. However, following the conventional breeding methods to improve potato is not simple due to male sterility, incompatibility and the autotetraploid genome of most cultivated potato varieties which resulted in slow genetic improvement of this crop. To overcome these problems, alternative approaches have been developed to improve commercial potato varieties. Somatic hybridization, mutagenesis and genetic transformation are *in vitro* techniques used to improve potato genotypes. Considering its importance and availability of adequate plant materials, different potato genotypes were used in this study to see the effects of different factors on improvement of *Agrobacterium*-mediated transformation efficiency.

Although different kind of factors can affect *Agrobacterium*-mediated transformation efficiency, improvement of tissue culturing conditions such as light, may enhance transformation efficiency. Light quality has effects on photosynthetic rate, growth and the number of leaf stomata on *in vitro* grown plants (Kim et al., 2003). The spectral quality of light affects horticultural plants in a way that light plays roles on accumulation of starch through photosynthesis, chloroplast development, chlorophyll formation and stomata opening. Although several studies demonstrate the effect of light quality on the physiology and morphology of plants, the response of plants to the different light types vary from species to species. Kim et al., (2003) demonstrate that blue light negatively affects the growth and net photosynthesis of chrysanthemum. In this study, it was also observed that leaf area and chlorophyll content were highest in plants grown under red and blue (RB) light and fluorescent (white) lights; and, the net photosynthetic rate was highest in RB and fluorescent light regimes. Plants grown under RB and fluorescent lights had larger size and small number of stomata, and these plants showed vigorous growth. The effect of spectral quality of light on growth and development was also confirmed on *in vitro* grown potato cultivars. Potato cultivars grown *in vitro* under blue light source had the shortest stem, whereas; longest stem was observed on cultivars grown under low-pressure sodium light source. The difference in stem length between cultivars illustrate that light affects plant cell elongation (Wilson et al., 1993). Plant cell elongation and proliferation is needed for effective plant transformation (Binns and Campbell, 2001). Growing plants *in vitro* in different light regimes and increasing their net photosynthetic rate, which, in turn, increases cell elongation and proliferation, may help to increase *Agrobacterium* mediated transformation efficiency. Improvement of growing media contents may also modify the physiology of the plant so that the efficiency of the plant to block pathogen attack may be reduced, which may, in turn, help the *Agrobacterium* to transfer its T-DNA easily into the plant cell. Therefore, this study was designed to see whether type of light, plant genotypes and inhibition of salicylic acid biosynthesis affects the transformation efficiency. In addition, experiment was designed to test the effect of plant transformation vectors and genotypes on the regeneration capacity of transformed genotypes.

2. Materials and methods

2.1. Plant materials

Two potato genotypes were used for the analysis of the effect of light on transformation efficiency. These genotypes were Karnico and VIP099-19. The genotypes were grown *in vitro, both* in white and blue LED light conditions (i.e., six pots of karnico and six pots of VIP099-19 in white TL light; and six pots of karnico and six pots of VIP099-19 in blue LED light). For the analysis of the effects of salicylic acid inhibitors on potato transformation efficiency, a potato variety which is called 'Desiree' was used. Desiree was grown *in vitro* under a normal white light condition. For the analysis of the regeneration capacity of potato genotypes, two genotypes were grown *in vitro* in a normal light condition. These genotypes were VIP038-1 and VIP097-27. For propagation and maintenance plants were cut and transferred to fresh MS medium every three weeks. Plants were grown at 24 °C and 16 hrs light; white light was provided by Philips cool white lamps with average intensity of 75 µmol/m^2/s; and, blue light by Philips Blue LED light type with average intensity of 65 µmol/m^2/s.

2.2. Agrobacterium tumefaciens culturing

In this study, one *Agrobacterium* strain with different constructs was used for transformation. For the analysis of the effects of light and salicylic acid inhibition on transformation efficiency, AGL0(pCambia1301) containing the plant binary vector equipped with the GUS reporter gene were used. For the analysis of the differences in regeneration capacity between potato genotypes, AGL0(pBin19-TT12) and AGL0(pBin19-TT19) were used. AGL0(pBin19-TT12) contained Transparent Testa 12 (TT12) gene, which is involved in the vascular accumulation of proanthocyanidin precursors in the seed (Marinova *et al*., 2007). AGL0(pBin19-TT19) construct contained Transparent Testa 19 (TT19), a glutathione S-transferase (GST) gene, which functions as a transporter of anthocyanin from cytosol to the tonoplast (Sun *et al*., 2012). Glycerol stock solutions were prepared, for Agl0(pCambia1301), to have adequate amount of bacterial suspension for the transformations done in this study. Glycerol stock solutions were prepared in a way that the 850µl bacterial suspension was added to a 2ml sterilized Eppendorf tube and 150µl sterile 98% glycerol was added to the bacterial suspension and vortexed to mix the solution. Then, the tubes were stored in -80^{0}C for long term storage. For the transformation, an overnight cultured *Agrobacterium* was prepared from these stock solutions. *Agrobacterium* was grown in Luria Broth (LB) medium containing filter sterilized kanamycin and rifampicin antibiotics at a concentration of 50mg/l

each, and acetosyringon was added at a concentration of 100 µM. The *Agrobacterium* suspension, with total volume of 10ml (LB + antibiotics + acetosyringon) in 50ml tube, was grown overnight at 30^0C in a shaker (150 rpm).

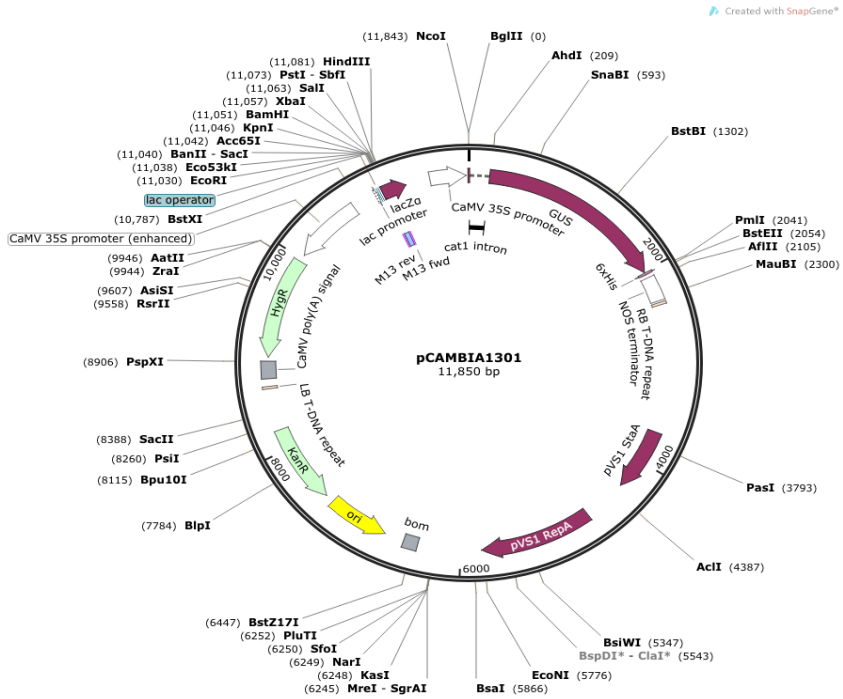

Figure 4. Schematic representation of pCAMBIA1301 vector

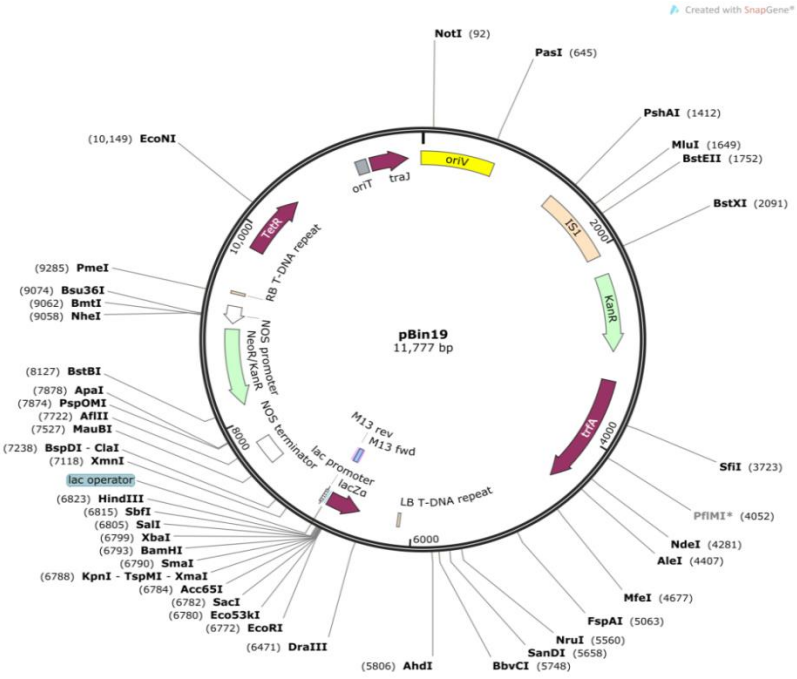

Figure 5. Schematic representation of pBin19 vector

2.3. Preculturing of explants:

Effect of light types

For this experiment, internodia explants with a size of 2-5mm were obtained from five weeks old plants grown in blue and white light conditions. Explants were precultured overnight in the same light as grown earlier at 24^0C on medium containing Murashige and Skoog (MS) basal salts & vitamins (4.4 g/l), sucrose (30g/l), micro agar (8 g/l), naphthalene acetic acid (NAA) (10.75µM) and benzylaminopurine (BAP) (4.4µM); and, the pH was adjusted to 5.8 for all media used in all experiments.

Effect of salicylic acid inhibitors (AIP and Paclobutrazol)

For these experiments, explants were exposed to the chemicals starting from the pre-culturing period. For the AIP experiment, eight week old plants were used as a source of explants, whereas, for the paclobutrazol experiment, four week old plants were used. The two experiments were done separately; and hence, they are not comparable to each other. Internodia explants with a size of 2-5mm were cut and precultured overnight at 24^0C on medium containing Murashige and Skoog (MS) basal salts (4.4 g/l), sucrose (30g/l), micro agar (8 g/l), naphthalene acetic acid (NAA) (10.75µM) and benzylaminopurine (BAP) (4.4µM). For these two experiments, preculturing of explants was done on medium containing AIP and paclobutrazol. Nine petridishes containing the above indicated medium were prepared for each of the two experiments (AIP and paclobutrazol). The three treatments (0µM, 10µM and 50µM) of each experiment were repeated three times; hence, three petridishes of each experiment were supplemented with one treatment, i.e., for AIP, three petridishes received 0µM, three petridishes received 10µM and three petridishes received 50µM. Similarly, for paclobutrazol, petridishes received the treatments with the same procedure as AIP.

Regeneration capacity of genotypes

For this experiment, internodia stem explants were obtained from five weeks old plants of each genotype and the explants were pre-cultured overnight at 24^0C. The composition of the medium used for preculturing of the explants of this experiment was similar to the basal medium used for the above experiments.

2.4. *Agrobacterium tumefaciens* mediated transformations and cocultivation

The same transformation procedure was applied for all experiments: after overnight pre-culturing, the explants were transferred to an overnight cultured *Agrobacterium* suspension. The explants were incubated in the *Agrobacterium* suspension for 15 minutes with occasional swirling. For the genotype regeneration capacity experiment, half of the precultured explants of each genotype were transformed with one of the two constructs (Agl0(pBin19-TT12)) and the remained half were transformed with the second type of construct (Agl0(pBin19-TT19)). After 15 minutes incubation, the explants were blotted dry on sterile filter paper and placed back to the medium where the explants were precultured. The petridishes were sealed and placed in a 24^0C growth chamber for two days cocultivation in a 16hr normal white light and 8hr dark light condition.

2.5. Transferring explants to selection medium

The *Agrobacterium* construct used for transformation of explants of the experiments: "effect of light types on transformation efficiency" and "effects of SA inhibitors on transformation efficiency" contained a gene (hpt) for selection on the antibiotic hygromycin but, for these experiments, kanamycin was used as a plant selectable agent instead of hygromycin (this was done by mistake but, normally, selection had to be done on hygromycin). The composition of selection medium is briefly explained below.

Effect of light

For this experiment, there were two light types (white TL light with average light intensity 75 µmol $m^{-2}s^{-1}$ and blue LED light with average light intensity 65 µmol $m^{-2}s^{-1}$), two genotypes (Karnico and VIP099-19) and two transformation efficiency evaluation time points (day-3 and day-14); there were a total of eight treatments per genotype each repeated three times (=24 experimental units per genotype). Hence, 24 plates were prepared for each genotype. After two days of cocultivation in normal white light, explants were transferred to selection medium containing Murashige and Skoog (MS) basal salts and vitamins (4.4 g/l), sucrose (20g/l), micro agar (8 g/l), zeatine (4.6µM), claforan (200 mg/l), vancomycin (200 mg/l), and kanamycin (100 mg/l). Out of the 24 plates, 12 plates were used to transfer those explants which were obtained from karnico grown in white light, and the remained 12 plates were used to transfer those explants which were obtained from karnico grown in blue LED light. Then, half of the plates containing explants of karnico grown in white light were transferred to blue LED light and the remaining half were kept in the white TL light. Similarly, half of the plates

containing explants of karnico grown in blue LED light were transferred to white TL light and the remained half were kept in the blue LED light. Each plate contained 15 explants. Placement of the treatments of VIP099-19 genotype in both light conditions was done in the same way as done for Karnico. Treatments of each genotype placed in each light type were, in turn, divided equally into two groups: one group was used for day-3 evaluation and the second group used for day-14 evaluation. Therefore, half of the treatments of each genotype placed in each light type were used to evaluate transient GUS gene expression (day-3), and the remaining half were used to evaluate the potential of stable transformation efficiency (day-14).

Effect of salicylic acid inhibitors (AIP)

Three concentrations of AIP (0 µM, 10 µM and 50 µM) were included in selection medium, as it was done in preculturing medium. Hence, after two days of cocultivation of inoculated explants in an MS medium containing the AIP treatments, explants were transferred to selection medium (the composition of the selection medium was similar to the selection medium used for the light type experiments) supplemented with 0 µM, 10 µM or 50 µM concentrations of AIP. There were two evaluation time points for this experiment, day-3 and day-14. Each of the three AIP concentration treatments was repeated three times per evaluation time point.

Effect of Paclobutrazol

Similar to the AIP, the effect of paclobutrazol (PBZ) on gene transfer was tested with three concentrations: 0 µM, 10 µM and 50 µM. These concentrations were also included in selection medium. Hence, after two days of cocultivation, explants were transferred to selection medium treated with 0 µM, 10 µM and 50 µM concentrations of PBZ. There were two evaluation time points for this experiment, too, day-3 after transferring to selection medium and day-14 after transferring to selection medium. Each of the three PBZ concentrations was replicated three times per evaluation time point.

Genotypes regeneration capacity

After two days of cocultivation, explants of each genotype were transferred to selection medium. Each treatment was repeated six times per construct and each plate contained 25 explants. Explants were transferred to new selection medium every two weeks (3 times). After two months of cultivation, regeneration capacity of genotypes was scored by counting the number of shoots regenerated in each plate.

2.6. GUS assay

The procedures followed for assaying GUS remained the same for all experiments of this study. GUS assays were conducted at two time points: after 3-days culturing and 14-days culturing of the transformed explants (after cocultivation). All the explants, including a positive control, were tested for β-glucuronidase (GUS) by incubation in x-gluc buffer overnight at 37^0C. The x-gluc buffer contained 5-bromo-4-chloro-3-indolyl glucuronide substrates, according to Jefferson (1987). After overnight incubation, the explants were washed with 80% ethanol and placed in the ethanol for a night before examining the explants under microscope. The explants were observed under a light microscope and those explants which showed blue GUS staining were counted.

2.7. Data analysis

Data analysis was conducted by Genstat 14^{th} edition and, mean separation was done by using Least Significant Difference (LSD) at 5%.

3. Results

3.1. Effect of light types

The effect of light types on transformation efficiency of potato genotypes was expressed as the percentage of explants that showed GUS staining at day-3 and day-14 (Table 1). In this study, day-3 and day-14 evaluation time points were used to refer the potential of transient and stable transformation efficiencies, respectively. The ANOVA showed that there was highly significant variation between genotypes and between time points. The interactions between time points and genotypes, and, light types and genotypes were also significantly different. For both transient and stable transformation events, Karnico showed better transformation efficiency than VIP099-19 in all light conditions except for blue to white light condition measured at day-3. It was also observed that the percentage of transformed explants was higher in 14 days cultivated explants than 3 days cultivated explants for both genotypes cultivated under all light conditions.

For the transient GUS gene expression, there was no significant difference between light types for VIP099-19 genotype, which showed lower transient transformation efficiency under all light conditions; whereas, for Karnico transient transformation efficiency, blue to white was significantly different from blue to blue and white to blue; and, white to white was also significantly different from white to blue light type. None of the inoculated explants of karnico showed transient transformation at blue to white light type. The transient transformation efficiency of karnico was also lower at white to white light type, which was not significantly different from blue to white light type. Significant differences were also not observed between white to white and blue to blue light types, and between blue to blue and white to blue. Comparing means suggested that, for karnico, transient transformation was more efficient under white to blue and blue to blue light conditions, however, the differences proved not to be statistically significant by ANOVA testing (Table 1).

For the stable transformation, transformation efficiency of karnico at white to white light type was significantly different from blue to white light type. There was no significant variation observed among other light types for the stable transformation efficiency of karnico. The result showed that Karnico had a higher transformation efficiency when plants are raised in white light and kept in the same light after transformation. On the other hand, VIP099-19 showed significantly improved transformation efficiency at blue to blue light type than white

to blue and white to white light types. For VIP099-19, growing of plants in blue and keeping the plants in the same light after transformation seems better (Figure 6).

Comparison of the differences in transformation efficiency between genotypes indicates that there was significant difference in transient transformation efficiency between genotypes at white to blue light type, at which Karnico showed better transient transformation efficiency. For the stable transformation efficiency, however, genotypes showed significant variations among all light types except blue to blue light type. Considering the overall performances, Karnico had better transformation efficiencies in almost all light types than VIP099-19 (Table 1).

Table 1. The effect of light types on GUS expression at day-3 and day-14 after cocultivation

Type of light	Total no. of explants tested	Transformed explants (%)			
		Day-3		Day-14	
		Karnico	VIP099-19	Karnico	VIP099-19
Blue to white	45	0.0^{a*}	2.2^{a}	26.7^{de}	6.7^{abc}
Blue to blue	45	15.6^{bcd}	4.4^{ab}	28.9^{ef}	17.8^{cde}
White to blue	45	24.5^{de}	0.0^{a}	28.9^{ef}	2.2^{a}
White to white	45	4.5^{ab}	2.2^{a}	40.0^{f}	4.4^{ab}

*means with the same letters are not significantly different at 5% LSD.

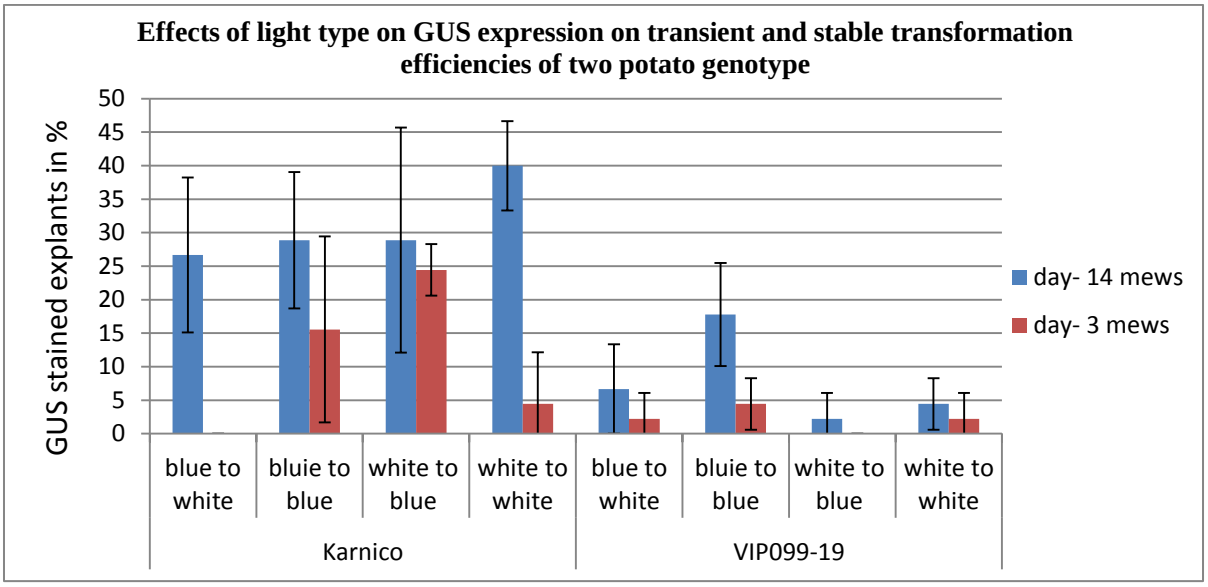

Figure 6. Effect of types of light on GUS expression of two potato genotypes at two evaluation days (day-3 and day-14).

3.2. Effect of salicylic acid inhibitors (AIP and paclobutrazol)

3.2.1. Effect of AIP on transient and stable GUS expression

The effect of AIP on transformation efficiency was also expressed as the percentage of explants that showed GUS staining at day-3 and day-14 (Table 2). For the transient transformation, the percentage of GUS stained explants showed a gradual increase from 0µM to 50µM concentration (Figure 7). A higher percentage of explants expressing GUS gene was observed at 50µM and 10µM than 0µM. The percentage of explants that expressed transient GUS gene was not significantly different at 10µM and 50µM AIP concentrations, but these two concentrations were significantly different from 0µM. For the stable transformation, however, the percentage of explants that expressed GUS gene showed reduction in the higher AIP concentration (50µM) (Figure 4). Similar to the transient transformation, there was no significant differences observed between 10µM and 50µM, and 0µM and 50µM AIP concentrations, for the stable transformation. However, the difference between 0µM and 10µM proved significant. As compared to 0µM and 50µM concentrations, the highest percentage stable transformants were obtained in 10µM AIP concentration. It was also observed that the percentage of GUS expressing explants differed between time points. At 0µM and 10µM concentrations of AIP, increment in percentage of GUS gene expression was seen on day-14 as compared to day-3 (Table 2) but, at 50µM concentration, the percentage of GUS gene expression was reduced.

The number and size of GUS expression spots on the explants were also different between concentrations. Figure 8 below shows the difference in GUS staining between explants supplemented with different AIP concentrations. It was observed on stable transformants that relatively higher number and/or larger sized GUS staining spots were observed on explants supplemented with 10µM than 0µM and 50µM AIP concentrations.

Table 2. Effect of AIP concentration on transformation efficiency of potato variety (Desiree).

AIP concentration	Total no. of explants tested		Explants showing GUS expression (%)	
	Day-3	Day-14	Day-3	Day-14
0 µM	45	45	0.00^{a*}	4.5^{ab}
10 µM	45	45	13.33^{bc}	17.8^{c}
50 µM	45	45	20.00^{c}	8.9^{abc}

*means with the same letters are not significantly different at 5% LSD.

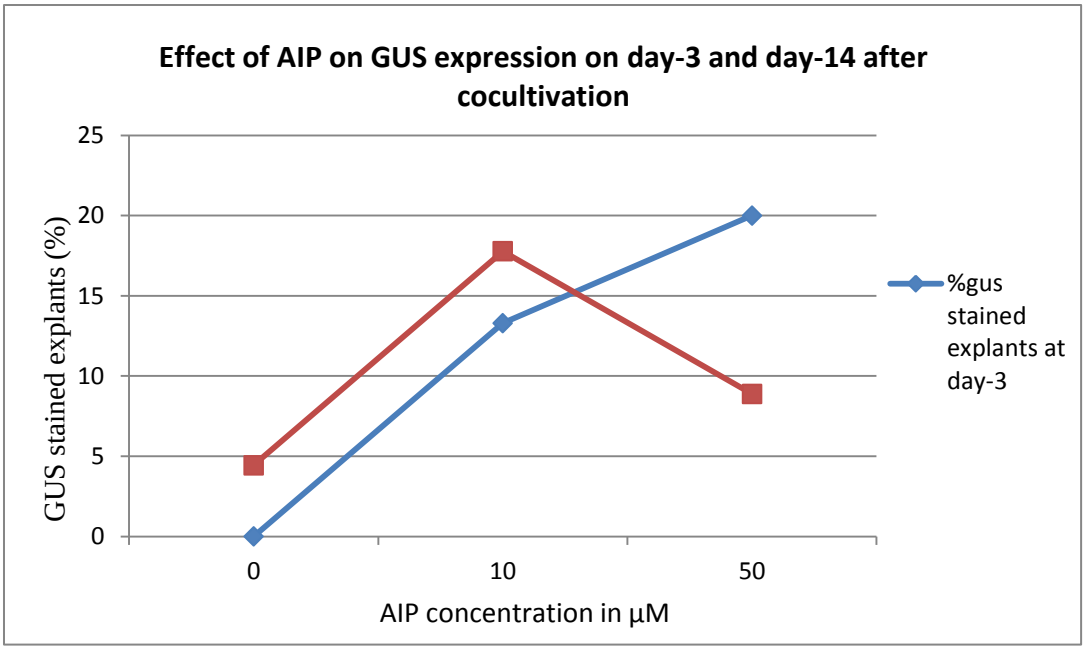

Figure 7. Effect of SA inhibitor (AIP) on GUS expression of potato genotype at two evaluation days (day-3 and day-14).

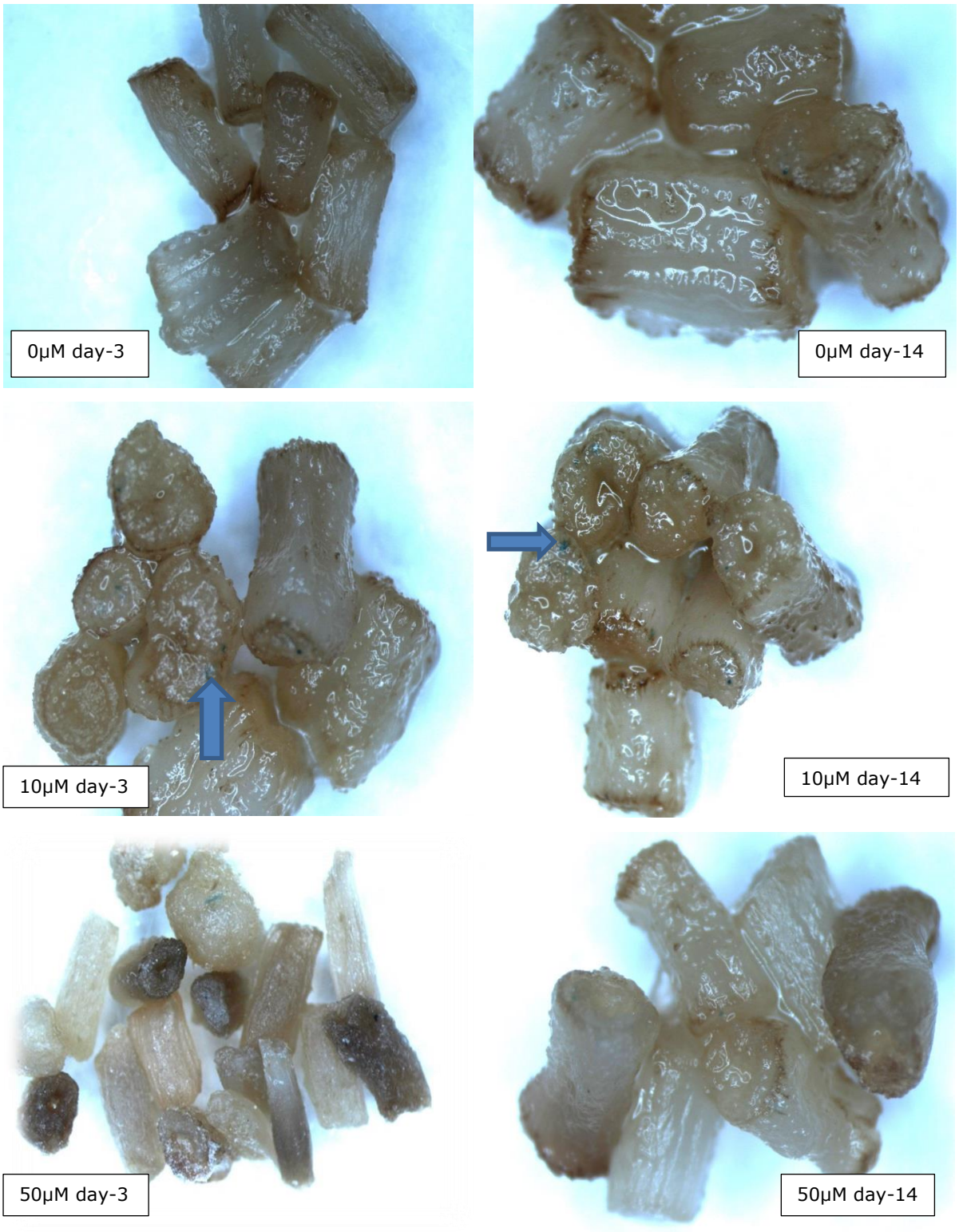

Figure 8. Transient (day-3) and stable (day-14) GUS gene expression of potato (Var. Desiree) supplemented with different concentrations of AIP.

3.2.2. Effect of paclobutrazol on transient and stable GUS expression

The study of the effect of paclobutrazol on transformation efficiency showed a lower effect. This experiment was done twice, with similar results. In the first experiment, none of the treatments showed any GUS spots on any of the explants except at 10µM concentration, at which 4.4% of the tested explants showed GUS gene expression. In the second repetition, similar result was obtained, 4.4% at 10µM concentration and 0 on all the other treatments.

3.3. Regeneration capacity of genotypes

The regeneration capacity of genotypes was expressed as the percent of explants regenerating shoots relative to the total number of explants tested. Difference in regeneration capacity was observed between genotypes transformed with the same *Agrobacterium* mediated transformation procedure. Genotypes showed significant differences for mean number of regenerated explants. VIP038-1 had a better regeneration capacity than VIP097-27, regardless of the type of construct used for transformation. There were no significant differences observed in genotypes for the different *Agrobacterium* constructs used for transformation. Figure 10 below, the photos of which were taken immediately after the third transferring of explants to selection medium, shows the differences in regeneration response as an indicator for transformation efficiency between the genotypes.

Table 3: Differences in regeneration capacity between potato genotypes transformed with the same *Agrobacterium* strain but different construct

Genotype	Construct Used for transformation	Mean number of explants tested	Mean number of explants regenerated
VIP097-27	Agl0(pBin19-TT12)	25	0.0
VIP097-27	Agl0(pBin19-TT19)	25	0.0
VIP038-1	Agl0(pBin19-TT12)	25	10.7
VIP038-1	Agl0(pBin19-TT19)	25	16.0

As it can be seen in Figure 10, the growth of calli was fast and vigorous in VIP038-1 but not in VIP097-27 genotype. The formation of calli was very low in VIP097-27 and the explants become brown coloured and started dying. So, comparing the two genotypes, VIP038-1 genotype was surviving healthier and had more regeneration rate than VIP097-27.

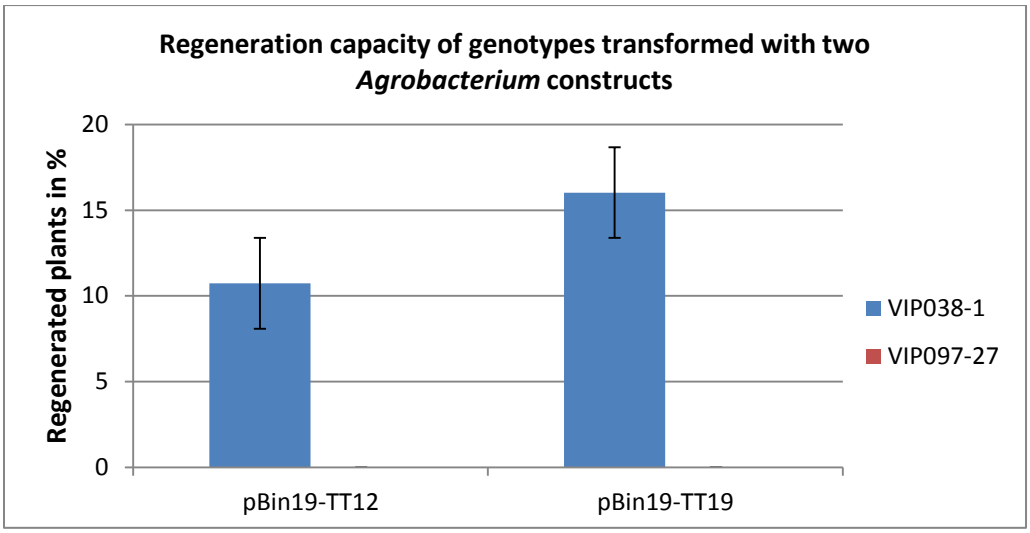

Figure 9. Regeneration capacity of genotypes transformed with two *Agrobacterium* constructs Agl0(pBin19-TT12) and Agl0(pBin19-TT19)

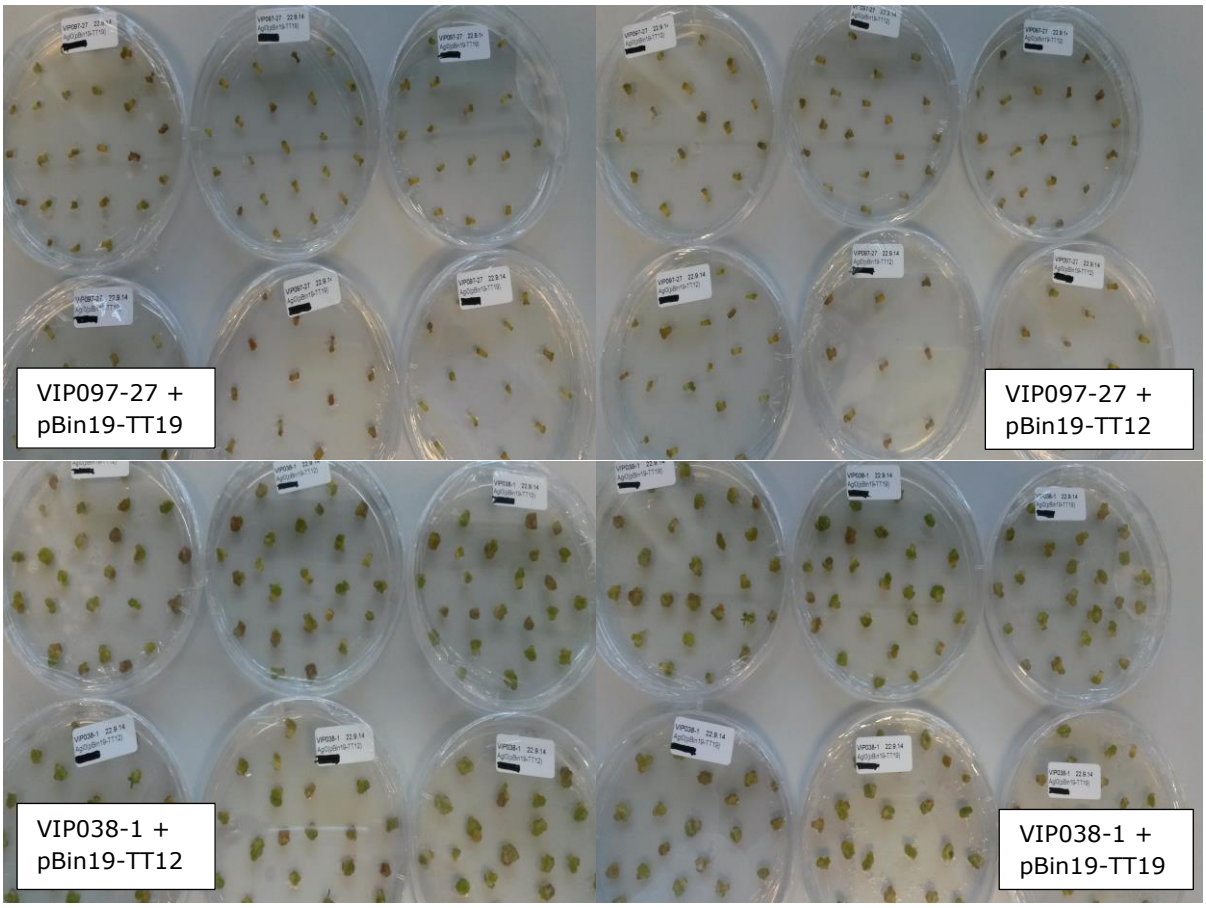

Figure 10. The difference in regeneration capacity of two potato genotypes (VIP097-27 and VIP038-1) after 45 days of cultivation of post transformation with two *Agrobacterium* constructs (Agl0(pBin19-TT12) and Agl0(pBin19-TT19)).

4. Discussion

4.1. Effect of light types

It is well known that the growth and development of plants are affected by the spectral quality of light. In this study, monochromatic lights were used to improve gene transfer from *Agrobacterium* to plant cells. The effect of the light on the efficiency of the *Agrobacterium* to transfer its T-DNA into plant cells was dependent on the genotype. Compared to VIP099-19, Karnico responded better for both transient (referring to day-3) and stable (referring to day-14) transformation efficiencies in all light types except in blue to white light, on which the transient transformation efficiency of this genotype was zero. Comparison of the effect of each light type on each genotype indicated that growing of karnico in white light and transferring of the explants to blue light after transformation resulted in the best transient transformation. For the stable transformation, however, growing of the donor plant (karnico, in this case) in blue light and keeping the transformed explants in the same light and/or growing of this genotype in white light and transferring of the transformed explants to blue light or keeping it in white light had no significant difference in transformation efficiency, however, transferring of karnico grown in blue light to white light resulted in significantly lower transformation efficiency as compared to growing of karnico in white light and keeping it in white light but, comparing the means difference, it seems that growing of karnico in white light and keeping the transformed explants in the same light is preferable to get better transformation efficiency (40%). For VIP099-19, the effect of light type on the transient transformation efficiency was not significant, but comparing the means of each light indicated that blue light had a slight positive effect. Stable transformation efficiency of VIP099-19 showed a significant difference (17.8%) when the donor plant (VIP099-19, in this case) was grown in blue light and cultivating the transformed explants in the same light. The variation in the transformation efficiency of each genotype between light conditions indicates that the growing conditions of the donor plant affects the transformation efficiency of genotypes differently (Carvalho et al., 2004; Mariana, 2012). This may be associated with the influence of the light on the physiology of the plant. This effect was confirmed in Arabidopsis mutants in which induction of SAR and salicylic acid production for systemic defense against *Pseudomonas syringae* depends on light. These mutants are blue light receptors, which could establish full SAR response. Arabidopsis double mutants, *cryptochrome1cryptochrome2 (cry1cry2)* and *phototropin1phototropin2 (phot1phot2)*, both of which are blue light receptors, accumulated high salicylic acid (SA) and establish full systemic acquired resistance

(SAR) response so that *Pseudomonas syringae* disease symptom was reduced. The presence of blue light may help the plants to produce photosynthetic metabolites and distribute to the whole plant to increase resistance in whole plant (Griebel and Zeier, 2008). However, light may also affect plants negatively by affecting their ability to defend pathogen attack through the reduction of salicylic acid accumulation. The effect of light on the physiology of plants is species dependent (Kim et al., 2003). The susceptibility of the plants is hypothesized to be favorable for *Agrobacterium* to transfer its T-DNA into the plant cell. So, from this experiment, blue light treatment enhances transformation efficiency of VIP097-27 genotype, which might be resulted due to the reduction in SAR caused by the effect of blue light on SA accumulation. This result contradicts the result obtained for Arabidopsis double mutants. For karnico, white light was important to enhance efficiency of stable transformation. However, the effect of white light on transient transformation efficiency of karnico was lower. This might be due to errors occurred during experimentation. I also observed that, eventhough it was not part of the objectives of this study, GUS staining had been mostly observed on large sized (in thickness) explants than small sized (thin) explants which might also be a reason for the lower transient transformation efficiency of karnico at white light. But, in general, it seems that the efficiency of gene transfer is more affected by the type of the light where the donor plants were grown, not by to which light type the explants were transferred after transformation. Although the mean differences were not significant, it was clearly seen that the percentage of GUS stained explants were reduced when explants were transferred to other light type than keeping the explants in the original light after inoculation but, this situation was different for day-3 treatments of karnico grown in white light and transferred to blue light (explants transferred to blue light showed better transformation efficiency than explants kept in white light after inoculation) (Table 1).

It was also observed that, comparison of the transformation efficiency at the two time points (day-3 and day-14) indicated that the transformation efficiency of both genotypes increased at day-14 in all light types. Earlier it was found that transient expression yielded more spots than stable expression (Van Kronenburg and Krens personal commun.). This might be the case here due to the effect of kanamycin on defense mechanisms of the explants against *Agrobacterium* attack. As it was stated above, the plants were selected on kanamycin but, they had to be selected on hygromycin. So, since the plants have no kanamycin resistance gene, long time exposure of the explants to selection medium may affect their ability to resist against pathogen attack and, this situation of the plants may favour the *Agrobacterium* to

transfer its T-DNA into plants cell, even though antibiotics to kill off *A.tumefaciens* were also present in the medium.

4.2. Effect of salicylic acid inhibitors

4.2.1. Effect of AIP on transient and stable GUS expression

2-Aminoindan-2-phosphonic acid (AIP) is known for its effect to inhibit salicylic acid production in plants, which is produced when plants experience stress in order to induce systemic acquired resistance (Mauch-Mani and Slusarenko, 1996; Lawton et al., 1995). As a result of inhibition of SA production, the susceptibility of plant cells to pathogen attacks becomes higher. The expression of the GUS gene in transformed explants, which were exposed to AIP, is an indicator for the transformation efficiency of plants. This implies that, because the plants were more susceptible to pathogens as a result of SA inhibition by AIP, the *Agrobacterium* could get a better chance to transfer its T-DNA into the plant cell. Because the plasmid, pCambia1301, contained a GUS gene intron and since *Agrobacterium* is a prokaryote, detection of the GUS gene in *Agrobacterium* is not expected due to lack of eukaryotic splicing apparatus in prokaryotes (Vancanneyt et al., 1990). Therefore, the observed differences in GUS expression efficiencies for the different concentrations of AIP indicated that AIP contributed significantly positive to gene transfer from *Agrobacterium* to plant cells. According to Nakai, 2013, increment of transient GUS expression was observed in *Nicotiana* with AIP concentration showing that an increase in transformation efficiency. In this study, transient GUS expression increased with AIP concentration, i.e., the highest transient GUS expression was observed in 50µM AIP concentration, but, for the stable transformation, the highest GUS expression was observed at 10µM and it decreased as the concentration of AIP was increased to 50µM. The expression of GUS gene was lower at 0µM AIP in both transient and stable transformations, which indicates that AIP is needed for better transformation efficiency of plants. The decrease in stable transformation efficiency at a higher AIP concentration resulted, may be, due to the toxicity effect of AIP, kanamycin or combination of the two. This is because, in this experiment, the plant selection antibiotics contained in the *Agrobacterium* construct was hygromycin but, plants transformed with this construct were selected on kanamycin containing medium. Kanamycin is toxic to plants so that the presence of kanamycin in selection medium may highly disturb the physiology of plants, (such as, inhibition of SA production, which, in turn, affect SAR induction during bacterial infection), and may cause plant cell death. The toxicity of the medium may also be the result from the higher concentration of AIP, or the combination of AIP and kanamycin.

The transformation efficiency observed in the 10µM AIP concentration may also be affected in a similar fashion but, in a lower degree than 50µM AIP.

4.2.2. Effect of paclobutrazol on transient and stable GUS expression

Paclobutrazol is believed to have a similar effect as AIP to inhibit the biosynthesis of salicylic acid (Xiang et al. 2011). This experiment was conducted twice and both experiments showed that the effect of paclobutrazol on transformation efficiency was low which may indicate that the SA inhibitory effect of paclobutrazol was low. But, as compared to the control (0µM) and 50µM, better transformation efficiency was obtained at 10µM paclobutrazol. The effect of paclobutrazol on transformation efficiency may also be affected by the plant selection antibiotics (kanamycin), as of AIP.

4.3. Genotype regeneration capacity

Previous studies showed that, not all genotypes have similar regeneration capacity and transformation efficiency (Heeres et al., 2002). In this study, the effect of genotypes on regeneration capacity of transformed plants was clearly observed. It was found in this study that VIP038-1 genotype showed a better regeneration capacity than VIP097-27. The difference in regeneration capacity between these genotypes may be associated with the difference in the physiological response of genotypes to stresses such as, induction of systemic acquired resistance (SAR) during pathogen attack. There are different defense mechanisms that plants activate to overcome the problem of pathogen attack; one of which is the induction of salicylic acid biosynthesis (Durner et al., 1997; Lawton et al., 1995). Failure of the plants to induce such mechanisms may result in susceptibility to pathogen attack. In this study, none of the tested explants of VIP097-27 genotype showed regeneration. This phenomenon may occur due to the physiological response of the plant against the pathogen attack, and this may affect the *Agrobacterium* to deliver its T-DNA into the plant cell. Plants which have not received T-DNA will not survive in the selection medium. This situation must have occurred in this genotype, which indicates that, this genotype was not suitable for the *Agrobacterium* to infect and transfer its T-DNA. On the other hand, VIP038-1 was better in regeneration capacity. This may illustrate us that this genotype was suitable for the *Agrobacterium* to infect and transfer its T-DNA into the plant cell. Since the T-DNA has plant selection antibiotics, plants that have the T-DNA will survive in selection medium and, this must have happened to this genotype.

5. Conclusions

The effect of light on transformation efficiency is different between genotypes and time points. For karnico, growing of the donor plant in white light and transferring to blue light after transformation is needed for better transient transformation efficiency. But, to obtain better stable transformation efficiency, it is needed to grow karnico in white light and keeping in the same light after transformation. For VIP099-19, however, both transient and stable transformation efficiencies were better when the donor plants were grown in blue light and kept in the same light. So, growing of VIP099-19 in blue light and keeping in the same light after transformation is needed to obtain better transient and stable transformants of this genotype. For the effect of SA biosynthesis inhibitors on transformation efficiency, addition of 10µM AIP to the growing medium is sufficient to obtain a higher stable transformation. For the transient transformation, however, addition of 50µM AIP to the growing medium will result in higher transient transformation efficiencies. Regarding the effect of paclobutrazol on inhibition of SA biosynthesis, its effect was lower in this study, but there is an indication that addition of 10µM PBZ in the growing medium could result in better transformation efficiency. The effect of genotypes on regeneration capacity after transformation was also clearly seen in this study. VIP038-1 was better in regeneration capacity than VIP097-27.

6. Recommendations

For the effect of light on transformation efficiency and effect of salicylic acid inhibitors (AIP and paclobutrazol) experiments, the transformed explants were selected on kanamycin instead of hygromycin. This situation may affect the result obtained in this study. So, to be sure that the results obtained in this study are true, it is recommended to redo the experiments by using the right selection antibiotics. Moreover, the experiments need to be repeated to see if the small differences observed are significant or not. The size of the explants may also have effects on results obtained in this study. Observation of the effects of explants size on transformation efficiency was not one of the objectives of this study so that care was not taken while distributing the cut explants to the plates. The large differences in the number of transformants between plates in all the experiments may also be resulted due to differences in size of explants. So, this should also be checked by using similar sized explants in each plate.

References:

Barampuram S. and Zhang Z.J., 2011. Recent advances in plant transformation. Methods in Molecular Biology 701: 1-35.

Binns A. and Campbell A., 2001. *Agrobacterium tumefaciens*-mediated transformation of plant cells. Encyclopedia of Life Sciences: 1-6.

Breitler J.C., Labeyrie A., Meynard D., Legavre T., and Guiderdoni E., 2002. Efficient microprojectile bombardment-mediated transformation of rice using gene cassettes. Theor. Appl. Genet 104: 709-719.

Carvalho C.H.S., Zehr U.B., Gunaratna N., Anderson J., Kononowicz H.H., Hodges T.K., and Axtell J.D., 2004. *Agrobacterium* mediated transformation of sorghum: factors that affect transformation efficiency. Genetics and Molecular Biology, 27: 259-269.

Clarke S.M., Mur L.A.J., Wood J.E., and Scott I.M. 2004. Salicylic acid dependent signaling promotes basal thermotolerance in *Arabidopsis thaliana.* Plant J 38: 432-447.

Dale P.J., and Hampson K.K., 1995. An assessment of morphogenic and transformation efficiency in a range of varieties of potato (*Solanum tuberosum* L.). Euphytica 85: 101-108.

Davey M.K., Anthony P., Power J.B., and Lowe K.C., 2005. Plant protoplasts: status and biotechnological perspectives. Biotechnology Advances 23: 131-171.

DeBondt A., Eggermont K., Druart P., Devi M., Goderis I., Vanderleyden J. and Broekaert W., 1994. Agrobacterium mediated transformation of apple (Malus x Domestica Borkh)- an assessment of factors affecting gene transfer efficiency during early transformation steps. Plant cell Reports 13, 587-593.

Durner J., Shah J., and Klessig D.F., 1997. Salicylic acid and disease resistance in plants. Trends in plant science, reviews 2: 1-9.

Gaffney T., Friedrich L., Vernooij B., Negrotto D., Nye G., et al., 1993. Requirement of salicylic acid for the induction of systemic acquired resistance. Science 261: 754-756.

Gelvin S.B., 2000. *Agrobacterium* and plant genes involved in T-DNA transfer and integration. Annual review. Plant physiology. Plant molecular biology 51:223-256

Griebel T., and Zeier J., 2008. Light regulation and day time dependency of inducible plant defences in Arabidopsis: Phytochrome signalling controls systemic acquired resistance rather than local defense. Plant physiology 147: 790-801.

Heeres P., Schippers-RozenboomM., Jacobsen E and Visser R.G.F., 2002. Transformation of a large number of potato varieties: genotype-dependent variation in efficiency and somaclonal variability. Euphytica 124: 13-22.

Janas K.M., Cvikrova M., Palagiewicz A., Szafranska K., Posmyk M.M. 2002. Constitutive elevated accumulation of phenylpropanoids in soybean roots at low temperature. Plant Sci 163: 369-373.

Jefferson R.A., 1987. Assaying chimeric genes in plants: the GUS gene fusion system. The Plant Molecular Biology Reporter 5: 387-405.

Kim S., Hahn E., Heo J and Paek K., 2004. Effect of LEDs on net photosynthetic rate, growth and leaf stomata of chrysanthemum plantlets *in vitro.* Scientia Horticulturae 101: 143-151.

Lawton K., Weymann K., Friedrich L., Vernooij B., Uknes S., and Ryals J., 1995. Systemic acquired resistance in Arabidopsis requires salicylic acid but not ethylene. The American Physiological Society 8: 863-870.

Lee S.H., Tewari R.K., Hahn E.J. and Paek K.Y., 2007. Photon flux density and light quality induce changes in growth, stomatal development, photosynthesis and transpiration of *Withania somfifera* (L.) Dunal. Plantlets. Plant Cell Tiss Organ cult 90:141-151.

Mariana B.D. 2012. Improving gene transfer in apple. MSc thesis report, Wageningen UR.

Marinova K., Pourcel L., Weder B., Schwaz M., Barron D., Routaboul J., Debeaujon I., and Klein M., 2007. The *Arabidopsis* MATE transporter TT12 acts as a vacuolar flavonoid/H+-antiporter active in proanthocyanidin-accumulating cells of the seed coat. The Plant cell 19: 2023-2038.

Mauch-Mani B. and Slusarenko A.J. 1996. Production of salicylic acid precursors is a major function of phenylalanine ammonia-lyase in the resistance of Arabidopsis to *Peronospora parasitica.* The plant cell 8: 203-212.

Paliyath G., Pinhero R.G., Rao M.V., Murr D.P., Fletcher R.A. 1997. Changes in activities of antioxidant enzymes and their relationship to genetic and paclobutrazol-induced chilling tolerance in maize seedlings. Plant Physiology 114: 695-704.

Salmenkallio-Marttila M., Aspegren K., Akerman S., Kurten U., Mannonen L., *et al*., 1995. Transgenic barley (*Hordeum vulgare* L.) by electroporation of protoplasts. Plant Cell Reports 15: 301-304.

Seabrook J.E.A., 2005. Light effects on the growth and morphogenesis of potato (*Solanum tuberosum*) *in vitro*: A review. American Journal of potato Research 82:353-367.

Sgarbi E., Fornasiero R.B., Lins A.P. and Bonatti P.M. 2003. Phenol metabolism is differentially affected by ozone in two cell lines from grape (*Vitis vinifera* L.) leaf. Plant science 165: 951-957

Soleka D. and Kacperska A., 2003. Phenylpropanoid deficiency effects the course of plant acclimation to cold. Physiol Plant 119: 253-262

Sun Y., Li H., and Huang J., 2012. Arabidopsis TT19 functions as a carrier to transport anthocyanin from the cytosol to tonoplasts. Molecular Plant 5: 387-400.

Tzfira T. and Citovsky V., 2006. *Agrobacterium*-mediated genetic transformation of plants: biology and biotechnology. Current Opinion in Biotechnology 17: 147-154.

Vancanneyt G., Schmidt R., O'Connor-Sanchez A., Willmitzer L., and Rocha-Sosa M., 1990. Construction of an intron-containing marker gene: Splicing of the intron in transgenic plants and its use in monitoring early events in *Agrobacterium*-mediated plant transformation. Mol Gen Genet 220:245-250.

Vernooij B., Friedrich L., Morse A., Reist R., Kolditz-Jawhar R., Ward E., et al., 1994. Salicylic acid is not the translocated signal responsible for inducing systemic acquired resistance but is required in signal transduction. The plant cell 6: 959-965

Wen P., Chen J., Wan S., Kong W., Zhang P., Wang W., Zhan J., Pan Q and Huang W. 2008. Salicylic acid activates phenylalanine ammonia-lyase in grape berry in response to high temperature stress. Plant Growth Regulator 55: 1-10.

Wenzler H., Mignery G., May G., and Park W., 1989. A rapid and efficient transformation method for the production of large numbers of transgenic potato plants. Plant Sciences 63: 79-85.

Wildermuth M.C., Dewdney J., Wu G., and Ausubel F.M., 2001. Isochorismate synthase is required to synthesize salicylic acid for plant defence. Nature, 414: 1-5.

Wilson D.A., Weigel R.C., Wheeler R.M., and Sager J.C., 1993. Light spectral quality effects on the growth of potato (*Solanum tuberosum* L.) nodal cuttings *in vitro*. In Vitro Cell Dev. Biol 29P:5-8

Xiang Y., Song M., Wei Z., Tong J., Zhang L., Xiao L., Ma Z., and Wang Y., 2011. A jacalin-related lectin-like gene in wheat is a component of the plant defence system. Journal of Experimental Botany 62:5471-5483.

Yepes L.M. and Aldwinckle H.S., 1994. Factors that affect leaf regeneration capacity in apple, and effect of antibiotics in morphogenesis. Plant Cell, Tissue and Organ Culture 37: 257-269.

Zambre M., Terryn N., De Clercq J., De Buck S., Dillen W., Montagu Van M., Straeten D.V.D and Angenon G. 2003. Light strongly promotes gene transfer from *Agrobacterium tumefaciens* to plant cells. Planta 216: 580-586.

Zupan J., Muth T.R., Draper O., and Zambryski P., 2000. The transfer of DNA from Agrobacterium tumefaciens into plants: a feast of fundamental insights. The Plant journal 23: 11-28

Website:

(http://faostat.fao.org/site/339/default.aspx) (FAO report, 2012)