Bachelor project in the Horticultural Science programme
2007-5, 10 p (15 ECTS)

Transformation of *E. coli* and *Agrobacterium* and molecular analysis of transformed apple (*Malus domestica)* rootstock A2

by

Carin Emanuelsson

Fakulteten för landskapsplanering, trädgårds- och jordbruksvetenskap

SLU-Alnarp

Transformation of *E. coli* and *Agrobacterium* and molecular analysis of transformed apple (*Malus domestica*) rootstock A2

Transformering av *E. coli* och *Agrobacterium* samt molekyläranalys av transformerad äpplegrundstam A2 (*Malus domestica)*

by

Carin Emanuelsson

Supervisor and examiner: Docent / Assoc. Prof. Li-Hua Zhu
Dept. of Plant breeding and biotechnology, SLU, Alnarp

Contents

Abstract

This project includes two parts. The first part was transformation of *Escherichia coli* and *Agrobacterium tumefaciens* with a vector containing the *GA2ox* gene. The second part was molecular analyses of the apple (*Malus domestica*) rootstock A2 transformed with the *rolC* gene. The *rolC* and *GA2ox* genes are two potential genes for dwarfing plants.

E. coli was transformed with a binary vector, which was supposed to contain the *GA2ox* and *nptII* genes. Transformation events were confirmed by restriction enzyme digestion and by PCR analysis, which led to the discovery that the *GA2ox* gene was not present in the original vector but the *gus* gene. *A. tumefaciens* was later transformed with the vector, isolated from the above transformed *E. coli*. Transformation of *A. tumefaciens* could not be confirmed by PCR analysis, even though the colonies grew well on the selectable medium containing kanamycin.

In the second part of the project Southern blot was conducted to further confirm integration of the *rolC* and *nptII* genes in the transgenic clones of the apple rootstock A2 and to determine the copy number of the transgenes. The Southern results confirmed that six clones contained the *nptII* and *rolC* genes. Two of the transformed clones have a single copy of the T-DNA integrated.

Sammanfattning

Det här projektet är uppdelat i två delar. Den första delen innefattar transformering av *Escherichia coli* och *Agrobacterium tumefaciens* med *GA2ox* genen. Den andra delen av projektet består av utförandet av molekyläranalyser av äpplegrundstammen A2 transformerad med *rolC* genen. *rolC* och *GA2ox* är två potentiella gener för att reducera plantstorleken.

E. coli blev transformerad med en plasmidvektor, vilken var tänkt att innehålla *GA2ox* och *nptII* generna. Genomförd PCR analys bekräftade transformering av *E. coli* men samtidigt upptäcktes det att vektorn innehöll *gus* genen istället för *GA2ox* genen. Vektorn som isolerats från *E. coli* transformerades senare in i *A. tumefaciens*. Transformering av vektorn i *A. tumefaciens* kunde inte bekräftas efter utförd PCR-analys, trots att bakteriekolonier växte normalt på medium med kanamycin.

I den andra delen av projektet analyserades A2 grundstamkloner som transformerats med *rolC* och *nptII* generna, genom utförandet av Southern blot hybridisering för att bestämma antalet kopior av transgenerna som integrerats i respektive klongenom. Det bekräftas att sex kloner är transformerade med *nptII* och *rolC* generna. Två av klonerna innehåller endast en kopia av de båda generna, som kan vara av intresse för tillämpningen i framtiden.

1. Introduction

This project includes two parts; the first part was transformation of *E. coli* and *A. tumefaciens* with the *GA2ox* gene. The second part was molecular analysis of the transgenic apple rootstock A2 with the *rolC* gene. The aim for the first part was to prepare a transformation vector containing the *GA2ox* gene. To achieve this aim, the following tasks were undertaken:

1) Preparation of competent cells of *E. coli* and *A. tumefaciens*.
2) Transformation with the binary vector pLARS120 containing the *GA2ox* gene into *E. coli* for cloning of the vector and *A. tumefaciens*, respectively.
3) Verification of transformed bacteria by restriction enzyme digestion and PCR analysis.

The aim of the second part was to further verify the transformation events and determine the copy number of the transgenes in the transgenic apple (*Malus domestica*) rootstock A2. To achieve this aim, Southern blot hybridization was preformed.

2. Background

2.1. The GA2ox gene

Plant hormone gibberellin (GA) plays an essential role in plant growth and development (Hedden 2000). GA-deficient mutants are usually much shorter than the wild type, revealing the important role of GA in stem elongation. Moreover, through involvement in signal transduction pathways, GA can also, in association with light signals, induce flowering, So far 126 different GA forms are known in higher plants, bacteria and fungi, but only a few of them have fundamental biological activity. Manipulation of the GA status in plants is widely practiced today in horticulture and agriculture. For instance, many crops are treated with chemical growth retardants that inhibit different enzymes involved in GA-biosynthesis. However, direct genetic manipulation of the GA metabolism would enable to reduce the use of chemicals. Another advantage of direct manipulation of GA biosynthesis through genetic transformation is that such a modification can be targeted to specific tissues in order to control GA-regulated developmental processes, individually.

The GA metabolism has a complex pathway, which has been thoroughly reviewed by MacMillan (1997). The dioxygenases are a group of enzymes that catalyse the later steps in the GA biosynthesis pathway. One dioxygenase, a multifunctional protein referred to as GA 2-oxidase (GA2ox), deactivates GAs by 2β-hydroxylation, a hydroxyl group is introduced to the

GA molecule, at a specific position, converting an active GA to an inactive one. This enzyme is encoded by *GA2ox*, a gene belonging to a small multigene family.

The *GA2ox* gene was first isolated by Lange (1997) in the pumpkin (*Curcurbita maxima* L.) seeds and later in runner bean (*Phaseolus coccineus* L.) by Thomas et al (1999), which also led to the discovery of homologous genes in *Arabidopsis thaliana*.

Schomburg et al. (2003) have for instance isolated the genes *AtGA2ox7* and *AtGA2ox8* from *A. thaliana*, which encode proteins that act as GA 2-oxidase. Over expression of these two genes in *A. thaliana* and *Nicotiana tobacum* resulted in dwarf phenotypes as a result of decreased levels of active GAs. These genes are conserved in most plant species and can therefore be useful for creating dominant dwarf varieties without the need to isolate the *GA2ox* gene from the particular species, thus over expression of these genes should cause dwarfing in a wide range of plant species.

2.2. The rolC gene

The *rol* (root locus) genes are isolated from *Agrobacterium rhizogenes*, a soil bacterium that causes the hairy root disease in many perennials in the Rosaceae family. The *rol* genes are able to modify different morphological, physiological and developmental processes in plants (Welander & Zhu 2006). Several attempts have been made to use these genes in breeding to obtain desired agronomic traits. The *rolC* gene (540bp) is believed to have both an indirect and direct effect on plant hormone levels when expressed in plants, e.g. down regulation of the GA biosynthesis and increased levels of the phytohormone cytokinin. Different crops e.g. 'Beurre Bosc' pear and apple rootstock 'Marubakaidou' etc. have been transformed with the *rolC* gene and some transformants have showed reduction in plant height, the number of nodes, leaf area, internode length and improved rooting ability (Welander & Zhu 2006) .

Kāle (2006) transformed the apple rootstock A2 with the *rolC* gene and obtained transgenic plants which showed reduced apical dominance and shortened internodes, and some of them had an altered leaf shape in vitro. Some transgenic shoots were also lighter green than control shoots. She concluded that the *rolC* gene seems to be effective in dwarfing the apple rootstock A2, which otherwise often results in trees that are too vigorous to be used in commercial production.

2.3. Bacterial transformation

The ability to transform bacteria with DNA is a prerequisite for most experiments on gene manipulation (Primrose & Twyman 2006). Bacterial transformation is the process by which bacterial cells take up naked DNA molecules. If the foreign DNA has an origin of replication recognized by the host cell DNA polymerases, the bacteria will replicate the foreign DNA along with their own DNA. In nature, bacteria can acquire new genetic information by three means: conjugation, transduction and transformation (Chen & Dubnau 2004). During conjugation, DNA is transferred directly from one organism to another, whereas in transduction, the DNA is carried by bacteriophages. Transformation involves the acquisition of naked DNA from the extracellular environment. Since the advent of recombinant DNA technology, biologists have transformed *E. coli*, using procedures that alter the permeability of the cell membrane, so that DNA can be introduced to the bacterial cell. Bacteria which are able to uptake DNA are called competent and are made so in laboratory by treatment with calcium chloride, a chemical method for bacterial transformation (Primrose & Twyman 2006). The bacterial cell membrane is permeable to chloride ions, but is non-permeable to calcium ions. As the chloride ions enter the cell, water molecules accompany the charged particle. This influx of water causes the cells to swell and is necessary for the uptake of DNA. The exact mechanism of this uptake is unknown. The calcium chloride treatment is followed by a heat shock, *E. coli* is subjected to 42 °C, while 37 °C for *A. tumefaciens*. The heat shock step is important for the uptake of DNA. At temperatures above 42°C, the bacteria's ability to uptake DNA becomes reduced, and at extreme temperatures the bacteria will die. When transformation is coupled with antibiotic selection techniques, bacteria can be induced to uptake certain DNA molecules, and those bacteria can be selected for that incorporation.

Electroporation is another mean for transforming bacterial cells. This method is based on that cells take up exogenous DNA when subjected to an electric shock, which can make poles on the cell membrane. The method is routinely used for bacterial transformation with high transformation efficiency.

2.4. Agrobacterium-*mediated transformation*

The soil phytopathogen *A. tumefaciens*, responsible for the Crown gall disease in plants, is widely used today as a tool for introduction of foreign genes into plants to improve

crop properties. Transformation of plant cells by the bacteria is based on the transfer of a small proportion of its DNA (T-DNA), located on its Tumor-inducing (Ti) plasmid into the plant nuclear genome (Gelvin 2003).

Except for the T-DNA, the virulence (*vir*) region located on the Ti plasmid is also important for transformation (Zupan et al. 2000). This region consists of a cluster of genes whose products generate the transfer intermediate (T-complex) and mediate the T-DNA transport. The expression of genes in the *vir* region is induced by the exudates like phenolics of wounded plants. The T-complex consists of a single stranded T-DNA with a single molecule of the Vir protein VirD2 covalently bound to the 5´ end, and coated along its length with the single stranded DNA binding protein VirE2. The *vir* genes of *A. tumefaciens* will help the transfer of any DNA between the flanking 25bp direct repeats (right and left borders) that delimit the T-DNA. This is the reason why *A. tumefaciens* has been developed into a tool for genetic engineering. Inside the plant cell, the T-complex is imported into the nucleus where the genes located between the border sequences become stably integrated into a plant chromosome.

A. tumefaciens has to be manipulated before being used for genetic engineering. Removal of all the genes within the T-DNA does not impede the ability of *A. tumefaciens* to transfer its T-DNA, but does prevent the formation of tumors, such modified plasmids are thus called disarmed plasmids (Hellens & Mullineaux 2000).

2.4.1. The binary vector system

The binary vector system has been developed after the findings of Hoekema et al. (1983) and de Frammond et al. (1983). They determined that the T-DNA and the *vir* genes could be located in two plasmids with two different replicons. When these replicons were within the same *A. tumefaciens* cell, products of the *vir* genes could act in *trans* on the T-region to affect DNA processing and transfer to a plant cell. Hoekema et al. called this a binary-vector system; the replicon harboring the T-region constituted the binary vector, whereas the vector containing the *vir* genes became known as the helper plasmid. T-DNA binary vectors have revolutionized the use of *A. tumefaciens* to introduce genes into plants (Gelvin 2003). These plasmids are small and easy to manipulate in both *E. coli* and *A. tumefaciens* as they can replicate themselves easily in *E. coli* and in *A. tumefacien*. The binary vector is generally containing multiple unique restriction endonuclease sites within the T-region into which genes of interest could be cloned.

Except for the development of the binary vectors, the development of a range of disarmed *A. tumefacien* strains is also the reason for the frequent use of this bacterium for transformation (Hellens & Mullineaux 2000).

2.4.2. Marker gene *nptII*

Selectable marker genes that confer resistance to antibiotics or herbicides are usually incorporated along with the gene of interest in a transformation process to allow the recognition of the transformed cells (Pawan et al. 2002). The neomycin phosphotransferase-II (*nptII*) gene that confers resistance to aminoglycoside antibiotics such as kanamycin and its analogues paromomycin and geneticin (G418) has successfully been used as the selectable marker in transformation of a variety of crops. When this gene is transferred into the host genome it offers advantages to the transformed cells/ tissues to grow in the presence of the antibiotics. The public concern against transgenics about their possible environment and human health effects is partly due to the concern of antibiotic resistant genes. The risk assessment of the *nptII* gene and its protein has resulted in a conclusion widely accepted in the scientific community that there are no human or animal health risks associated with the use of this particular marker gene in the transgenic crops. The products of marker genes may not be necessarily harmful, but their presence in transgenic plants may increase the chance of their escape through pollen or seed dispersal to the wild and weed relatives. However there are strategies available today to eliminate the selectable marker genes from the transgenic plants for the improved safety for both the environment and consumers. One method is the MAT vector system developed by Ebinuma et al. (1997).

2.5. Polymerase chain reaction (PCR)

The PCR reaction leads to amplification of a specific DNA sequence (Primrose & Twyman 2006). PCR techniques are used in many areas of biology, for instance for amplification of foreign genes from plant tissues as an analytical tool for detecting transformed cells (Hamill et. al 1991).

In a PCR reaction two primers (short single stranded DNAs) are used that are complementary to opposite strands of the desired specific DNA sequence to be amplified (Baumforth et.al. 1999). After heat mediated denaturation (95° C) of the template DNA, the primers hybridize to their respective sequences (annealing) on the template DNA and a thermostable DNA polymerase synthesizes a complementary strand in the 5' to 3' direction (extension). With completion of each denaturation, annealing, and extension one PCR cycle is

finished. The amount of the desired specific template DNA sequence doubles theoretically with each cycle. Therefore, after 10 cycles the target sequence of the template DNA is multiplied by a factor of one thousand and after 20 cycles by a factor of more than 1 million. Eventually additional cycles will not lead to any further increase in amplified product, because of the exhaustion of reagents such as the dNTPs and primers. The optimum temperature at which each of these steps (denaturation, annealing, and extension) proceeds is different and therefore the reaction is best performed in a thermal cycler, which automatically makes the temperature change as required.

The thermostable *Taq* DNA polymerase, first found in *Thermus aquaticus* is commonly used in PCR (Bermingham & Luettich 2003). Optimal PCR extension occurs at 72° C with *Taq* polymerase in the presence of magnesium ions and dNTPs, and the enzyme remains active at this temperature even after repeated exposures to 95° C during the denaturation step.

2.5.1. Factors affecting PCR

A typical PCR has the following compositions; sterile water, 10 x reaction buffer, deoxynuceotide triphostphate (dNTP), upstream- and downstream primers, DNA polymerase, and template DNA.

2.5.1.1. Primers

The primer design and the optimal use of the primers are crucial for amplification of a specific DNA sequence (Baumforth et.al. 1999). There are some rules that should be followed when designing PCR primers: a length can be 15-30 bp; 50-60 % of the primers should consist of the bases cytosine and guanine; not more than three to four bases of the same kind next to each other; secondary structures like hairpin loops should be avoided; primers should not contain sequences that are complementary to each other to avoid hybridization with each other, causing primer-dimer formation; palindromic sequences should be avoided and primer melting temperature T_m is preferred between 55-80° C. Primer sequence should be checked out in databases to make sure that it is not homologous to other sequences, to avoid amplification of undesired DNA.

2.5.1.2. Annealing temperature

The annealing temperature in PCR reactions is dependent on the composition and length of the primers. The annealing temperature is preferred 1-5° C lower than the lowest T_m

value of the primers. Too low annealing temperatures result in non specific annealing, while too high annealing temperatures result in a reduced yield of the product. Primer T_m can be calculated using the formula below, where n is equivalent to the length of the primer:

$T_m = 64.9 + 0.41(\%guanine + \%cytosine)-600/n$ ° C.

2.5.1.3. dNTP concentration

The concentration of dNTP affects the ability of DNA amplification during PCR. Each dNTP is usually used at a concentration between 50 μM and 200 μM, higher concentrations encourage misincorporation by the DNA polymerase.

2.5.1.4. Reaction buffer

The reaction buffer affects PCR results, 10 mM Tris buffer is the most often used one, with a pH range between 8.5 and 9.0 at 25°C. The pH of Tris buffers decreases by 0.3 units for each 10°C rise in temperature, a buffer made to pH 8.8 at 25°C will have a pH value of 7.4 at 72°C, and this pH value is optimal for the activity of *Taq* polymerase at this temperature.

2.5.1.5. Magnesium ions

The appropriate concentration of magnesium ions in the reaction buffer is also important for maximal *Taq* polymerase activity. Because the dNTPs bind magnesium ions, the reaction mixture must contain an excess of magnesium ions. As a rule of thumb, the magnesium concentration in the reaction mixture is generally 0.5–2.5 mM greater than the concentration of dNTPs.

The concentration of magnesium ions also influences the efficiency of primers to template annealing. As a result, it is possible to modify the magnesium concentration rather than the annealing temperature to regulate primer specificity. Other salts, for example, KCl or NaCl may help facilitate primer annealing, but concentrations in excess of 50 mM will inhibit *Taq* polymerase activity. Phosphate salts should be avoided because they might precipitate magnesium ions at the high temperatures used in the PCR. Detergents such as Tween 20, Triton X-100, or Nonidet P-40, 13 14 and/or extra protein (for example, gelatin or bovine serum albumin) may also be added to the reaction buffer. The addition of these reagents helps to prevent precipitation of the hydrophobic *Taq* polymerase in aqueous solutions.

2.5.1.6. Template DNA

The sequence to be amplified must be present in the initial PCR reaction solution for a successful PCR. Regarding DNA quality PCR generally does tolerate poor quality DNA, but the extraction method with phenol:chloroform usually results in the best quality of template DNA. It is important to add low concentrations of the DNA, while both the primers and dNTPs should be present in excess. An overabundance of template DNA will favor the annealing of the two strands of the template sequence, rather than their annealing to the two primer pairs and will also increase the chance of forming non specific products.

2.5.1.7. Contamination

Contamination of the sample in any step of preparation or during the PCR reaction can result in amplification of an exogenous sequence and may lead to a false positive result. The contamination can be from the person conducting the PCR or from previously amplified sequences present in the laboratory working area.

2.6. Southern blot hybridization

Southern (1975) developed a method for detection of specific sequences among DNA fragments after gel electrophoresis. This method was first conducted by transferring the separated fragments of DNA from agarose gel to a nitrocellulose membrane. The DNA fragments were then fixed to the nitrocellulose membrane and hybridized to a radioactive labeled DNA, called probe. The fragments in the DNA sample that hybridized with the probe could then be detected as sharp bands by radio autography on X-ray film.

The original method developed by Southern has been improved over the years. The supported nylon membrane has replaced the nitrocellulose membrane, which have the drawback of being very fragile. Engler-Blum et al. (1993) have developed an improved chemiluminescence (generation of light as a result of a chemical reaction) -based RNA/DNA detection procedure using digoxigenin (DIG)-labeled nucleic acid. This method offers a good alternative to radioactive ^{32}P labeling.

The Southern blot method has been frequently used in plant transformation experiments to confirm transformation events and to determine the copy number of the transgenes (Holefors et al. 1998; Zhu et al. 2001).

2.6.1. Common procedure

The DNA to be analyzed is digested with restriction enzymes and the digested DNA fragments are then separated by agarose gel electrophoresis (Primrose & Twyman 2006). Before transfer of the DNA from the gel to a nylon membrane, the gel is exposed to pretreatments. The pretreatments consists of depurination with HCl which breaks DNA into small pieces, followed by an alkali treatment, which denaturates the double-stranded DNA and gives rise to single DNA strands for later hybridization and destroy any RNA residues. The gel is then rinsed in a neutralization buffer (0,5 M Tris-HCl pH 7,5; 2,5 M NaCl) (www.roche-applied-science.com). After this, the DNA is transferred to the nylon membrane by capillary transfer using 20 x SSC buffer (3M NaCl; 300mM Na-citrate, pH 7,0). Upon completion of the transfer, the DNA is fixed to the membrane by baking at 120° C for 30 minutes and the membrane is then ready for prehybridization. The prehybridization is to block nonspecific nucleic acid-binding sites on the membrane to reduce the background. This is conducted by incubating the membrane in the hybridization buffer without the probe in a hybridization oven at the hybridization temperature for at least 2 h. The prehybridization solution is then replaced with the hybridization solution, containing either DIG labeled or radioactive-labeled DNA, complementary to the specific DNA sequence of interest. Conditions are chosen so that the labeled nucleic acid hybridizes with the target DNA on the membrane, usually overnight.

The optimal hybridization temperature for a specific probe depends on the length of the probe and the extent of sequence homology with the target sequence. Stringency can be regarded as the specificity with which a particular target sequence is detected after hybridization with a probe (Primrose & Twyman 2006). Stringency is usually controlled by the temperature and salt concentration in the post- hybridization washes but it could also be manipulated during the hybridization step. The melting temperature (T_m) of a probe gives a starting point for further determination of correct stringency. For probes longer than 100 bp:

$$T_m = 81{,}5\ ^\circ C + 16{,}6 \log M + 0{,}41\ (\%\ G+C)$$

Where M = ionic strength of buffer in mol/liter. For long probes, the hybridization is usually carried out at T_m-25° C. For every 1 ° C reduction in temperature, 1 % of the probe sequence is hybridized non-specifically.

After hybridization the membrane is first washed to remove unhybridized probe (www.roche-applied-science.com). Before detection the membrane is equilibrated in the washing buffer (100mM maleic acid, 150mM NaCl, pH 7,5).

When using chemiluminescent detection a light signal is produced on the site of the hybridized probe. Chemiluminent detection is based on a three-step method. In the first step the membrane carrying the hybridized probe is treated with Blocking Reagent to prevent non specific attraction of antibody to the membrane. In the next step the membrane is incubated with an antibody solution which conjugates to alkaline phosphates. In the third step a chemiluminescent substrate is added to the membrane, which will react with the antibody conjugates bound to the hybridized probe. The membrane is then exposed to X-ray film to record the chemiluminescent signal.

3. Materials and methods

3.1. Bacterial transformation

3.1.1. *E. coli*

3.1.1.1. *E. coli* strain and plasmid vector

The recombinant plasmid, binary vector pLARS120 containing the *GA2ox* gene under the 35S promoter and the *nptII* gene under the control of the *nos* promoter was kindly obtained from Dr. Andy Phillips in UK. The *E. coli* strain XL-Blue, was used for transformation. The *GA2ox* gene in the vector was isolated from runner bean.

The XL-Blue strain is a host strain that can be used for routine cloning of plasmid vectors (www.stratagene.com). This strain is endonuclease deficient (*endA*), which improves the quality of miniprep DNA and is recombination (*recA*) deficient, improving insert stability. The strain is also *hsdR* mutant which prevents the cleavage of cloned DNA by the *EcoK* endonuclease system. The strain is resistant to the antibiotic tetracycline.

3.1.1.2. Competent cell preparation

The bacteria, stored in the freezer, were first activated on solid LB medium with antibiotic (12,5 mg l^{-1} tetracycline) overnight at 37ºC, and then stored at 4ºC for future use. The competent cells were prepared as described by Inoue et al. (1990). Competent cells were frozen in liquid nitrogen and stored in -80ºC for future use.

3.1.1.3. Transformation of the vector into *E. coli*

For transformation, 200 µl competent cells, 1µl of pLARS120 vector DNA were added into a sterile tube, which was then incubated on ice for 40 minutes, followed by a heat shock at 42 ºC for 2 minutes and further incubation on ice for 20 minutes. Then 0,5 ml of LB medium was added and the mixture was incubated at 37 ºC with 250 rpm for 1 h. The bacterial solution was then poured on the solid LB medium with antibiotics (tetracycline 12,5 mg l^{-1}, kanamycin 50 mg l^{-1}) for selection of transformed cells at 37ºC until single colonies appeared. Randomly chosen colonies were then cultured on solid LB medium and stored at 4ºC for later use.

3.1.2. *Agrobacterium*

3.1.2.1. *A. tumefaciens* strain and plasmid vector

A. tumefaciens strain C58C1 containing the helper plasmid pGV3850 was used as the target for insertion of the binary vector pLARS120. The strain is resistant to rifampicillin and the helper plasmid is resistant to carbenicillin.

3.1.2.2. Competent cell preparation

The bacteria, stored in the freezer, were activated on solid LB medium with antibiotics (50mg l^{-1} rifampicillin, 100mg l^{-1} carbenicillin) at 28 ºC overnight. The competent cells were prepared in a similar way as for *E. coli* and stored at -80 ºC.

3.1.2.3. Transformation

For transformation 200 µl competent cells were used. One µl of plasmid DNA of the pLARS120 binary vector cloned in *E. coli* was added. The plasmid DNA had a concentration of 2500 ng/µl measured with the spectrophotometer. Three different amounts of DNA were added: 250 ng, 50 ng and 25 ng. An extra vial was included for 25 ng using competent cells from another batch. The vials were incubated on ice for 40 minutes, followed by a heat shock at 37 ºC for 5 minutes and a further incubation on ice for 20 minutes. Afterwards 0,5 ml of liquid LB medium was added and cells were incubated for 3 h at 30 ºC with shaking at 150 rpm. The bacterial solutions were poured on solid LB medium with antibiotics (50mg l^{-1} rifampicillin, 50 mg l^{-1} carbenicillin, kanamycin 50 mg l^{-1}) for selection of transformed cells at 28ºC for 3 days until single colonies appeared. Six single colonies from the plate with 25ng of DNA were chosen and further cultured for later use. PCR analyses of the *nptII* and *gus* genes were conducted as described.

3.1.3. Small scale preparation of plasmid DNA

Plasmid DNA was prepared from 4 single colonies for verification of transformation events. Single colonies were cultured in tubes with 3 ml of solid LB medium with antibiotics (tetracycline 12,5 mg l^{-1}, kanamycin 50 mg l^{-1}) and cultured at 37 ºC for *E. coli* and 28 ºC for *A. tumefaciens* with 200 rpm overnight. Bacterial solution was centrifuged at 4 ºC 12000 rpm for 30 sec. Supernatant was poured off and 100 µl Solution I (50mM Glucose, 25mM Tris-HCl pH 8.0, 10mM EDTA pH 8.0) was added. Pellet was dispersed by vortexing then 200 µl of Solution II (0,2 M NaOH, 1% SDS) was added. Samples were gently mixed and stored on ice for 5 minutes, followed by addition of 150 µl of Solution III (5 M CH_3COOK 60ml, $C_2H_4O_2$ 11,5 ml, H_2O 28,5 ml) and a gentle mixing. Samples were then stored for 5 minutes on ice before centrifugation for 5 minutes. Supernatant was transferred to clean tubes and 450 µl of phenol:chloroform was added and samples were mixed by shaking vigorously. Samples were then centrifuged for 5 minutes. Supernatant was transferred to new tubes and 2 volumes of 99,5% ethanol was added, samples were vortexed and left at room temperature for 2 minutes and then centrifuged for 5 minutes. Supernatant was removed and pellet was rinsed in 1 ml of 70% ethanol at 4 ºC. Pellet was dried and then dissolved in 50 µl of TE buffer containing RNase (100 µg/ml).

3.1.4. Restriction enzyme digestion and electrophoresis

Plasmid DNA of the selected single colonies was cut with the restriction endonuclease *Sac*I in a total reaction volume of 10 µl at 37 ºC for 3 h. The digested DNA was separated on agarose gel. Products were evaluated under UV light after ethidium bromide staining.

3.1.5. PCR analysis

PCR on plasmid DNA of the selected single colonies was carried out for examining the presence of the *nptII*, *GA2ox* and *gus* genes. The primers used for amplification of the *nptII* gene were 5′-GCC CTG AAT GAA CTG CAG GAC GAG GC-3′ and 5′-GCA GGC ATC GCC ATG GGT CAC GAC GA-3′, generating a 411 bp product. Four different primer pairs were used to amplify the *GA2ox* gene, GA2ox1-2: 5′-CAA CCA TGG TTG TTC TGT CTC A-3′ and 5′-CTA GAG TTG CGT TCC AT GAC A-3′, resulting in a 1203 bp product, GA2oxa1-2: 5′-CAA CCA TGG TTG TTC TGT CTC A-3′ and 5′-CGC TTC CAT TGA

CAA TAG AGG A-3′, with a 1203 bp product, GA2oxb1-2: 5′-TTG TTC TGT CTC AGC CAG CA-3′ and 5′-CTA GAG TTG CGT TCC ATT GAC A-3′, with a 1203 bp product and GA2oxc1-2: 5′-CGA CAA CAA CAA CAA CAA CCA-3′ and 5′-CTC CCC AAA CCC AAC CAA-3’ generating a 632 bp product. The primers used for amplification of the *gus* gene were: 5′-CCT GTA GAA ACC CCA ACC CGT G-3′ and 5′-CCC GGC AAT ACC ATA CGG CGT G-3′, with a product of 365 bp. Sample DNA was used in a reaction volume of 10 µl with the following program: initial denaturation, 5 min at 95 ºC, followed by 30 cycles of 15 s denaturation at 95 ºC, 15 s annealing at 65 ºC for *nptII* and *gus* or 52 ºC for *GA2ox* primers, 30 s elongation at 72 ºC. The program was ended with a final elongation step, 5 min at 72 ºC. PCR products were stored at 4 ºC until the samples were evaluated on agarose gel under UV light after ethidium bromide staining.

3.2. Southern blot analysis of transgenic apple

3.2.1. Plant material

The plant material was in vitro grown shoots of transformed apple rootstock A2 with the *rolC* and *nptII* genes (Kāle 2006).

3.2.2. Southern blot hybridization

Genomic DNA was extracted from four weeks old in vitro propagated transformed clones and untransformed control shoots according to Aldrich (1993).

The Southern hybridization was based on a non-radioactive digoxigenin method as described by Holefors et al. (1998). Thirteen different samples originating from different transgenic shoots were analyzed. Twenty µl of DNA from each sample was digested with the restriction endonuclease *Eco*RI in a total restriction volume of 100 µl for 6 h. The digested DNA was separated on 0,7 % agarose gel and transferred to a positively charged nylon membrane (Boeheringer and Mannheim). The hybridization was carried out in 10 ml of standard hybridization buffer at 68°C for 18 h. Probes used had been produced by PCR with *nptII* and *rolC* primers the same as for PCR analyses. After hybridization, the membrane was washed and the hybridized signals were detected by chemiluminiscent detection using the CSPD solution at 37°C for 15 minutes and the membrane was exposed to an X-ray film for 6 h or longer.

4. Results

4.1. Bacterial transformation

Many single *E. coli* colonies were obtained on the selectable medium. Ten of them were further cultured on the selection medium. From these 10 colonies 4 were chosen for molecular analyses, including restriction enzyme digestion and PCR. *A. tumefaciens* grew well on the selectable medium after transformation. Ten colonies were further cultured on the selectable medium and six of them were analyzed by PCR. Due to time limitation, only PCR analysis was performed on the DNA of the selected colonies.

4.1.1. Verification of transformation events by restriction enzyme digestion

E. coli colonies were digested with the restriction enzyme *Sac*I (Fig.1). The digestion resulted in a weak band in lanes 4, 6 and 7 representing *E. coli* single colonies 1, 3 and 4. The size of the band is slightly more than 1000 bp and the expected size of the *GA2ox* gene is 1006 bp. This result indicates the presence of the *GA2ox* gene in the vector and the successful of transformation of the vector into the bacteria.

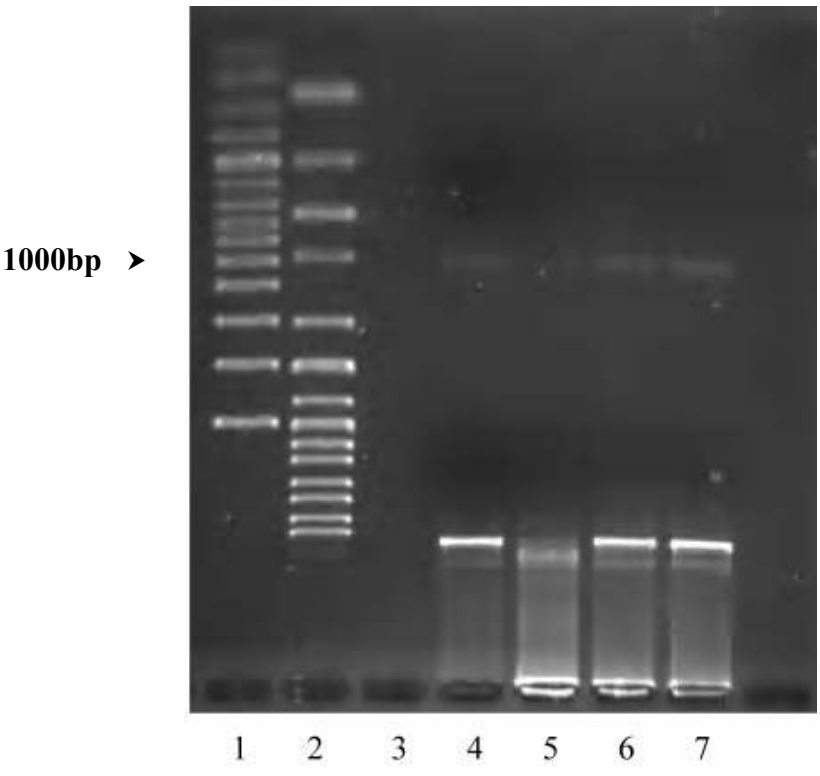

Figure 1. The 1006 bp product digested by *Sac*I. From left to right: 100bp molecular weight marker (lane 1); 1000bp molecular weight marker (lane 2); empty (lane 3); *E. coli* single colonies 1-4 (lanes 4-7).

4.1.2. Verification of transformation events by PCR analyses

For the *E. coli* transformation, all 4 single colonies showed a PCR positive for the *gus* gene (Fig.2) and *nptII* (Fig.3). PCR results showed negative for the *GA2ox* gene for all 4 colonies (Fig.4).

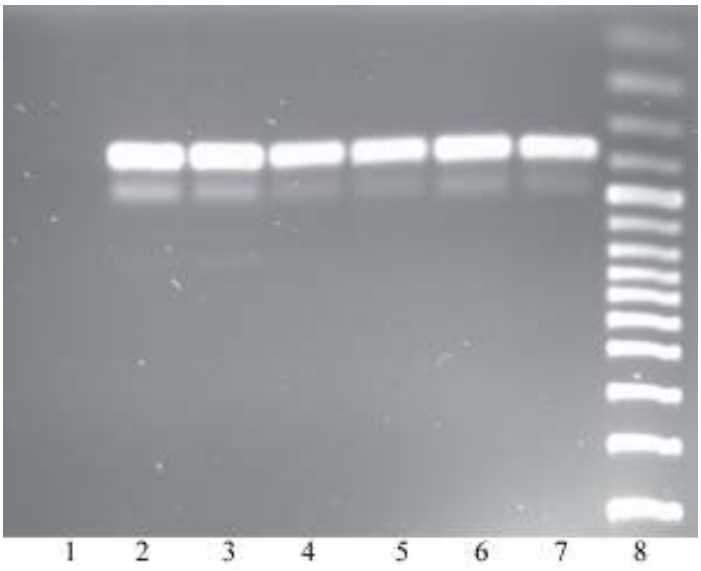

Figure 2. The 365 bp DNA fragment of the *gus* gene amplified by PCR. From left to right: Blank negative control (lane 1); *GA2ox* original DNA (lane2); positive control (lane 3); transformed *E. coli* single colonies 4-1 (lane 4-7); DNA molecular weight marker (lane 8).

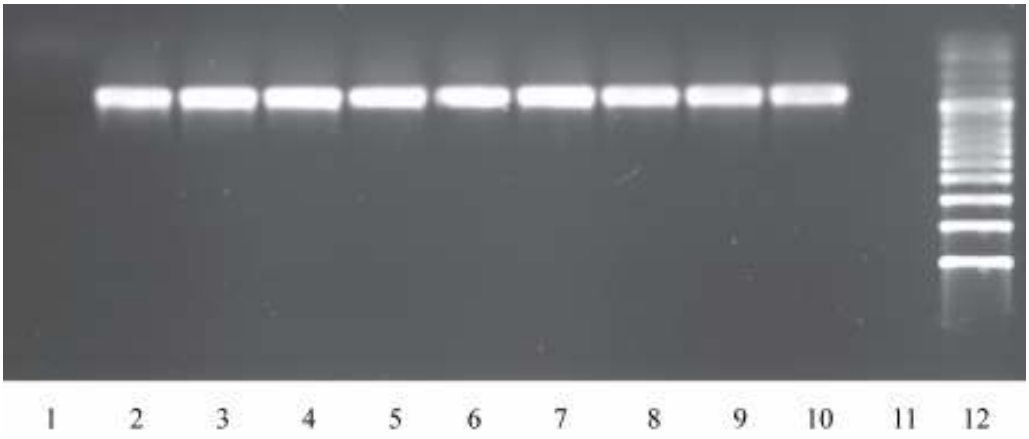

Figure 3. The 411 bp DNA fragment of the *nptII* gene amplified by PCR. From left to right: Blank negative control (lane 1); positive control (lane 2); *E. coli* single colonies 4-1, 100x

dilution (lane 3-6); *E. coli* single colonies 4-1, 50x dilution (lane 7-10); empty (lane 11); DNA molecular weight marker (lane 12).

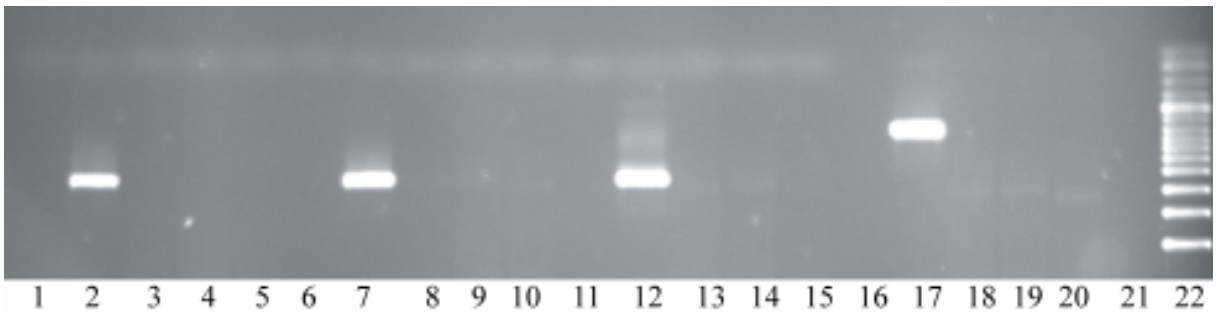

Figure 4. *GA2ox* gene amplified by PCR with four different primers. From left to right: GA2oxa primers with a 1203 bp product (lanes 1-5): blank negative control (lane 1); positive control (lane 2); *E. coli* single colonies 3-1 (lanes 3-5), GA2ox primers with a 1203 bp product (lanes 6-10): blank negative control (lane 6); positive control (lane 7); bacterial colonies 3-1 (lanes 8-10), GA20oxb primers with a 1203 bp product (lanes 11-15): blank positive control (lane 11); positive control (lane 12); bacterial colonies 3-1 (lanes 12-15), GA2oxc primers with a 632 bp product (lanes 16-20): blank negative control (lane 16); positive control (lane 17); bacterial colonies 3-1 (lanes 18-20); empty (lane 21); molecular weight marker (lane 22).

4.2. Southern blot analysis of transgenic apple

The transgenic plants obtained by Kāle, 2006 were analyzed by Southern blot hybridization to further confirm the transformation events and to determine the copy number of the transgenes. Fig. 5.A and B show the Southern results of the copy number of the *nptII* and *rolC* genes, respectively. From the results it can be suggested that the two clones named rolC(1)-rbcS3-6 and rolC(1)-rbcs3-5, lane 11 and 12 have four copy numbers of both *nptII* gene and *rolC* genes. These two clones were originated from the same leaf explant, the band patterns are the same, indicating that they were from the same transformation event. Clone rolC(1)-rbcS5-7, lane 10 probably has one copy number of the *nptII* and *rolC* genes, respectively, as shown in Fig. 5.A and Fig. 5.B Clone rolC(1)-rbcS5-1, lane 9 was originated from the same leaf explant as clone rolC(1)-rbcS5-7, lane 10. However, the different band patterns indicate that they were from different transformation events. Clone rolC(1)-rbcS5-1, lane 9 has at least 3 copy numbers of both genes. Clone rolC(1)-rbcS4-2, lane 8 has four copy numbers of both genes. Clone rolC(1)-rbcS9-1, lane 4 has one copy number of the two transgenes. Lane 13 is an untransformed A2-control clone. Lane 2, 3, and 5 representing

clones rolC(1)-rbcs12-14, 5-5 and 5-4 show no indication of having been transformed, not Lane 6 and 7 either, representing clones rolC(1)-35S1 and rolC(1)-35S2.

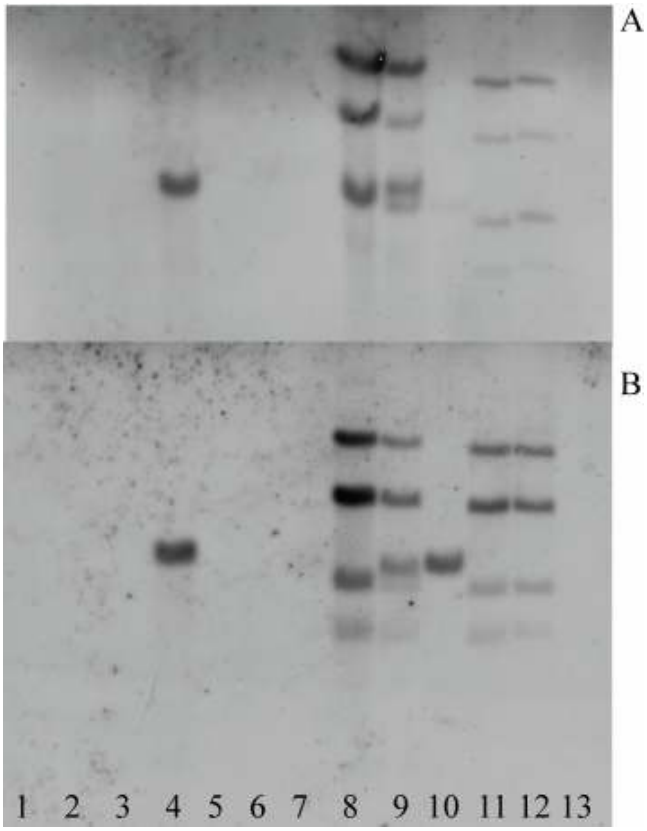

Figure 5. Southern analyses of copy numbers of the *nptII* and *rolC* genes after digestion with *Eco*RI and hybridisation with the *nptII* probe (A) and the *rolC* probe (B). Lane 1-12 transgenic clones, lane 13 untransformed control. Lane 6 and 7 with the 35S promoter, lane 1-5 and lane 8-12 with the rbcS (ribulose-1,5-bisphosphate carboxylase small subunit) promoter.

5. Discussion

5.1. Bacterial transformation

One of the aims with this project was to transform a binary vector containing the *GA2ox* gene into *A. tumefaciens* for preparing a transformation vector for future use. To achieve this aim *E. coli* was first transformed with the binary vector carrying the gene for quick multiplication of the plasmid. Transformation of *E. coli* was confirmed by PCR and restriction enzyme digestion with the endonuclease *Sac*I. A PCR analysis was first performed to confirm the presence of the *GA2ox* gene, but no positives were found among the four *E. coli* colonies analyzed. Later the presence of the *nptII* gene was confirmed in all the selected

E. coli colonies. This result was expected as all colonies grew well on LB medium with 50 mg l^{-1} kanamycin. If the *nptII* gene has been transformed into the bacteria the *GA2ox* gene should also have been transformed too, because they are supposed to have been cloned into the same vector. At the beginning it was thought that the PCR condition for *GA2ox* might not be optimal, some parameters affecting PCR, such as DNA template concentration, different primers and annealing temperature were tested, but still no positives were observed among the tested colonies. Since in the original vector the *gus* gene was replaced by the *GA2ox* gene. We suspect that the vector is not modified. We then performed PCR on the *gus* gene and found out the presence of the *gus* gene instead of the *GA2ox* gene. Our result suggests either that the *GA2ox* gene was not cloned into the vector pLARS120 or that the DNA sequence of *GA2ox* cloned into the vector was not from the runner bean. In our PCR analyses, we used the sequence from runner bean for designing *GA2ox* primers. To identify the problem with the vector, further studies are needed.

A. tumefaciens was transformed with the binary vector pLARS120 DNA isolated from *E. coli* to prepare a transformation vector. Single colonies were obtained on LB medium with kanamycin 50 mg l^{-1}, suggesting that transformation was successful. However, transformation was not confirmed by PCR. This gives a doubtable conclusion that the bacteria colonies were transformed. It happens that *A. tumefaciens* sometimes give rise to false transformation events. More detailed studies are required for *Agrobacterium* transformation. However there was no time for further investigation of this within the project period.

5.2. Southern blot analysis

The Southern blot analysis showed that one to four copies of the *rolC* and *nptII* genes were integrated into the genome of six clones out of total twelve previously transformed clones confirmed only by PCR by Kāle 2006.

The copy number of a transgene integrated into the genome of a transformed plant and the site of integration can not be controlled (Vaucheret et. al. 1998). Single-copy transgene integration seems to be a prerequisite for the stable expression of the transgene (Kumar & Fladung 2001). Epigenic transgene inactivation may occur when several copies of the transgene is integrated into the genome. It is suggested that the host genome combined with environmental factors may affect the long-term transgene expression. The insertion of foreign DNA into a plant genome may lead to changes in its structure, which can affect the host and transgene expression. Transgene silencing can be due to the position effect where the flanking plant DNA and chromosomal position of the transgene negatively influence the expression of

the transgene. Stable expression of transgenes is important for commercial use of plant transformation in long lived tree species and also important for ecological risk assessment studies.

In this study two clones rolC(1)-rbcs9-1 and rolC(1)-rbcs5-7 had a single copy of the transgenes. These two clones might be the ones useful for further investigation in greenhouse and field trials, as they will probably have the better chance for stable expression of the transgenes.

6. Conclusions

Transformation of *E. coli* with the binary vector pLARS 120 was confirmed by PCR. It was confirmed that the pLARS120 vector did not contain the *GA2ox* gene, or at least not the sequence from the runner bean. It was shown that the vector contained the *gus* gene. Transformation of the vector into *A. tumefaciens* was not confirmed by PCR even though bacteria grew well on kanamycin containing LB medium. Further studies are required.

The Southern blot analyses confirmed transformation of 6 clones of the apple rootstock A2 with the *nptII* and *rolC* genes. Two of the clones show single copy insertion of both genes. These two clones are preferable for future growth analysis in greenhouse and field trials for eventual commercialization.

Acknowledgements

I thank my supervisor and examiner Li-Hua Zhu for letting me work with this interesting project, for helpful discussion and for offering suggestions and corrections on the thesis and Annelie Ahlman for invaluable explaining and guidance in the laboratory. I would also like to thank the people working in the Department of Plant breeding and Biotechnology for being inspiring.

References

Aldrich & Cullis.(1993), RAPD analysis in flax: optimization of yield and reproducibility using Klen*Taq*I DNA polymerase, Chelax 100, and gel purification of genomic DNA. *PlantMolecular Biology Reporter* ll (2): 128-141

Baumforth, K. R. N., Nelson, P. N., Digby, J. E., O'Neil, J. D., & Murray, P. G. (1999), Demystified…The polymerase chain reaction. *J Clin Pathol: Mol Patho*, 52: 1–10

Bermingham, N., & Luettich, K. (2003), Polymerase chain reaction and its applications. *Current Diagnostic Pathology,* 9: 159-164

Chen, I. & Dubnau, D. (2004), DNA uptake during bacterial transformation. *Nature reviews Microbiology ,*2:241-249

de Frammond, A. J., Barton, K. A., & Chilton, M. D. (1983), Mini-Ti-: a new vector strategy for plant genetic engineering. *Bio/Technology,* 5:262-269

Ebinuma, H., Sugita, K., Matsunaga, E. and Yamakado, M. (1997), Selection of marker-free transgenic plants using the isopentenyl transferase gene. *Proc. Natl. Acad. Sci. USA,* 94: 2117-2121

Engler-Blum, G., Meier, M., Frank, J., & Müller G. A. (1993), Reduction of Background Problems in Nonradioactive Northern and Southern Blot Analyses Enables Higher Sensitivity Than ^{32}P-Based Hybridizations. *Analytical Biochemistry,* 210:235-244

Gelvin, S. B. (2003), *Agrobacterium*-Mediated Plant Transformation: the Biology behind the "Gene-Jockeying" Tool. *Microbiology and Molecular Biology Reviews*, 67 (1): 16-37.

Hamill, J. D., Rounsley, S., Spencer, A., Todd, G., & Rhodes, M. J. C. (1991), The use of the polymerase chain reaction in plant transformation studies. *Plant Cell Reports,* 10: 221-224

Hedden, P. & Phillips, A. L. (2000), Gibberellin metabolism: new insights revealed by the genes. *Trends in plant science Reviews*, 5 (12): 523-530

Hellens, R., & Mullineaux, P. (2000), A guide to Agrobacterium binary Ti vectors. *Trends in plant science,* 5 (10) :446-451

Hoekema, A.,. Hirsh, P. R., Hooykaas, P. J. J., & Schilperoort, R. A. (1983), A binary plant vector strategy based on separation of *vir*- and T-region of the *Agribacterium tumefaciens* Ti-plasmid. *Nature,* 303:179-180.

Holefors, A., Xue, Z-T., & Welander, M., (1998), Transformation of apple rootstock M26 with the *rol* A gene and its influence on growth. *Plant Science,* 136: 69-78

Inoue, H., Nojima, H., & Okayama H. (1990), High efficiency transformation of *Escherichia coli* with plasmids. *Gene,* 96: 23-28

Kumar, S., & Fladung, M. (2001), Gene stability in transgenic aspen (*Populus*). II. Molecular characterization of variable expression of trangene in wild and hybrid aspen. *Planta,* 213: 731-740

Kāle, L. (2006), Master Thesis in Biology: Transformation of apple (*Malus domestica*) rootstock A2 with either *GA20ox* or *RolC* gene to reduce plant size. Department of crop science, Sveriges Lantbruksuniversitet (SLU).

Lange, T. (1997), Cloning gibberellin dioxygenase genes from pumkin endosperm by heterologous expression of enzyme activities in *Escherichia coli*. *Proc. Nat., Aca., Sci. USA,* 94: 6553-6558

MacMillan, J. (1997), Biosynthesis of the gibberellin plant hormones. *Natural Product Reports*, 14: 221-244

Pawan,. K. J., Sahoo, L., Singh, N. D., & Singh, R. P. (2002), Startegies to deal with the concern about marker genes in transgenic plants: Some environment-friendly approaches. *Current Science,* 83 (2): 128-136

Primrose, S.B., & Twyman, R. (2006), *Principles of Gene Manipulation and Genomics,* 7nd edition. Blackwell Publishing Professional, Blackwell. p 18-20, 24-26

Schomburg, F. M., Bizzell, C. M., Lee, D. J., Zeevaart, J. A. D., & Amasino, R. M. (2003), Overexpression of a Novel Class of Gibberellin 2-Oxidases Decreases Gibberellin Levels and Creates Dwarf Plants. *The Plant Cell*, 15: 151-163

Southern, E. M (1975), Detection of Specific Sequences Among DNA Fragments Separated by Gel Electrophoresis. *J. Mol. Biol.,* 98:503-517

Thomas, S. G., Phillips A. L., & Hedden, P. (1999), Molecular cloning and functional expression of gibberellin 2-oxidases, multifunctional enzymes involved gibberellin deactivation. *Plant Biology*, 96: 4698-4703

Vaucheret, H., Béclin, C., Elmayan, T., Feuerbach, F., Godon, C., Morel, J-B., Mourrain, P., Palauqui, J-C., & Vernhettes, S. (1998), Transgene-induced gene silencing in plants. *The Plant Journal,* 16 (6): 651-659

Welander, M. & Zhu, L-H. (2006), Rol Genes: Molecular Biology, Physiology, Morphology, Breeding Uses. *Plant Breeding Reviews,* 26: 79-103

Zhu, L-H., Holefors, A., Ahlman, A., Xue, Z-T. & Welander, M. (2001), Transformation of the apple rootstock M.9/29 with the *rol*B gene and its influence on rooting and growth. *Plant Science,*160:433-439

Zupan, J., Muth, T. R., Draper, O., & Zambryski, P. (2000), The transfer of DNA from *Agrobacterium tumefaciens* into plants: a feast of fundamental insights. *The Plant Journal,* 23 (1): 11-28

Internet:

Stratagene http://www.stratagene.com/manuals/200249.pdf 4/4-07

Roche www.roche-applied-science.com 1/5 -07