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AGROBACTERIUM-MEDIATED GENETIC TRANSFORMATION OF APPLE (*MALUS DOMESTICA* BORKH.)

A Thesis in

Plant Physiology

by

Siela N. Maximova

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Submitted in Partial Fulfillment

of the Requirements

for the Degree of

Doctor of Philosophy

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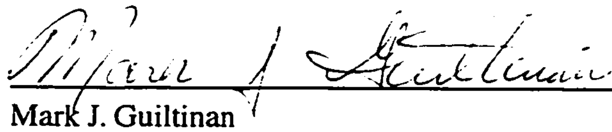

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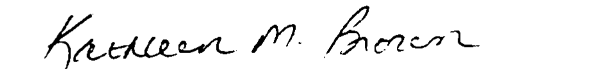

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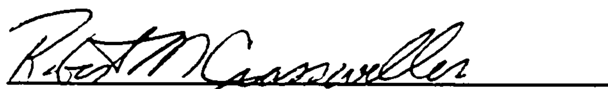

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ABSTRACT

Among deciduous fruit tree species, apple is an attractive candidate for gene transfer because of its economic importance (Dandekar *et al.*, 1990), ability to easily regenerate from somatic tissue (Belaizi *et al.*, 1991; Fasolo *et al.*, 1989; James *et al.*, 1988b), long juvenile period, and high level of self incompatibility. Using the classical selection methods, most improvements in apple are achieved by hybridization and mutagenesis, but for many woody perennial crops these methods are hindered by a long generation time and high heterozygosity. Unlike sexual breeding, molecular genetic strategies involving gene transfer allow direct modification of existing cultivars.

The importance of developing efficient micropropagation, regeneration, and transformation systems for introducing foreign genes into plant species is paramount for the successful application of genetic engineering. *Agrobacterium*-mediated gene transformation has become an important tool for genetic improvement of agriculturally important crop species. Studies in several laboratories have successfully produced transgenic apple plants; however, the transformation frequencies (based on the number of transformants divided by the number of control nontransformed and nonselected regenerants) have been low (0.2% to 2.8%) (James *et al.*, 1989; Maheswaran *et al.*, 1992; Yao *et al.*, 1995). Additionally, transformants will not always express inserted genes at desired levels, which may be the result of positional effects causing variation in expression levels between independent transformants, unintact inserted genes, or gene silencing. Therefore, 20-50 independent transformants are needed for each gene introduced to ensure that at least one line exhibits high level stable expression.

There are four main components of the *Agrobacterium* genetic transformation process: initiation (selection of explants), infection, selection of transformed cells, and

regeneration of transformed plants. The success of this process is influenced by many factors including bacterial strain and plasmid type, bacteria virulence induction, co-cultivation time and media, preculture of the explants and age of the tissue. The objectives of this thesis research were to investigate the main factors influencing the genetic transformation of apple, and to use this knowledge to develop a high efficiency transformation system.

The first step for optimizing the system is to establish a highly efficient rooting protocol for apple scion cultivars, using axillary shoots from *in vitro* proliferating cultures. Such *in vitro* propagated and rooted plants provide a large supply of leaf tissue for regeneration and transformation experiments. Rooting of *in vitro* propagated shoots was induced using a simplified liquid culture medium containing 15 μM indole-3-butyric acid (IBA), followed by a root elongation phase on an agar solidified medium. Under optimal conditions (root induction for two days at 25°C), rooting frequencies (percentage of rooted plants) of 'Delicious', 'Golden Delicious', 'Fuji', 'Royal Gala' and 'Empire' varied from 60% to 98%, with the mean number of roots per rooted plant ranging from 4.2 to 20.7. Considerable variation among cultivars was observed with respect to their response to culture conditions. The duration of the root induction period in the presence of IBA (15 μM) and incubation temperature strongly affected rooting of all apple cultivars studied. A basipetal V-shaped cut with an angle of approximately 30° to the shoot axis enhanced root formation and increased the number of roots per shoot in all cultivars studied. This system allows for rooting of apple shoots *in vitro* within 10-20 days of culture initiation and rooted plants can be rapidly transferred to greenhouse conditions or utilized for genetic manipulations.

A important part of the transformation procedure was to optimize the adventitious shoot regeneration system for four apple scion cultivars. The explants, 7 mm diameter leaf discs from three-week old *in vitro* rooted plants were cultured on BNZ511 medium (James, 1991) and shoot regeneration occurred in 14 to 30 days. Shoot regeneration was significantly enhanced when 3% sorbitol was substituted for sucrose as a carbohydrate source in the regeneration medium. Sorbitol induced the formation of large numbers of healthy shoots. The substitution of PhytaGel™ in place of agar as the solidifying agent in the regeneration media also enhanced regeneration. No significant variation in response to this system was observed among the four cultivars originally tested and subsequently the procedure has been successfully applied for regeneration of other apple cultivars.

To increase the transformation and regeneration efficiencies of the *Agrobacterium*-mediated gene transfer system, preculture of 'Fuji', leaf explants with 3 mg/l IBA for various times prior to bacterial infection was evaluated. The *Agrobacterium tumefaciens* disarmed strain C58C1 containing plasmid p35S GUS INTR (Vancanneyt *et al.*, 1990) with a chimeric β-glucuronidase (GUS) reporter gene and a neomycin transferase (*nptII*) gene conferring resistance to the antibiotic kanamycin was used. Percentage of transformed explants and mean number of GUS expressing areas were measured by GUS histochemical assay. Preliminary results indicated that IBA preculture of 'Fuji' leaf explants did not enhance transformation frequency.

To investigate early events of *Agrobacterium*-mediated transformation of apple cultivars, a synthetic green fluorescent protein gene (SGFP) was used as a reporter gene. Leaf explants from four apple cultivars ('Delicious', 'Golden Delicious', 'Royal Gala' and 'Greensleeves') were transformed with *Agrobacterium* EHA101 harboring plasmid pDM96.0501. SGFP expression was first detected 48 hours after co-cultivation and the results indicated a very high T-DNA transfer rate. Plant cells with stably incorporated T-

DNA exhibited cell division and developed transgenic calli, followed by formation of transgenic shoots at low frequencies. The detection of SGFP expression with an epi-fluorescence stereo microscope confirmed the effectiveness of GFP as a reporter gene for detection of very early transformation events and for screening of putative transformants. The efficiency of the transformation and regeneration process decreased approximately 10, 000 fold from *Agrobacterium* infection to transgenic shoot regeneration, suggesting that factors other than *Agrobacterium* interaction and T-DNA transfer are rate limiting steps in *Agrobacterium*-mediated transformation of apple.

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Chapter 1

LITERATURE REVIEW

1.1 *Tissue culture of apple*

1.1.1 *Rooting apple cultivars and rootstocks in vitro*

Micropropagation of apple involves culture of isolated buds on cytokinin-rich media, multiplication of these buds through the outgrowth and excision of lateral meristems, and rooting of these shoots through adventitious root formation on auxin containing media. Extensive efforts have been made to develop efficient micropropagation and rooting procedures for different apple cultivars and rootstocks (James & Thurbon, 1981a; James & Thurbon, 1981b; Sriskandarjah *et al.*, 1982; Zimmerman, 1983; Zimmerman & Broome, 1981). Some of the factors that influence rooting of apple shoots *in vitro* are the presence of the phenolic compound phloroglucinol (PG) in the propagation and root induction media, auxin concentration in the root induction media, etiolation of proliferating shoots, sucrose and agar concentration, the nature and concentration of the auxin applied, the number of subcultures of the shoots prior to root induction, cultivar differences, temperature, light conditions, and others. PG has been reported to stimulate rooting of M.9, 'Spartan', 'Redspur Delicious' and 'Delicious' (James & Thurbon, 1981a; James & Thurbon, 1981b; Sriskandarjah et al., 1982; Zimmerman, 1983; Zimmerman & Broome, 1981). A synergism between auxin and PG was investigated for two different

rootstocks, M.9 (with low rooting potential) and M.26 (with high rooting potential) which were induced to root by exposure to 5 mg/l IAA and/or 162 mg/l PG for 6 days in the dark (James & Thurbon, 1981a). The presence of PG during both shoot induction and root initiation resulted in the highest rooting percentage in both rootstocks tested - M.9 (90%) and M.26 (75%). The influence of this compound (in combination with IBA) on the rooting of eight other apple cultivars was tested and a positive effect was recorded for only one cultivar, 'Spartan', where PG significantly increased the number of roots per cutting (Zimmerman & Broome, 1981).

The research group at the U.S. Department of Agriculture, Agricultural Research Service, Beltsville, MD, under the leadership of Dr. Richard Zimmerman has made major contributions to the optimization of rooting procedures for a large number of apple cultivars. In a summary of five years of research on factors affecting rooting of 17 apple cultivars, this group reported that optimum rooting was attained at a concentration range from 0.1 to 0.3 mg/l auxin (IBA or NAA). PG had no effect on 12 of the cultivars tested, agar concentrations from 4.5 to 8.5 mg/l did affect the rooting of two cultivars, and etiolation of the proliferating cultures, followed by several days of re-greening improved the rooting of four cultivars (Zimmerman, 1983). The time for rooting recorded for the different cultivars varied from 2 to 4 weeks (Zimmerman & Broome, 1981). The interactions among light, temperature, PG and auxin were also studied and several strains of 'Delicious' apple cultivar were successfully rooted with up to 100% efficiency when shoots were placed in the dark at 30°C for one week on PG and IBA media (Zimmerman, 1984). Perhaps one of the most popular methods for rooting apple cultivars *in vitro* was published in 1985 (Zimmerman & Fordham, 1985). Shoots from proliferated shoot cultures were incubated for 7 days in the dark on a simplified liquid root induction media containing only 43.8 mM sucrose and 1.5 μM IBA at 30°C. After root initiation, the

shoots were transferred into non-sterile preformed peat plugs for 3 weeks, acclimatized and then moved to greenhouse conditions. The method was applied to 12 cultivars and the rooting percentages varied from 12 to 100%, emphasizing genotypic effects. The same group observed that depending on the apple cultivar, rooting on agar solidified media could be more advantageous (Zimmerman *et al.*, 1987). In the same report it was shown that a 6 hour soak treatment at higher auxin concentrations can substitute for a 7-day incubation in very low concentrations.

The importance of sucrose as a rooting media component was demonstrated with the apple rootstock 'Antonovka 313' (Travers *et al.*, 1985). One hundred percent rooting was consistently achieved with shoots cultured on modified Murashige and Skoog (MS) salt medium (Murashige & Skoog, 1962) supplemented with 0.25 μM IBA and 3% sucrose. When shoots were placed on a medium without sucrose the rooting percentages decreased dramatically.

Although high root induction frequencies have been achieved, most of the published procedures are time consuming and efficient only for a limited number of rootstock or scion cultivars. For the *in vitro* propagation of a large number of apple plants of varying genotypes for marketing, as well as for the production of rooted plants for genetic manipulations, a more efficient, rapid and less genotypically dependent rooting protocol would be advantageous.

1.1.2 *Regeneration of adventitious shoots from apple somatic leaf tissue*

High frequency regeneration of adventitious shoots from somatic tissues of fruit trees is a major prerequisite for the success of organogenesis-based genetic engineering

methods such as *Agrobacterium*-mediated genetic transformation (Dandekar, 1993; James & Dandekar, 1991; James *et al.*, 1988a). For this purpose, leaf explants from apple plants grown *in vitro* have often been used. Leaf tissue contains no pre-existing shoot primordia and the tissue is more likely to produce non-chimeral plants (Broertjes & Van Harten, 1988).

Apple leaves regenerated *in vitro* are influenced by many factors (Fasolo et al., 1989). The effect of phytohormones in the media on regeneration of adventitious shoots appears to be related not only to concentration but also to treatment length and time of application (Fasolo & Predieri, 1990). Close to 100% shoot regeneration from *in vitro* propagated leaves of M.26 rootstock was achieved on media with 4.4 μM BA and 0.5 μM NAA (Predieri & Fabbri Malavasi, 1989). A dark period also proved beneficial to regeneraiion frequencies (Predieri & Fabbri Malavasi, 1989; Welander, 1988). Welander (1988) reported up to 85% regeneration of adventitious shoots from mature leaves of the cultivar McIntosh after a three week dark treatment. *Malus domestica* leaf regeneration also depends on the position of the leaf on the mother-shoot and its age (Druart, 1990). The three youngest fully unfolded leaves of the apple plants showed the highest regeneration potential (James, 1991).

Regeneration of adventitious buds has been very successful for various scion and rootstock cultivars, but there is a need for improvement of the system for the important apple scion and rootstock cultivars (Ancherani *et al.*, 1990; Predieri & Fabbri Malavasi, 1989; Welander, 1988).

1.1.3 *Influence of the carbohydrate source on regeneration*

The influence of the type and concentration of carbohydrate source in tissue culture media on growth and shoot formation of apple also has been investigated (Chong & Taper, 1974; Pua & Chong, 1984; Pua & Chong, 1985). Sorbitol is one of the important carbon sources in tissue culture of fruit trees. It is required for the survival of *in vitro* shoot cultures of *Malus robusta* Mill. (Pua & Chong, 1984), and induces high frequency shoot formation from explant sources in *Prunus cerasus* L. (Borowska & Szczerba, 1991), *Malus pumila* Mill.(apple) (Pua & Chong, 1985), *Actinidia deliciosa* (actinidia) (Leva & Bertocci, 1988), *Annona cherimola* (cherimoya), *Solanum muricatum* (pepino), and *Cyphomandra betacea* (tamarillo) (Jordan *et al.*, 1992). Chong and Taper (Chong & Taper, 1974) discovered that sorbitol is most effective for the growth of callus derived from the stem of *Malus robusta.*

In apples, pears and apricots, sorbitol (a sugar alcohol) is the main product of photosynthesis and the main carbohydrate translocated in the phloem (Bieleski, 1982; Moorby, 1982). Bielski (1982) demonstrated that actively growing apple tissues contains mainly sorbitol, while dormant tissues contain mainly sucrose. This is consistent with the results of Loescher et al. (1982), who observed that the young apple leaves convert predominantly sorbitol into CO_2. Subsequently it has been shown that adventitious shoot regeneration in apple leaf tissue occurs at the highest frequencies in actively growing tissues (Fasolo & Predieri, 1990; James & Dandekar, 1991; James et al., 1989; Welander, 1988). The substitution of sorbitol for sucrose in the regeneration media could provide a carbohydrate source more easily metabolized by the young apple leaf tissue and contribute to increased regeneration frequencies.

1.2 *Methods for plant genetic engineering*

Due to the long juvenile and reproductive phase, high heterozygosity, and self incompatibilities, the improvement of woody plants by conventional breeding methods is difficult and time and labor consuming. Perhaps the greatest potential for the improvement of tree species is in the application of genetic engineering technology. Genetic transformation involves the transfer of foreign genes into the genome of the recipient plant cells and recovery of transgenic plants that stably express the foreign genes.

The two major methods of gene transfer utilize either a bacterial vector or direct introduction of foreign DNA into isolated plant tissues, cells or protoplasts by various physical means.

1.2.1 *Agrobacterium-mediated genetic transformation*

Agrobacterium tumefaciens and *A. rhizogenes* are gram-negative soil bacteria which, upon wounding and infection, induce crown gall and hairy root disease respectively in many dicotyledonous plants. Tumor or hairy root induction are associated with the presence within the bacteria of a megaplasmid, the tumor-inducing (Ti) plasmid of *A. tumefaciens* or the root-inducing (Ri) plasmid of *A. rhizogenes*. Both plasmids contain a defined region known as transfer-DNA (T-DNA), which is transferred into the plant cell and integrated into the plant nuclear genome. The transfer of T-DNA is controlled by virulence (*vir*) genes located on the Ti/Ri plasmids, which synthesize enzymes responsible for excision of the T-DNA and which also mediate T-strand formation (Zambryski, 1992).

One or more copies of the T-DNA can be transferred to the plant cell. T-DNA can occur in tandem repeats or can be separated and linked to different regions of the plant genome. The site of T-DNA integration into the host nuclear DNA is apparently random.

T-DNA contains structural genes, preceded by eukaryotic promoter sequences which control the synthesis of phytohormones. The new genes products override the normal regulation of the plant metabolic pathways involved in the synthesis of phytohormones, resulting in an oncogenic phenotype (Draper *et al.*, 1988). Hence, these genes are considered to be oncogenes (*onc*). Genes are also present on the T-DNA for the synthesis of novel amino acids and sugar derivatives known as opines, which are carbon and nitrogen sources for *Agrobacterium.* The production of different opines by the plant cell depends on the strain of *Agrobacterium.* Octopine and nopaline are two types of opines that are among the easiest to detect in tumor tissue. The biology of the Ti and Ri plasmids and the mechanism of T-DNA transfer have been reviewed by several authors (Draper et al., 1988; Klee *et al.*, 1987; Schell *et al.*, 1979; Zambryski, 1992).

An important discovery was that the *onc* genes are not required for T-DNA transfer to the plant cell nor for its integration into the nuclear DNA. Therefore these genes can be deleted from the Ti/Ri plasmid and replaced. Another advance was the development of plant selectable marker genes that confer resistance to antibiotics, which allow for the selection of transformed cells from mixed populations.

Non-oncogenic or disarmed plasmids can be divided into two types, depending on whether the T-DNA regions are located on the same replicon as the *vir* genes (*cis*-acting *vir* genes) or on a separate plasmid (*trans*-acting *vir* genes). The *cis* vectors are also referred to as co-integrative vectors and the *trans* vectors as binary vectors. *Cis* vectors are derivatives of wild-type Ti plasmids in which the *onc* genes were replaced by DNA with a region of homology to a small cloning vector that can replicate only in *E. coli.* An example of such a

co-integrative vector is plasmid pGV3850, which is a derivative of the T37 nopaline Ti plasmid and contains pBR322 in place of most of the T-DNA (Zambryski *et al.*, 1983). One disadvantage of the co-integrative vectors is the relatively low frequency (10^{-4} and 10^{-7}) of cointegrate formation (Klee et al., 1987). *Trans* or binary vectors are based on plasmids that can replicate in both *E. coli* and *Agrobacterium*. The binary component can be manipulated in *E. coli* and transferred to *Agrobacterium* strains that contain a Ti plasmid with a *vir* region, but lacking T-DNA borders. The binary vectors are maintained in both, *E. coli* and *Agrobacterium* by antibiotic selection (Draper et al., 1988). A commonly used binary vector is pBin19, based on the wide host range plasmid pRK252 (Bevan, 1984). To date, the *Agrobacterium*-mediated gene transformation system has been utilized for a great range of herbaceous and woody plant species including fruit trees such as apple, cherry, plum (James *et al.*, 1985), and pear (Browning *et al.*, 1985).

1.2.2 *Direct transfer of foreign DNA into plant cells*

Agrobacterium-mediated transformation is a natural and simple method for gene transfer, but many plant species, including corn, rice, and wheat are not its natural hosts and are not readily transformed by the method. The difficulties in woody plant transformation are often a result of low susceptibility of the plant tissue to *Agrobacterium* or the inability of the tissue to regenerate shoots, even when the transformation has been successful. As an alternative approach, methods for direct transfer of foreign DNA into plant tissues, cells or protoplasts were developed. Considerable efforts have been directed towards introducing free DNA into isolated plant protoplasts. Protoplasts have been transformed at high pH using a polyethyleneglycol (PEG)-mediated uptake procedure

(Krens *et al.*, 1982; Paszkowski *et al.*, 1984), electroporation (Fromm *et al.*, 1985; Hauptmann *et al.*, 1987) or microinjection (Kost *et al.*, 1995). One problem with these techniques is that they require efficient protoplast regeneration systems, which have not been well developed for many species.

The discovery of the high-velocity particle bombardment method (gene gun) eliminated the necessity to use protoplasts for direct gene transfer by allowing the transfer/bombardment of intact cells in plant tissues (Klein *et al.*, 1987). The method involves acceleration of DNA coated gold or tungsten microprojectiles that penetrate the walls of intact cells and deliver the DNA. This DNA delivery system has been used for transformation of corn, wheat, soybean, beans and many other plant species (Chrispeels & Sadava, 1994; Gasser & Fraley, 1992). The first report of a transgenic, economically important tree transformed via particle bombardment was published in 1991 by McCown et al.. Transgenic poplar plants were produced, containing a GUS reporter gene, a *nptII* selectable marker gene and a *Bt* endotoxin gene for resistance to lepidopteran larvae. However the application of the gene gun for transformation of woody plants is still limited due to difficulties related to regeneration of shoots from the bombarded tissues. In addition, reports of gene silencing and/or multiple scrambled DNA insertions in plants generated via particle bombardment have made *Agrobacterium*-mediated transformation the first method of choice for plants.

1.3 *Selectable marker and reporter genes for identification of transgenic plant cells*

1.3.1 *Common selectable marker and reporter genes*

In addition to the incorporation of agriculturally important genes within the T-DNA, it is necessary to include chimeric genes that function as dominant selectable markers, along with reporter genes for rapid detection of transformed cells. In the first report of a chimeric gene functioning in plant tissue by Herrera-Estrela et al. (1983), the authors confirmed expression of a functional octopine synthase (OCS) protein in transgenic tissue as a result of transcription of a chimeric gene consisting of a promoter from a nopaline synthase gene (*nos* promoter) joined to OCS coding sequence and *nos* polyadeniyation signals. The activity of the gene was assayed by the production of octopine in the transgenic tissue. This study was followed by three reports on the construction of a chimeric gene consisting of a *nos* promoter joined to the bacterial Tn5 neomycin phosphotransferase II (*nptII*) coding sequence and *nos* polyadenylation signals (Bevan *et al.*, 1983; Fraley *et al.*, 1983; Herrera-Estrella *et al.*, 1983a). The expression of the *nptII* gene conferred resistance of the transgenic tissue to aminoglycoside antibiotics (kanamycin, neomycin, and G-418). Based on DNA and RNA blots and *nptII* activity in transformed cell extracts, kanamycin resistance was established as the first dominant, selectable marker for plant transformation. To date, kanamycin resistance is the most commonly used selectable marker gene and has been utilized in the production of a large number of transgenic plants of various species, including apple (James et al., 1989).

Other selectable marker genes subsequently been developed. Herrera-Estrella et al. (1983a) reported that the methotrexate-insensitive dihydrofolate reductase (*dhfr*) coding

sequence from the R67 resistance plasmid conferred resistance to methotrexate when fused to the *nos* expression signals. Hygromycin B (*hyg*) is a selectable marker system that was developed for yeast and animal cells (Gritz & Davies, 1983; Kaster *et al.*, 1984). Hygromycin is an aminocyclitol antibiotic that inhibits protein synthesis in prokaryotic and eukaryotic cells. A bacterial hygromycin phosphotransferase (*hpt*) coding sequence has been expressed under the control of CaMV 35S promoter and *nos*3' signals and conferred resistance to hyg in transformed petunia and tobacco cells (Fraley *et al.*, 1983). The biolaphos resistance gene (bar) coding for phosphinothricin acetyltransferase has been used for transformation of different dicotyledonous and monocotyledonous species (Akama *et al.*, 1995; Cabrera-Ponce *et al.*, 1995; Dekeyser *et al.*, 1989). The *bar* gene was cloned from *Strepomyces hygroscopicus* and confers resistance to herbicides such as biolaphos and Basta (Thompson & Murakami, 1986). Phosphinothricin, the active component of bialaphos and Basta, is analog of plant glutamic acid and inhibits the action of glutamine synthetase in plant cells (Tachibana & Kaneko, 1986). This causes ammonia accumulation in the cells leading to plant death (Tachibana *et al.*, 1986). Other selectable markers used are bleomycin resistance, chloramphenicol resistance, aminoethyl cysteine resistance, and adenine phosphoribosyltransferase (Dahl & Tempe, 1983; Herrera-Estrella et al., 1983b; Schaff, 1994).

Reporter genes encode easily detectable products and are often used for fast detection of gene expression. The *E. coli* β-glucuronidase gene (GUS) has been the reporter gene of choice since its introduction (Jefferson *et al.*, 1986). β-glucuronidase is an acid hydrolase that catalyzes the cleavage of a wide variety of β-glucuronides. Substrates for β-glucuronidase are commercially available and can be used for spectrophotometric, fluorometric, or histochemical analysis. When β-glucuronidase in transgenic tissue interacts with X-Gluc (5-bromo-4-chloro-3-indolyl-β-D-glucuronic acid) substrate, the

reaction product is blue and easily detectable (Jefferson *et al.*, 1987). One problem with the assay is that β-glucuronidase gene is active in *Agrobacterium*, thus preventing the precise determination and localization of T-DNA transfer into plant cells. To avoid this obstacle the wild type β-glucuronidase gene was modified by the introduction of a plant derived portable intron, IV2 of the St-LS1 gene (Vancanneyt et al., 1990). Due to the lack of a splicing apparatus in prokaryotes, *Agrobacterium* can not produce GUS enzymatic activity from the GUS-INT gene, where transgenic plants containing this chimeric gene can efficiently splice the intron and give rise to GUS activity. A second non-invasive reporter gene is firefly luciferase (LUC), which allows screening for transformed cells by virtue of their bioluminescence (Millar *et al.*, 1992). After the substrate luciferin is added to the transformed tissue, bioluminescence can be monitored by luminometry or by low-light video microscopy in combination with a real-time photon imaging technique (Chia *et al.*, 1994).

1.3.2 *Green fluorescent protein as a novel reporter gene for monitoring gene expression in living cells and organisms*

Green fluorescent protein (GFP) from the jellyfish *Aequorea victoria* has been established as a novel genetic reporter system (Kitts *et al.*, 1995). *Aequorea victoria* emits a bluish-green light from the margin of its umbrella (Harvey, 1952). The blue light is produced when calcium binds to the photoprotein aequorin. GFP derives its excitation energy from aequorin and emits green light (l=509 nm). When expressed in either eukaryotic or prokaryotic cells and illuminated by UV light (l=395 nm), GFP yields a bright green fluorescence. This fluorescence is stable, species-independent, and can be

monitored non-destructively in living cells, or whole organisms. Light stimulated GFP fluorescence does not require any co-factors, substrates, or additional gene products.

GFP has been expressed and showed fluorescence in *Escherichia coli*, yeast, the nematode *Caenorhabditis elegant* (Chalfie *et al.*, 1994), *Drosophila melanogaster* (Wang & Hazelrig, 1994), *Arabidopsis thaliana* (Haseloff & Amos, 1995), and *Nicotiana tabacum* (Pang *et al.*, 1996). In these organisms, GFP did not have a toxic effect. However, the preliminary results from the transformation of *Arabidopsis* with the GFP gene suggested a mild toxicity or inhibition of regeneration in very brightly fluorescing tissues. GFP transformed tissue can be isolated and monitored through selection. Potentially, the new marker gene permits the immediate nondestructive mass screening of putative transformants.

1.4 *Factors affecting the efficiency of* Agrobacterium*-mediated genetic transformation*

The main factors affecting the efficiency of *Agrobacterium*-mediated genetic transformation can be grouped into five functional categories: explant tissue type and propagation method, genetic background and virulence induction of Agrobacterium, selection system for transformed cells, and regeneration of transformed plants. Many different factors that influence these steps have been reviewed by James and Dandekar (1991), Dandekar et al. (1990), and De Bondt et al. (1992; 1994). The pertinent literature presented next concerns the effects of explant preculture on transformation efficiency.

1.4.1 *Effect of phytohormone treatment prior to bacterial infection on plant transformation and regeneration*

One of the approaches that has been applied for increasing transformation frequency is the treatment of explants with different phytohormones prior to bacterial infection. Auxin pretreatment of *Arabidopsis* explants resulted in high transformation and regeneration frequencies (Sangwan *et al.*, 1992). Schmidt and Willmitzer (1988) reported that the pretreatment of *Arabidopsis* leaf explants for 4 d with 1 mg/l 2,4-D and 0.2 mg/l kinetin resulted in a 55% transformation frequency (the number of explants producing resistant calli per the total number of explants); however, only 20% of the resistant calli regenerated shoots. These authors concluded that the pretreatment resulted in an enhanced transformation frequency.

Transformation frequencies of 68% to 79% have been reported after 5 days preculture of *Arabidopsis* zygotic embryos on 1 mg/l BAP and 1 mg/l NAA or 1 mg/l 2,4-D and 1 mg/l kinetin (Sangwan *et al.*, 1991). After 5 days preculture and 2 days co-culture with *Agrobacterium*, embryo explants produced green calli resistant to kanamycin. These calli produced numerous transgenic shoots after additional 2-3 weeks of culture. These results were confirmed with several transformation vectors. The same authors reported 92% transformation frequency for cotyledon explants after 5 days preculture on 1 mg/l BAP and 1 mg/l NAA or 1 mg/l 2,4-D and 1 mg/l Kinetin (Sangwan et al., 1992). They speculated that a newly synthesized cell wall or an auxin-modified cell wall in dedifferentiating cells may promote the passage of macromolecules like T-DNA into the cytoplasm.

There are several reports of phytohormone pretreatment of apple explants. Yao et. al. (1995), found that pretreatment of 'Royal Gala' leaf explants with 5 mg/l BA and 0.2

mg/l NAA for up to 3 days did not influence the transformation frequency (number of GUS stains at 5 days post inoculation). Pretreatments of 1 to 3 days, showed very high levels of transient expression (97-91%). Five days preculture resulted in very low transient expression. After 21 days of culture, the number of GUS positive explants for these treatments was strongly reduced.

'Delicious' leaf explants treated with auxin (1.86 mg/l to 7.44 mg/l NAA or 1.75 mg/l to 7 mg/l IAA) for up to 48 hours before inoculation and selection produced several shoot primordia that later died on selection medium (Sriskandarjah *et al.*, 1994). These data suggested that pretreatment of explants may contribute to increased regeneration of non-transformed shoots on selection media (escapes), which are initiated in the tissue during the pretreatment period.

1.5 *Chronicles and current status of apple genetic transformation*

Although transgenic apple plants with marker genes, such as nopaline synthase (*nos*) and neomycin phosphotransferase (*nptII*), have been reported (James *et al.*, 1990; James et al., 1989), it has been difficult to produce transgenic apple plants in large numbers. The first successful apple *Agrobacterium*-mediated transformation was reported in 1988 with the cultivar 'Greensleeves' (James et al., 1988a), using the leaf disc technique for transformation and selection defined by Horsch (1985). During the last nine years a number of variables affecting shoot regeneration after transformation have been studied, including the effect of *Agrobacterium* strain and transformation vectors (Dandekar et al., 1990), the effect of the plant phenolic acetosyringone on bacterial virulence (Stachel *et al.*, 1985), length of the co-cultivation period, type and quality of agar or agarose, choice of

phytohormones during regeneration, the length of the selection period on kanamycin, and the age and condition of the explant material (James, 1991; James et al., 1989; James et al., 1988b; Lambert & Tepfer, 1992; Maheswaran et al., 1992; Stachel et al., 1985; Welander & Maheswaran, 1991).

James et al. (1988), transformed leaf pieces and shoots from the apple cultivar 'Greensleeves' with *Agrobacterium tumefaciens* and *Agrobacterium rhizogenes* (LBA 9402/19) wild-type strains and *Agrobacterium tumefaciens* (LBA4404) carrying the disarmed binary plasmid pBin 6 (Bevan, 1984). The tissue transformed with wild-type *Agrobacterium tumefaciens* regenerated nontransformed plants. These plants were negative for nopaline production, suggesting selection for untransformed cells and cross-feeding from transformed cells. Eighteen separate experiments were performed over a 15 month period, involving 31 separate infections (50-100 explants per infection). As a result, the tissue transformed with pBin 6 regenerated three transformed plants on 60 μg/ml kanamycin-containing media that were positive for *nos* (nopaline synthase) activity. From these plants only two were successfully rooted on kanamycin media.

Using the same apple cultivar, Dandekar et al. (1990) investigated the factors influencing virulence in *Agrobacterium* which in turn influence the frequency of transformation. Two important factors which were identified are the *Agrobacterium* strain used for infection and the site of infection on the target plants. In the same study, measurement of the frequency of tumor and root formation on leaves obtained from *in vitro* propagated apple plants indicated that the difference in virulence could be attributed to the differences in the vir regions of the different plasmids. Leaf position on the mother plant also influenced the transformation efficiency. The data suggested that the leaves closest to the meristem produced the maximum number of tumors or roots when compared to more distal leaves.

The *E. coli* β-glucuronidase gene (GUS) was introduced as a reporter gene system for transformation of plants in 1986 (Jefferson et al., 1986). The GUS-containing plasmid pBI121 was inserted into two bacterial strains of *Agrobacterium rhizogenes* and eight strains of *A. tumefaciens*, and used in transformation experiments with apple seedling stems, apple seedlings grown in greenhouse, and apple xylem parenchyma from the third internode from the apex (Martin *et al.*, 1990). The seedlings infected with pBI121, or wild-type strains, grown in the greenhouse did not produce tumors. The callus initiated from xylem parenchyma survived on kanamycin plates (50-400 μg/ml), and was positive for GUS, opines, and *nptII* enzymatic activity (Martin et al., 1990). From the ten strains of *Agrobacterium* tested, A281 was identified as the best.

Transformed shoots were regenerated from leaf segments (73-78%) from apple rootstocks M.26 after infection with *Agrobacterium tumefaciens* C58 containing the binary vector pBI121 (Welander & Maheswaran, 1991). The shoots formed on medium containing 100 mg/l kanamycin and 500 mg/l cefotaxime, five weeks after co-cultivation. Apple rootstock (M.26) has also been transformed with *Agrobacterium rhizogenes* (A4) (Lambert & Tepfer, 1992). Micro-cuttings were pricked at their base, dipped in the bacterial solution, and transferred to rooting medium. Roots induced by *Agrobacterium* were excised and cultured *in vitro* in phytohormone-free liquid media and later exhibited the transformation phenotype observed in other species. Transformed roots that were still attached to the mother plant regenerated shoots on medium containing 0.5 mg/l BAP.

Successful transformation of M.26 with disarmed binary vectors in different strains of *A. tumefaciens* was reported by Maheswaran et al. (1992). Adventitious shoots were regenerated from leaf explants on media with MS micronutrients and organic compounds (30g/l sucrose, 22 μM BAP, and 1 μM NAA) (Murashige & Skoog, 1962). One of the conclusions from this study was that the media solidifying agent and transformation vector

affect the transformation and regeneration of transgenic plants in different fashions. The explants did not regenerate shoots on agar media, while on Gelrite the tissue produced 5 transgenic shoots for the explants transformed with pCGP527:CZ707 and 3 transgenic shoots for the explants transformed with pBI121:1104. However, the experiment comparing agar with Gelrite was performed only once. Putative transformants originating from tissue transformed with pBI121 had lower GUS and *nptII* activity than those transformed with pCGP257. Since the GUS gene in both constructs had CaMV 35S promoters, this differential expression may have been due to genomic positional effects (Jones *et al.*, 1985), copy number effects (Sanders *et al.*, 1987), or incomplete gene transfer (Parrot *et al.*, 1989). The *nptII* gene driven by the *nos* promoter (pBI121) expressed little or undetectable enzyme activity, whereas *nptII* driven by the CaMV35S (pCGP257) promoter had high levels of expression. This demonstrates the importance of the promoters driving the *nptII* gene. The putative transformants from this experiment were rooted on media with 50 mg/l kanamycin and gene integration (GUS and *nptII*) was confirmed by Southern blot analysis.

The GUS reporter gene was also used to determine the optimal antibiotic concentration for the selection of *nptII*-transformed apple cells (Norelli & Aldwinkle, 1993). Apple leaf pieces from M.26 rootstock and its transgenic derivative T1 were inoculated with *Agrobacterium tumefaciens* strain C58sZ707 (p35SGUS-INT) and placed on regeneration medium with various concentrations of kanamycin, neomycin, geneticin, and paromomycin. Comparison of the growth of the leaf pieces and the level of GUS activity under different selective conditions indicated that neomycin and paromomycin were more useful for selection of *nptII*-transgenic apple plants than kanamycin and geneticin. Regeneration was absent after infection of the plant material.

The plant phenolic compound acetosyringone (AS) and the osmoprotectant beatine phosphate (BP) have been shown to increase the efficiency of apple transformation (James *et al.*, 1993b). AS is produced during wounding of plant cells and has been shown to induce the transcription of the virulence genes of *Agrobacterium tumefaciens* by interacting with a transmembrane receptor protein encoded by *vir* A (Melchers *et al.*, 1989; Stachel et al., 1985). BP has been shown to increase the expression of several virulence genes in *Agrobacterium* (Vernade *et al.*, 1988). James et al. (1993), used the two compounds either in the presence of simplified virulence induction media (Alt-Moerbe *et al.*, 1988) or with MS20 (Murashige & Skoog, 1962) medium. GUS activity of cells transformed with *Agrobacterium* was measured fluorometrically. A significant synergistic response between AS and BP in increasing GUS activity compared with either compound alone was demonstrated. More than half (53%) of the leaf discs in the presence of BP and AS showed GUS activity in range of 10-100 pmoles/min/disc. The data indicated that the positive effects of these compounds on the bacterial virulence are confined to the virulence induction phase and have no effect if they are applied during the co-cultivation phase. Using 0.1 mM AS and 1 mM BP to induce *Agrobacterium* virulence, a gene encoding a cowpea trypsin inhibitor (CpTI) was transferred into apple and strawberry (James *et al.*, 1993a). CpTI was introduced in *Agrobacterium tumefaciens* strain LBA4404::pROCpTI+5 (Hilder *et al.*, 1987). Sixty clones of the apple cultivar 'Greensleeves' regenerated after selection on kanamycin, and half of these rooted on kanamycin after micropropagation. Six of the clones were chosen randomly for Southern blot analysis and proved the expected DNA integration. Similarly, trees expressing different combinations of the marker gene *nptII*, reporter gene GUS, nopaline synthase (*nos*), and CryIA gene from *Bacillus thuringensis* were produced and were planted in field trials in California (James et al., 1993a). The transgenic 'Greensleeves' clones accumulated up to 0.004% Bt toxin protein,

which was sufficient to cause up to 100% mortality of first instar codling moth larvae during a codling moth laboratory bioassay (Dandekar *et al.*, 1992).

Transformation of the commercially important apple cultivar 'Delicious' was firs reported in 1993 (Goodwin *et al.*). The *Agrobacterium* transformation was performed with various strains carrying different plasmid vectors. In this experiment the selectable agents evaluated were kanamycin, G418, and phosphinothricin. From these, kanamycin was established as the best selective agent. Callus produced after transformation was maintained on 100 mg/l kanamycin in darkness and within a period of 4 to 50 weeks shoots appeared at a rate 1-2 per 100 initial transplants. The plants were grown and rooted on kanamycin. After two years, these plants had all the characteristics of the control trees and the upper leaves of the transformed trees showed β-glucuronidase and nopaline synthase activity. Southern blot analysis was used to prove gene insertion into the plant genome. The same group of researchers produced nine independently transformed shoot lines from 600 callus explants over a 6 month period (Sriskandarjah et al., 1994). Four of these originated from 150 explants co-cultured with pGV3850::1103gus, three from 210 explants co-cultured with pGV3850::1103neo, and one each from pGV3111XKIWI (100 explants) and pGV3850::35SKN (75 explants). The plants originated from transformed calli produced on various auxins. Different concentrations of TDZ, BA, NAA and IAA hormones were tested for shoot initiation, but shoots were formed in low frequency on calli growing on regeneration media containing 15 μM TDZ, 5.4 μM NAA, and 100 mg/l kanamycin.

Using a binary transformation vector containing a chimeric GUS-intron gene, De Bondt et al. (1994) studied different factors affecting apple gene transformation efficiency during early transformation steps: influence of the bacterial vector and strain; cell density and growth; period of co-cultivation; influence of the carbon source and phytohormonal

composition; and the influence of the explant age and genotype. The evaluation of transformation efficiency was measured based on histochemical analysis of the number of GUS expressing zones immediately after the co-cultivation period. The results suggest that from all the bacterial strains tested, EHA101 yielded twice as much GUS expression as strain LBA4404, whereas with strain C58C1 no expression was observed. Co-cultivation periods of up to four days increased the number of zones per leaf explant, and the highest number of GUS expression zones were obtained upon inoculation with a bacterial suspension at 0.5 OD or $0.2X10^9$ cells/ml. EHA101 also produced the highest number of transformed calli. In this experiment the leaf discs were precultivated (prior to infection) and postcultivated (after co-cultivation) on auxin shoot induction media for 0 to 8 days. Precultivation for up to 6 days enhanced the number of GUS expressing zones, but decreased the number of transformed calli. Therefore, the authors concluded that the physiological status of the tissue optimal for DNA uptake was not necessarily optimal for integration of foreign DNA into the host genome. High concentrations of cytokinin in the co-cultivation media contributed for a higher number of GUS expressing zones in the transformed leaf discs. Maximal transformation and regeneration efficiency for leaf explants was shown when the shoot cultures were between 20 and 40 days old. For determination of genotype influence on the GUS expression, six different apple cultivars were tested ('Jonagold', 'Elstar', 'Gala', 'Braeburn', 'Merlijn', and 'Fuji'). The explants were infected with EHA101 and GUS expressing zones were counted immediately after co-cultivation and after six weeks on kanamycin selection media. The highest levels of transient expression (blue zones immediately after 4 days of co-cultivation) were observed for the cultivars 'Braeburn', 'Jonagold' and 'Gala'. After six weeks on selection media the number of GUS expressing calli was highest for the cultivars 'Merlijn', 'Jonagold' and

'Elstar'. The authors suggested that genotype dependent factors may have affected DNA uptake and integration differentially.

The highest regeneration frequency of transformed shoots has been reported for the cultivar 'Royal Gala' (Yao et al., 1995). Twenty nine independently derived kanamycin resistant plants were tested for GUS expression and analyzed by Southern blot analysis. It was established that 0-day preculture, 2-day co-cultivation and 2-day delayed selection were optimal for regeneration of transgenic plants. The transgenic plants were regenerated on media containing MS salts, Gamborg B_5 vitamins, BA 5 mg/l, NAA 0.2 mg/l, 30g/l sucrose and 2.5 g/l PhytaGel™. Thirteen transgenic plants from 4 independent transformants were produced after transformation with pKIWI110 containing a gene conferring a resistance to chlorsulfuron. These plants were shown to be resistant to herbicide Glean™.

Seven years following the initial transformation of cultivar 'Greensleeves', James et al. (1996) reported stable expression and Mendelian segregation of the transgenes in fruit and vegetative tissues from the transgenic clones. The shoots regenerated in 1986 were transgenic for the *nos* reporter gene and *nptII* selectable marker gene. Five of these clones were phenotypicaly normal and one exhibited reduced rooting capacity, and premature leaf senescence. Annual tests of leaves from the six clones for the seven year growth period confirmed stable expression of the *nos* gene. Fruit analyses were performed five years after the initial experiment, when two of the five clones with normal phenotype flowered. The flowers were pollinated using pollen from apple cultivar 'Baskatong', which carries a dominant, homozygous gene for anthocyanin pigmentation. The fruit flesh assays verified *nptII* and *nos* gene expression in one clone and only *nptII* gene expression in the second clone, which was known to lack *nos*, but had several *nptII* copies. PCR segregation

analysis of the two genes were conducted with a population of 47 randomly chosen seedlings. Three different genotypes were designated - *nos+/nptII+*, *nos-/nptII+*, and *nos-/nptII-*. The inheritance data indicated 3:1 segregation for *nptII* and 1:1 segregation for *nos*. The 3:1 segregation for *nptII* was consistent with the original parent carrying two unlinked copies of the gene. The 1:1 ratio for *nos* was due to segregation of an allele heterozygous at a single locus. This was the first report on long term stability of a transgene with Mendelian segregation in a tree species.

In summary, while genetic transformation and regeneration systems for apple have been developed, they suffer from low efficiency and genotypic variability. To realize the potential of biotechnology in apple, these methods must be improved to levels sufficient for commercial development. To this end, research aimed at increasing the efficiency of apple transformation and development of genotype-specific conditions to improve transformation frequencies were performed. This included optimization of *in vitro* rooting, adventitious shoot regeneration and transformation procedures. This work is particularly unique in the use of the green fluorescent protein reporter gene, which allowed for the *in vivo* observation of very early events during transformation.

Chapter 2

RAPID PROTOCOL FOR ROOTING APPLE CULTIVARS *IN VITRO*

2.1 Introduction

The formation of a functional root system on microcuttings is required for the successful micropropagation of a number of fruit tree species (Van Telgan *et al.*, 1992). Additionally, it was found that leaf tissue from plants rooted *in vitro* are more susceptible to *Agrobacterium*-mediated gene transfer than leaves from unrooted plants (Dandekar et al., 1990) and (James & Dandekar, 1991). It was proposed that high shoot regeneration frequencies were due to active cell division in the rapidly expanding young leaves of rooted plants. Successful procedures for rooting some apple cultivars *in vitro*, mainly in rootstocks, have been established (De Fussard, 1976; James & Dandekar, 1991; Jones *et al.*, 1979; Sriskandarjah et al., 1982; Zimmerman & Fordham, 1985). In most of the protocols, root formation is induced by different auxin treatments on solid agar media (Dandekar et al., 1990) or liquid media (Zimmerman & Fordham, 1985). In some of the procedures shoots were stabilized with paper bridges (Abbot & Whitely, 1976; Sriskandarjah et al., 1982) or perlite (Zimmerman & Broome, 1980). After root induction, the plants can be transferred to peat plugs (Zimmerman & Fordham, 1985) or to agar solidified media for root elongation (Dandekar et al., 1990), further adaptation and transfer to a greenhouse, or for use in genetic engineering experiments.

The objectives of this study were to evaluate the influence of the incubation temperature and IBA exposure time on root induction of shoots propagated *in vitro*, and to develop a rapid, highly efficient rooting protocol for five different apple cultivars. The establishment of such a protocol will assure successful acclimatization and transfer of plants to greenhouse conditions and will provide sufficient quantities of leaf material from rooted plants for genetic transformation experiments.

2.2 Materials and Methods

In vitro shoot multiplication of the apple cultivars 'Golden Delicious', 'Delicious', 'Empire', 'Fuji', and 'Royal Gala', was done by the method of James and Dandekar (1991). Single, 1.5-2.0 cm long, axillary shoots were excised aseptically from shoot clusters. At the basal end of the stems, the shoots were cut in a V-shape at an angle of approximately 30° to the shoot axis. Three to five shoots were placed upright in disposable 20 ml blood dilution vials (Fisher Scientific) containing 5 ml of a simplified liquid root induction medium (RI) with 1.5% sucrose and 15 μM IBA. The shoots were incubated in the dark for either 2 days at 25°C, 2 days at 30°C, 7 days at 25°C or 7 days at 30°C. After root induction all shoots were transferred to an agar-solidified root elongation medium (RE) containing half strength macro, micro salts and vitamins of Murashige and Skoog (1962) basal medium, 2% sucrose, 0.58% Agar type A (Sigma Chemical Company, St. Louis, MO) at pH 5.7. Root elongation cultures were exposed to cool-white light at a 16/8 h photoperiod (110 $\mu mol/m^2/sec$) and 25°C. Three replicates of fifteen shoots were used per treatment/cultivar combination. To evaluate the effect of the V-cut on rooting efficiency, control plants with perpendicular cuts were incubated on root initiation media for two days

at 25°C and transferred to root elongation media. Only roots longer than 0.5 cm were counted. Data were collected 10 days after the plants were transferred to root elongation media. Mean percentages of rooted plants and mean numbers of roots per shoot were calculated. Analysis of variance and mean separation were performed with Student-Newman-Keuls statistics; significance was determined at $p \leq 0.5$.

For evaluating the influence of the IBA concentration on root induction, two additional treatments with 0.5 and 5 µM IBA were performed. Three replicates with fifteen shoots from the cultivars 'Delicious' and Golden 'Delicious' were incubated on root induction media with 0.5 or 5 µM IBA for two days at 25°C.

2.3 *Results*

2.3.1 Effect of auxin treatment duration and incubation temperature on adventitious root formation

To evaluate the effect of the duration of auxin treatment on root initiation in combination with the incubation temperature, two different exposure times were tested in combination with two temperature regimes (Table 2.1). When shoots were incubated on root initiation media with 15 µM IBA for 2 days at 25°C, the first roots (2-4 mm long) were observed 4-6 days after transfer to root elongation medium. This was followed by rapid root elongation during the next 10 days and simultaneous leaf expansion. The highest rooting frequencies were obtained for 'Delicious', 'Empire', and 'Royal Gala', which in

Table 2.1 Rooting percentages obtained for various apple cultivars after root induction at different temperatures and 15μM IBA.

Cultivar	2 days at 25°C + Vcut	7 days at 25°C + Vcut	2 days at 30°C + Vcut	7 days at 30°C + Vcut	2 days at 25°C - V-cut	Mean % of rooting shoots per cultivar
RD	86.7±3.8	57.7±9.6	45.3±3.9	69.0±9.9	58.7±5.7	63.5±4.5^{b}
GD	60.0±4.0	42.0±5.9	37.7±4.7	51.0±17.4	30.0±5.8	44.1±4.4^{a}
FJ	69.0±5.9	44.3±5.9	63.7±3.7	28.7±4.3	42.3±15.7	49.6±5.1^{a}
RG	97.7±2.3	75.7±14.4	68.7±2.2	66.7±13.9	64.3±8.1	74.6±4.9^{b}
EM	88.7±4.3	66.7±20.2	69.0±11.0	68.7±8.1	44.0±9.0	67.4±5.8^{b}
Mean % per treatment	80.4±4.0^{b}	57.3±5.8^{a}	56.9±4.1^{a}	56.8±6.5^{a}	47.9±6.9^{a}	

The mean percentages of rooted shoots for each treatment and cultivar combination are presented ± SE (n=3). Data were collected 10 days after the shoot were transferred to RE medium. The means of treatments and cultivars were separated using Student-Newman-Keuls test ($p \leq 0.5$). Means followed by the same letter (a, b) are not significantly different. Abbreviations: RD - 'Delicious'; GD - 'Golden Delicious'; FJ - 'Fuji'; RG - 'Royal Gala'; EM - 'Empire'

10-15 days had well developed roots and large, actively expanding top leaves (Fig. 2.1). 'Golden Delicious' and 'Fuji' showed lower rooting frequencies for this treatment.

When treated with 15 μM IBA for 7 days at 25°C and 2 days at 30°C all cultivars produced lower percentages of rooted plant compared to the 2 day at 25°C treatment (Table 2.1). During both treatments minor browning of the root induction medium was observed, which did not appear to affect root initiation.

All of the shoots incubated in liquid root induction medium for 7 days at 30°C secreted substances into the root induction medium, causing intensive browning. This seemed to reduce the percentage of successfully rooted shoots. Most of the shoots exposed to auxin treatment for 7 days formed roots through an extensive callus phase. The roots emerging through the calli were thin and hairy, displaying abnormal morphology. As a result of this treatment, all apple cultivars tested produced lower percentages of rooted plants (Table 2.1).

The mean separation for the treatments, indicated that treatment 2 days at 25°C was significantly different from the rest of the treatments (Table 2.1). This supports the observation that root induction with 15 μM IBA for two days at 25°C produced significantly higher percentages of rooted plants for all cultivars. The separation of the means for the cultivars indicated that 'Golden Delicious' produced the lowest number of rooted plants independently from the treatment and no difference was detected among the rest of the cultivars.

No significant differences in the average numbers of adventitious roots produced from cultivars 'Golden Delicious', 'Fuji' and 'Royal Gala' were observed among the different treatments (Table 2.2). The highest average number of adventitious roots produced from 'Delicious' were recorded after 15 μM IBA treatments for 2 days at 25°C

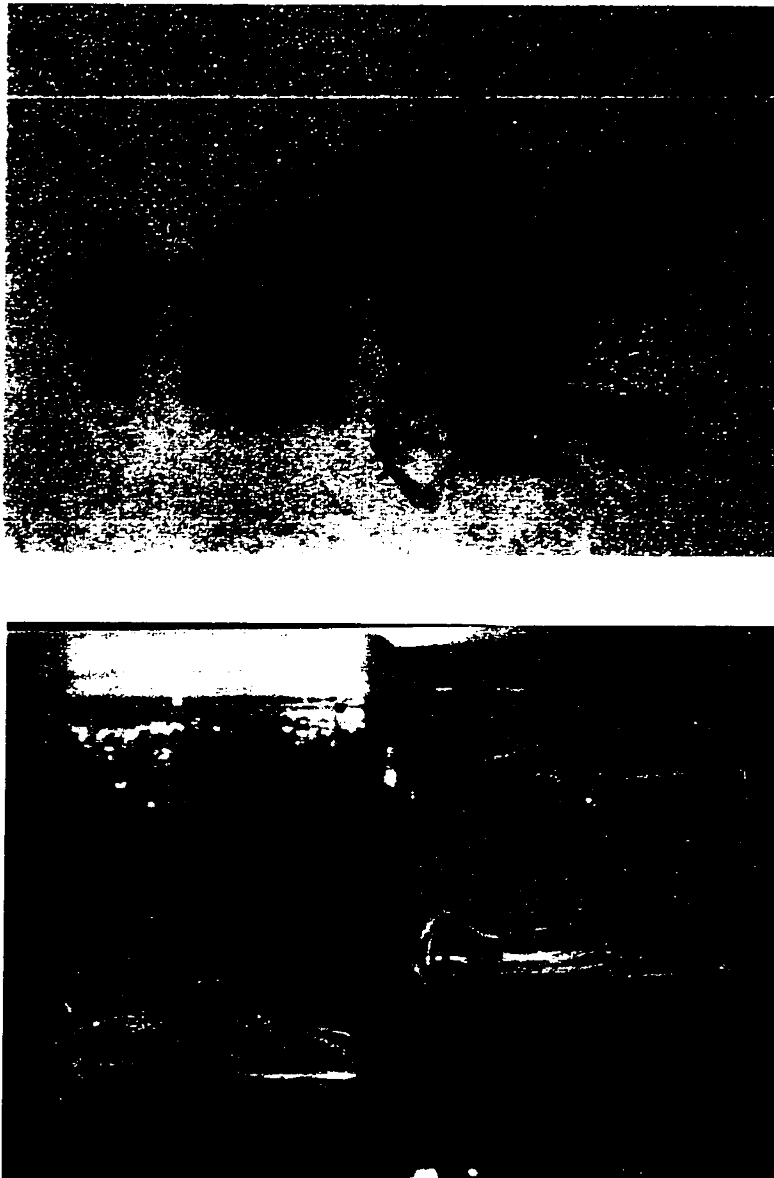


Figure 2.1 Adventitious root formation of apple shoots propagated *in vitro*. One to three cm shoots were incubated on root induction medium for 2 days at 25oC and then transferred to phytohormone free MS root elongation medium. Roots emerged along the cut surfaces and within 10 to 15 days the leaves enlarged and a strong, well balanced root system was formed A. Different stages of *in vitro* root development (note the V-cut incision on first shoot at the left); B. Fully developed rooted plants on agar solidified RE medium fifteen days after transfer from RI to RE medium.

and 7 days at 25°C. For this cultivar, the number of roots decreased after exposure to 15 μM IBA for 2 days at 30°C and 7 days at 30°C. The highest number of roots was produced by 'Empire' after treatment with 15 μM IBA for 2 days at 30°C. The separation of the means for the cultivars indicated that 'Empire' and 'Royal Gala' produced the highest number of roots under all treatments, followed by 'Delicious' and 'Fuji'. 'Golden Delicious' produced the lowest number of roots independently of the treatment.

2.3.2 Effect of basal V-cut on root initiation

To evaluate the effect of different shoot excision treatments, shoots were incubated for 2 days at 25C and rooting of shoots with basal V-cuts was compared with those with perpendicular cuts. The application of a basal V-cut contributed to increased percentages (27 to 45% higher) of rooted plants compared to the perpendicular cut for all cultivars tested (Table 2.1). The highest increase of the rooting frequency was recorded for 'Empire'. Without a V-cut, this cultivar produced only 44% rooted plants, which is 45% less than the 89% rooting frequency recorded after a V-cut was applied. The perpendicular cut also resulted in a higher variation in percentage of rooting among the replicates for all cultivars tested, as indicated by the higher standard errors (Table 2.1). The average number of roots produced by apple

Table 2.2 Mean number of roots produced by various apple cultivars after root induction at different temperatures and 15μM IBA.

Cultivar	2 days at 25°C + V-cut	7 days at 25°C + V-cut	2 days at 30°C + V-cut	7 days at 30°C + V-cut	2 days at 25°C - V-cut	Mean # of roots per cultivar
RD	19.5±1.9	25.3±13.0	8.4±1.6	7.8±2.0	7.7±1.1	13.7±3.0^{bc}
GD	4.2±0.3	2.6±0.6	3.2±1.6	2.3±0.7	3.7±1.0	3.2±0.4^{a}
FJ	7.9±3.0	6.0±1.9	11.6±1.2	9.6±2.1	4.2±2.0	7.8±1.0^{ab}
RG	15.9±3.0	17.3±5.1	14.1±3.8	15.1±4.9	13.8±1.3	16.0±1.5^{c}
EM	20.7±7.7	22.6±7.7	24.7±13.3	9.3±1.6	17.8±6.2	19.0±3.1^{c}
Mean # of roots per treatment	13.6±2.3^{a}	14.8±3.4^{a}	12.4±3.0^{a}	8.8±1.5^{a}	9.4±1.9^{a}	

The mean numbers of rooted shoots for each treatment and cultivar combination are presented ± SE (n=3). Data were collected 10 days after the shoots were transferred to RE medium . The means of treatments and cultivars were separated using Student-Newman-Keuls test ($p \leq 0.5$). Means followed by the same letter (a, b) are not significantly different. Abbreviations: RD - 'Delicious'; GD - 'Golden Delicious'; FJ - 'Fuji'; RG - 'Royal Gala'; EM - 'Empire'.

shoots with V-cuts compared to no V-cut was significantly enhanced only in 'Delicious' (Table 2.2).

Roots emerged directly from the central cylinder along the cut surfaces on both sides of the stem, forming a strong, well balanced root system. In contrast to plants produced without the V-cut treatment, the leaves expanded faster and did not display chlorotic symptoms when cultivated on root elongation medium.

When shoot were incubated for 7 days at 30°C however, the large wounded areas of the V-cut increased the secretion of compounds into the root induction medium and subsequently browning, thus decreasing the percentage of rooted plants. Under these conditions, the large wounded areas of the stem initially produced massive calli followed by the differentiation of roots with abnormal morphology.

2.3.3 Effect of IBA concentration on the rooting of apple cultivars in vitro

To test the influence of IBA concentration in the root induction medium on adventitious root formation, three different phytohormone concentrations were tested: 0.5, 5, and 15 μM IBA. Both cultivars tested had enhanced rooting of plants with increased IBA concentration (Fig. 2.3 a). The highest percentages of rooted plants for 'Delicious' (86%) and 'Golden Delicious' (60%) were recorded after treatment with 15 μM IBA (Fig. 2.3 a). The difference among the treatments was significant at $p \leq 0.8$, which could be of practical importance for large scale *in vitro* production of apple plants.

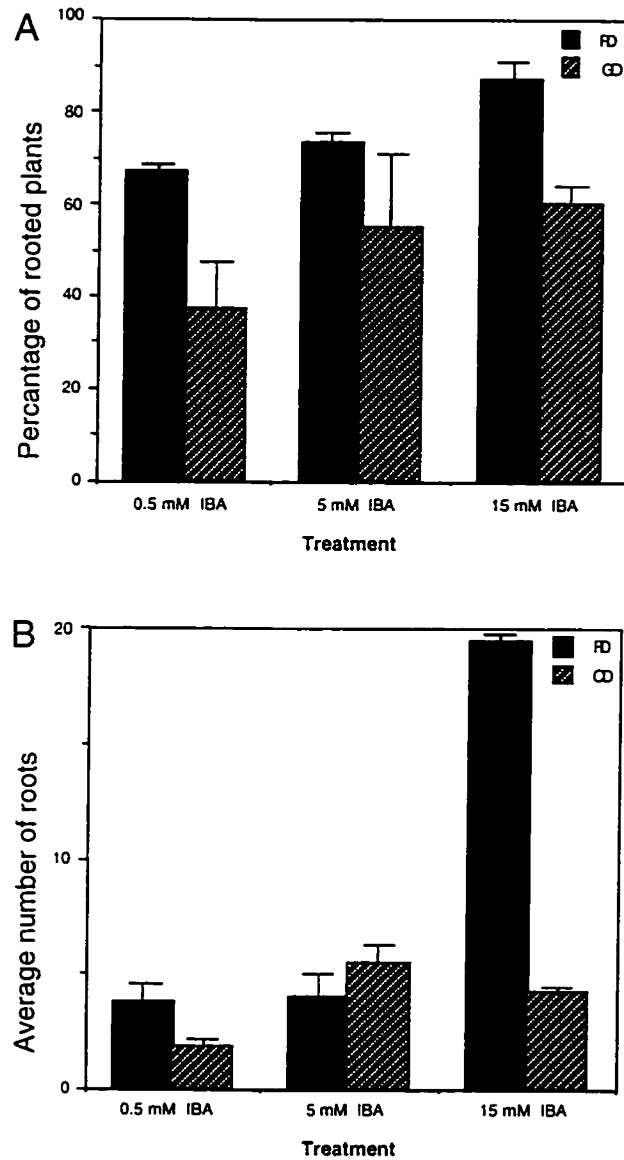


Figure 2.2 Influence of IBA concentration on adventitious root formation of cultivars 'Delicious' (RD) and 'Golden Delicious' (GD). A. Mean percentages of rooted plants ± SE (n=3) and B. Mean numbers of adventitious roots ± SE (n=3), produced under treatments with different concentrations of IBA during the root initiation phase.

The increased concentration of IBA contributed to a significant increase in the mean number of roots produced by 'Delicious', but not by 'Golden Delicious' (Fig. 2.3 b).

2.4 Discussion

The successful utilization of liquid root induction media has been previously reported for the induction of roots on *in vitro* cultured apple shoots in combination with different stationary techniques like filter paper bridges (Abbot & Whitely, 1976), perlite (Zimmerman & Broome, 1980) or shell vials (Zimmerman & Fordham, 1985). Higher rooting percentages (up to 80%) for some apple cultivars on liquid medium have been reported by Zimmerman & Fordham (1985), however several varieties of 'Delicious' responded better to agar solidified media. Dandekar et al., (1990) demonstrated that leaves of three-week old plants from cultivar 'Greensleeves' rooted on 1/2 MS medium after a two-day incubation at 25°C on solid medium supplemented with IBA (15 μM). The combination of liquid induction media reported by Zimmerman & Fordham (1985) and the two-day incubation period with 15 μM IBA during root induction, followed by an agar-solidified root elongation medium resulted in higher rooting frequencies than previously reported. Perhaps the most important advantage of this system is the rapid production of plants with functionally developed leaves and root systems. Using this rooting method, roots are visible 4 to 6 days after the root induction phase and after 10 to 15 days on root elongation medium the root system is fully functional with secondary branching. James and Thurbon (1979) and James and Dandekar (1990) reported that during the two-phase *in vitro* method for rooting of apple shoots, roots appear within 10-14 days after culture initiation. According to Zimmerman and Fordham (1985), the time for detection of root

initials after treatment on liquid root induction medium was 7 to 10 days and roots were developed after 3 weeks in peat plugs. Compared to these methods, the new procedure requires significantly less time for root initiation and development (10 to 15 days). The V-shaped cut at the basal end of the shoot stems exposed a large number of cambium cells to direct contact with the auxin medium resulting in development of more adventitious roots per shoot. Additionally, the use of agar-solidified medium for root elongation instead of peat plugs is advantageous for the production of aseptic plant tissues *in vitro*, for shoot regeneration and genetic transformation experiments. After root initiation on 15 μM IBA, plants were transferred to peat plugs or agar solidified media for elongation. After the root system was fully developed the plants were acclimatized and moved to a greenhouse conditions with high survival rates (data not shown).

As a result of this experiment some cultivar specificities were observed. 'Golden Delicious' produced the lowest percentage of rooted plants and the lowest number of roots under all treatments, indicating lower rooting potential. Furthermore, 'Empire' produced the highest number of roots independent of the treatment applied.

Despite the cultivar specificity, in general all cultivars achieved the highest rooting frequencies after induction with 15 μM IBA for two days at 25°C with a basal V-cut. This simplified rooting procedure allows the production of healthy plants with well developed functional root systems in a considerably shorter period of time then previously reported. Apple plants ready for transplanting to soil or for excision of leaf discs for gene transfer experiments can be obtained within 12-20 days. This system is highly efficient for the cultivars tested in this study. Additionally it has been successfully applied for rooting of the cultivars 'Greensleeves' and rootstock M.9 (data not shown).

Chapter 3

ADVENTITIOUS SHOOT REGENERATION OF APPLE CULTIVARS

3.1 *Introduction*

The regeneration of adventitious shoots from apple leaf explants has been previously reported (Fasolo & Predieri, 1990; Fasolo et al., 1989; James et al., 1990; James et al., 1988a; Welander, 1988). However, the reported results are inconsistent and cultivar dependent. Leaves obtained from shoots grown under strictly controlled environmental conditions exhibited differences in their regeneration potential (James & Dandekar, 1991). This can be overcome by using leaf explants at the 'correct' physiological age and developmental stage (James et al., 1990). High regeneration levels have been obtained for several cultivars using full strength MS salt supplemented with the cytokinin BAP and the auxin NAA, but the concentrations need to be optimized for individual cultivars (James & Dandekar, 1991; Welander, 1988). Adventitious shoot formation has been induced from cultivars 'McIntosh', 'Triple Red Delicious', and 'Paladino Spur McIntosh' after the addition of 10 μM of TDZ (a synthetic cytokinin) to the regeneration media (Fasolo et al., 1989).

In apples, sorbitol (a sugar alcohol) is the main product of photosynthesis (Bieleski, 1982; Moorby, 1982). Young apple leaves convert sorbitol primarily to CO_2, and actively growing apple tissues contain mainly sorbitol, while dormant tissues contain mainly sucrose (Bieleski, 1982; Loescher et al., 1982). It has been shown that

adventitious shoot regeneration in apple leaf tissue occurs at the highest frequencies in actively growing tissues (Fasolo & Predieri, 1990; James & Dandekar, 1991; James et al., 1989; Welander, 1988). Based on these observations, my hypothesis is that substitution of sorbitol for sucrose as a carbon source in the regeneration media will contribute to increased regeneration frequency of young apple tissue. The objectives of the experiments were to compare the effects of sorbitol and sucrose on regeneration of apple leaf explants and to optimize the regeneration protocol for apple cultivars 'Royal Gala', 'Golden Delicious', 'Empire', and 'Fuji'. In addition, the effect of the regeneration media solidifying agent agar was compared to PhytaGel™. PhytaGel™ is an agar substitute produced from bacterial substrate composed of glucuronic acid, rhamnose and glucose (Sigma Chemical Company, St. Louis, MO). It is a product with high purity, that provides an economical alternative to agar.

3.2 *Materials and Methods*

In vitro clonally propagated shoots of apple cultivars 'Royal Gala', 'Golden Delicious', 'Empire', and 'Fuji' were held on maintenance medium A17 at 25°C (James, 1991) with a 16/8 hour day photoperiod under cool-white fluorescent light (110 $\mu mol/m^2/sec$). Individual shoots were excised and incubated in 14 ml disposable test tubes with 3 ml liquid root induction medium in the dark at 25°C (see Chapter 2). After 2 days, the shoots were transferred to root elongation medium (see Chapter 2) for 10 to 20 days before leaf explants were removed (James et al., 1988a). Only the three youngest fully

unfolded leaves were used for the regeneration experiments, and 7 mm diameter leaf discs were excised with a sterile cork borer.

Adventitious shoot regeneration was induced on basic culture medium containing MS (Murashige & Skoog, 1962) mineral and organic nutrients, 5 mg/l BA, 1 mg/l TDZ, 1 mg/l NAA (BNZ511), pH 5.5 adjusted prior to autoclaving as previously described (James et al., 1993b). The original medium was supplemented with either sucrose or sorbitol at 3% and solidified with PhytaGel™. The effect of the solidifying agent was evaluated by incubating leaf discs on BNZ511:sorbitol medium solidified with either agar (5.8 g/l) or PhytaGel™ (3.0 g/l). Five replications, each consisting of 25 leaf discs were evaluated per treatment. All cultures were incubated in darkness at 25°C until the first adventitious primordia were visible. They were then transferred to cool white light (16/8 h photoperiod, 110 $\mu mol/m^2/sec$).

Shoots were counted every 10 days after culture initiation for two months, only data from 30 and 40 days after culture initiation are presented. Percentage of leaf discs which regenerated one or more shoots and mean number of regenerated shoots per leaf disc (for explants with shoots only) were calculated.

3.3 *Results*

3.3.1 *Effect of carbon source on adventitious shoot regeneration*

'Golden Delicious', 'Fuji', 'Royal Gala', and 'Empire' leaf discs cultured on sorbitol media had higher regeneration percentages than leaf discs cultured on sucrose

Table 3.1 Effect of carbohydrate source and solidifying agent on the regeneration of apple leaf discs

Cultivar	3% sucrose-PhytaGel™ medium	3% sorbitol-PhytaGel™ medium	3% sorbitol-agar medium	Mean % of shoots per cultivar
GD	84%	100%	94%	92.67%±4.67
FJ	70%	95%	86%	83.67%±7.31
RG	85%	100%	92%	92.33%±4.33
EM	73%	88%	77%	79.33%±4.48
Mean % of shoots per treatment	78%±3.81	95.75%±2.84	87.25%±3.82	

The mean percentages of leaf explants regenerating one or more shoots are represented ± SE

Abbreviations: GD - 'Golden Delicious'; FJ - 'Fuji'; RG - 'Royal Gala';

containing media (Table 3.1). No differences were observed among the six cultivars with respect to percent shoot regeneration.

The substitution of sorbitol for sucrose as a carbon source resulted in an approximately 2 fold increase in shoot production in all genotypes (Fig. 3.1). Sorbitol also reduced the time required for shoot development. The first adventitious shoot primordia were detected 30 to 35 days after culture initiation on sucrose medium, while on sorbitol medium primordia arose after 19 to 21 days culturing (Table 3.2). The four cultivars did not differ in the number of days to first primordia appearance (Table 3.2).

The leaf discs in the treatments with 3% sucrose expanded in volume almost two fold during the first two weeks of dark incubation and produced large amounts of calli, while those cultured on 3% sorbitol remained almost unchanged in size and the shoots arose directly from the leaf tissue without intermediate callus phase (data not shown). The shoots regenerated from explants incubated on sorbitol medium elongated faster than those on sucrose and did not display symptoms of hyperhydricity (accumulation of excess water in inter-cellular spaces).

3.3.2 *Effect of media solidifying agent on adventitious shoot regeneration*

The utilization of PhytaGel™ as a solidifying agent in the regeneration media resulted in higher regeneration percentages compared to agar-solidified media for all cultivars (average 9% increase) (Table 3.1). The leaf discs cultured on PhytaGel™ containing media appeared to produce less callus and to have better contact with the culture

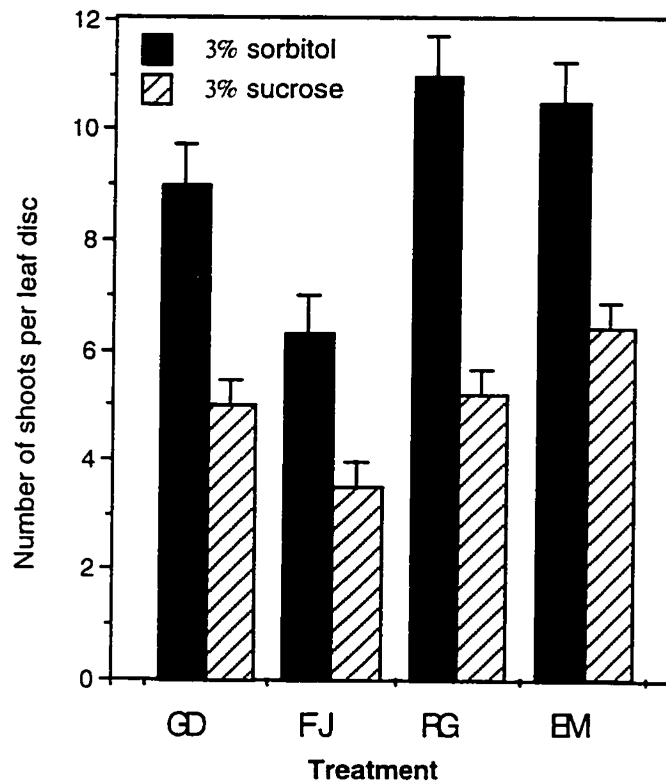


Figure 3.1 Influence of sucrose and sorbitol on the mean number of adventitious shoots produced by 'Golden Delicious', 'Fuji', 'Royal Gala' and 'Empire'. Only leaf discs producing one or more shoots were counted. The data are presented as the mean numbers of shoots per regenerating leaf explant ± SE.

Table 3.2 Influence of the carbohydrate source on time required for formation of adventitious shoot primordia

Apple Cultivar	Time from culture initiation to first visible primordia (days)	
	3% sucrose	3% sorbitol
GD	30	19
FJ	44	22
RG	28	20
EM	35	21
Mean±SE	34±1.34	20±0.65

Means of the treatments are presented ± SE.

Abbreviations: GD - 'Golden Delicious'; FJ - 'Fuji'; RG - 'Royal Gala'; EM - 'Empire'

medium. Compared to the explants grown on agar, PhytaGel™ grown explants produced less browning of the regeneration medium.

3.4 *Discussion*

Development of a highly efficient regeneration system from somatic apple tissues is important for the application of gene transfer technology and for selecting for somaclonal variation. The most important factors influencing the frequency of shoot regeneration has been phytohormone type and concentration in the media (James et al., 1988b). Both cytokinins and auxin are necessary and BA and IBA have proven effective (James et al., 1988b). Although sorbitol has been previously established to be an effective energy source for growing cells and calli from various apple genotypes and related woody plants (Chong & Taper, 1974; Coffin *et al.*, 1976), there are no previous reports on the comparative influence of sorbitol during organogenesis. The BNZ511 medium was previously developed for regeneration of the apple cultivar 'Greensleeves'. For this experiment BNZ511 was supplemented with sorbitol as a carbon source instead of the commonly used sucrose. In comparison with sucrose, sorbitol containing medium increased the percentage of leaf explants regenerating shoots for the four cultivars tested. The mean number of shoots was also dramatically affected with an average increase of approximately 2 fold.

In apple, as in many members of *Rosacea*, sorbitol is the major translocated sugar and metabolite of photosynthesis, as well as an intermediate metabolite, respiratory substrate, and storage compound (Chong & Taper, 1972). This needs to be considered in adventitious shoot regeneration procedures where young leaves are the main tissue source. Previous evidence suggested that sorbitol may play a role in *in vitro* organogenesis of

Malus robusta (Pua & Chong, 1985). The results of this study support the hypothesis that sorbitol has a positive effect on regeneration of shoots for all of the cultivars evaluated. The use of sorbitol decreased the time necessary for the initiation of new meristems. This process is thought to involve a shift in metabolism in which new enzymes are synthesized, or enzymes already present show increased synthesis (Thorpe, 1980).

The use of PhytaGel™ as a solidifying agent has been reported to result in regeneration of transgenic shoots from apple leaf explants from rootstock M.26 (Maheswaran et al., 1992) and to increase the genetic transformation efficiency of several commercial apple cultivars (De Bondt et al., 1992). In combination with sorbitol as a carbon source, PhytaGel™ contributed to high regeneration frequencies for all cultivars. The regeneration percentages varied from 88% for 'Empire' to 100% for 'Golden Delicious' and 'Royal Gala'. The conformity of the response suggests that the medium can be successfully used for all the genotypes. This regeneration procedure has also been used with similar results for 'Delicious', 'Greensleeves' and 'M.9' rootstock (data not shown).

Leaf explants cultured on the BNZ511 with sorbitol and PhytaGel™ produced adventitious shoots 2 to 3 weeks after culture initiation. The shoots were formed directly from the explant surface without visible calli intermediate, suggesting a mechanism of direct organogenesis where adventitious shoot meristems form from epidermal and sub-epidermal leaf cells (Hinchee & Horsch, 1986). Regeneration of shoots from somatic tissues without an intermediate callus phase reduces the levels of somaclonal variation, and benefits the selection of transformants with normal development (D'Amato, 1985).

The modified BNZ511 system developed in this study is simpler, faster and more efficient than previously described methods, providing an optimized step for the application of genetic engineering to apple.

Chapter 4

EFFECT OF AUXIN PRETREATMENT OF APPLE EXPLANTS ON *AGROBACTERIUM*-MEDIATED TRANSFORMATION

4.1 *Introduction*

The introduction of a preculture step preceding *Agrobacterium* infection and co-cultivation has a positive effect on transformation frequencies for several plant species (De Bondt et al., 1994; Sangwan et al., 1992; Sangwan et al., 1991; Yao et al., 1995). During a preculture step, the plant tissue to be transformed is cultured under a set of conditions to induce or increase the competency of the cells for interaction with *Agrobacterium*. In previous studies with apple, experiments with explants precultured on regeneration media with various phytohormones demonstrated that preculture conditions contribute to high transient expression levels and result in a reduction, or had no effect on transgenic calli production. When 'Greensleeves' leaf discs were exposed to regeneration media for periods of 0 to 8 days prior to inoculation, all preculture treatments halved the number of explants that eventually produced kanamycin resistant calli (James, 1991). Additionally, when explants were precultured for six days on regeneration media there was an enhancement of the number of gene transfer events for apple cultivar 'Jonagold', but a decrease in the number of stably transformed calli. One to three days preculture of 'Royal Gala' stimulated very high transient expression levels, but had no effect on the production of transformed calli (De Bondt et al., 1994; Yao et al., 1995). Additionally, preculture of

'Royal Gala' for 5 days resulted in inhibition of transformation (Yao et al., 1995). These experiments tested different cytokinins and combinations of cytokinins and auxins but none of these reports evaluated the effect of auxin pretreatment alone.

Preculture with auxin alone has been shown to stimulate very high transformation and regeneration frequencies in *Arabidopsis thaliana* (Sangwan et al., 1992). The authors concluded that preculturing cotyledon and leaf explants for 5 days on IAA containing medium led to induction of active cell division and dedifferentiation of cells competent for *Agrobacterium*-mediated transformation. IBA is a synthetic auxin, which is widely utilized in plant tissue culture technology for rooting of shoots, micropropagation, regeneration, and embryogenesis. The objectives of this experiment were to test the influence of IBA pretreatment on *Agrobacterium* -mediated transformation and regeneration frequency of the apple cultivar 'Fuji'.

4.2 *Materials and Methods*

4.2.1 *Plant Material*

Leaf explants were obtained from *in vitro* clonally propagated and rooted shoots of 'Fuji' apple (see Chapter 2). From the top three fully unfolded leaves of the rooted shoots, explants were cut with a 7 mm diameter cork borer as described by James & Dandekar (1991) and cultured on one of three different media (see below) in 9 cm Petri dishes with the abaxial side down. The leaf discs were taken from the proximal end of the leaf, excluding the leaf petiole (Welander, 1988).

Prior to bacterial infection, leaf explants were precultured on medium with 3 mg/l IBA (Sigma Chemical Company, St. Louis, MO), 4.43 g/l Murashige and Skoog (MS) salts and vitamins (Sigma Chemical Company, St. Louis, MO), 3% w/v sorbitol (Sigma Chemical Company, St. Louis, MO), and 3 g/l PhytaGel™ (Sigma Chemical Company, St. Louis, MO). The medium was adjusted to pH 5.2 with 1 M NaOH and 1 M HCl prior to autoclaving. Each of the nine treatments consisted of five Petri dishes with 25 explants each. The treatments applied were 7, 6, 5, 4, 3, 2, 1 day, and 6 hours on solid medium and 6 hours on liquid medium. For the 0 hour treatment (no pretreatment control) leaf explants were excised and placed directly into *Agrobacterium* solution. Also, a non-infected, regeneration control was established to determine explant regeneration potential on BNZ511 regeneration medium without selection. The treatments were performed during three individual transformation experiments with three Agrobacterial infections (T1, T2, and T3). T1 includes of treatments 0 hours, 6 hours on liquid medium, 1 day, and 2 day. T2 includes pretreatment 0 hours, 6 days, and 7 days. T3 includes pretreatments 0 hours, 6 hours on solid medium, 3 day, 4 day, and 5 day.

4.2.2 *Bacterial Strain*

The disarmed *Agrobacterium tumefaciens* strain C58C1/GV2260 harboring the binary vector p35S GUS INTR (Vancanneyt et al., 1990) was used in all experiments. The vector included the neomycin phosphotransferase II (*nptII*) gene conferring resistance to the antibiotic kanamycin and the β-glucuronidase (GUS) gene carrying the second intron (IV2) of the St-LS1 gene. The *nptII* gene is under the control of the *nos* promoter; the GUS gene is under the control of the *CaMV35S* promoter.

4.2.3 *Growth and Virulence Induction of Agrobacterium*

A. tumefaciens C58C1/pG2260::p35S GUS INTR was maintained on a selection plate at 4°C. Two loops of bacteria were used to inoculate 5 ml LB broth in a 50 ml screw top centrifuge tube. The culture was incubated for 3 hours at 28°C in the dark with rotary agitation of 200 rpm without selection. Filtered sterilized kanamycin sulfate (Sigma Chemical Company, St. Louis, MO) was added to the 5 ml culture to a concentration of 50 μg/ml and the culture was grown overnight. A 10 ml overnight culture was prepared with 9 ml LB broth, 50 μg/ml kanamycin, and 1 ml bacteria from the overnight 5 ml culture and grown overnight in the dark at 28°C, shaken at 200 rpm.

The 10 ml overnight solution was centrifuged for 20 minutes at 1600xg and resuspended in 1 ml of induction medium (MS20, 1 mM acetosyringone, 1 mM proline, pH 5.2, filtered sterilized) and then adjusted to a final concentration of 0.5 OD at 420 nm. The culture was incubated for 5 hours at 25°C in the dark with shaking at 200 rpm.

4.2.4 *Transformation and Regeneration*

Explants were infected with 5 ml of bacterial solution for 10-20 minutes in a Petri dish with periodic gentle shaking. After infection, the leaf discs were blotted dry on sterile filter paper and placed on co-cultivation medium (BNZ511 covered with sterile filter paper soaked with 1 ml of induction medium) for two days of dark culture at 25°C. After co-cultivation, the explants were transferred to BNZ511 with 300 mg/l cefotaxime and no kanamycin for two days (Yao et al., 1995).

After the two days delayed selection period, the explants were transferred to selection media (BNZ511 with 25 mg/l kanamycin and 200 mg/l cefotaxime) and cultured at 25°C in the dark. Every ten days, explants were transferred to fresh selection medium. Three weeks after initiation of selection, the explants were released from selection and placed on shoot elongation medium (4.43 g/l MS, 3 mg/l BA, 0.1 mg/l GA, 3.0 g/l PhytaGel™, 30 mg/l sorbitol, pH 5.5). The cultures were transferred to light with 16/8 hours photoperiod after a number of shoot primordia arose. Shoots were counted every 20 days after co-cultivation.

4.2.5 *GUS Histochemical Assay*

GUS histochemical assays were performed 20 and 40 days after co-cultivation. Ten random samples from each treatment were placed in microtiter plates with X-Gluc solution prepared by dissolving 10.4 mg X-Gluc in 240 µl dimethyl formamide and adding 10 ml GUS incubation buffer to a final concentration of 2 mM (Draper et al., 1988; Gallagher, 1992b). The explants were incubated overnight at 37°C in the dark, then washed with 70% ethanol to bleach the chlorophyll from the tissue. The percentage of assayed leaf discs showing GUS expression (transformed discs) and the mean number of blue areas per total number of leaf discs with GUS expression were then calculated.

4.3 *Results*

Twenty days after co-cultivation (DAC), leaf discs from the various treatments were assayed for GUS expression. The 6 hour solidified medium treatment showed an increase in the percentage of transformed discs (80%) compared to the no pretreatment control (25%) (Fig. 4.1). Similar results were observed with the 1 and 2 days treatments. The 6 and 7 day treatments resulted in no transformation.

After 40 days, 90% of the leaf discs from the no pretreatment control showed GUS activity. Although some of the auxin pretreatments resulted in higher percent transformation at 20 days, in general these treatments had little effect, or at longer treatments, a negative effect on the transformation.

From the 20 to 40 DAC assay, the mean numbers of blue areas decreased for all treatments except for no the pretreatment control, 6 days, and 7 days (Fig. 4.2). For the 20 day assay, the highest mean number of 45 areas per disc was recorded for 1 day auxin treatment. The lowest mean numbers of blue areas were reported for the 6 and 7 day treatments with no observed blue areas after 20 days of culture.

At 40 days after co-cultivation, a high number of blue areas per leaf disc with GUS expression was observed again for the no pretreatment control. The 1 and 2 days treatments also had a high mean numbers of blue areas. The lowest values reported were with 3 to 6 days treatment with a mean of 1.7. From 20 to 40 days after co-cultivation, most treatments showed a decline in the mean number of blue areas, however the no pretreatment control, 6 days, and 7 days treatments showed improvement over this period.

All treatments with the exception of 3, 4, 5, and 7 day treatments regenerated adventitious shoots that died on selection medium within a period of 6 months, indicating that they were most likely non-transgenic shoots that survived selection.

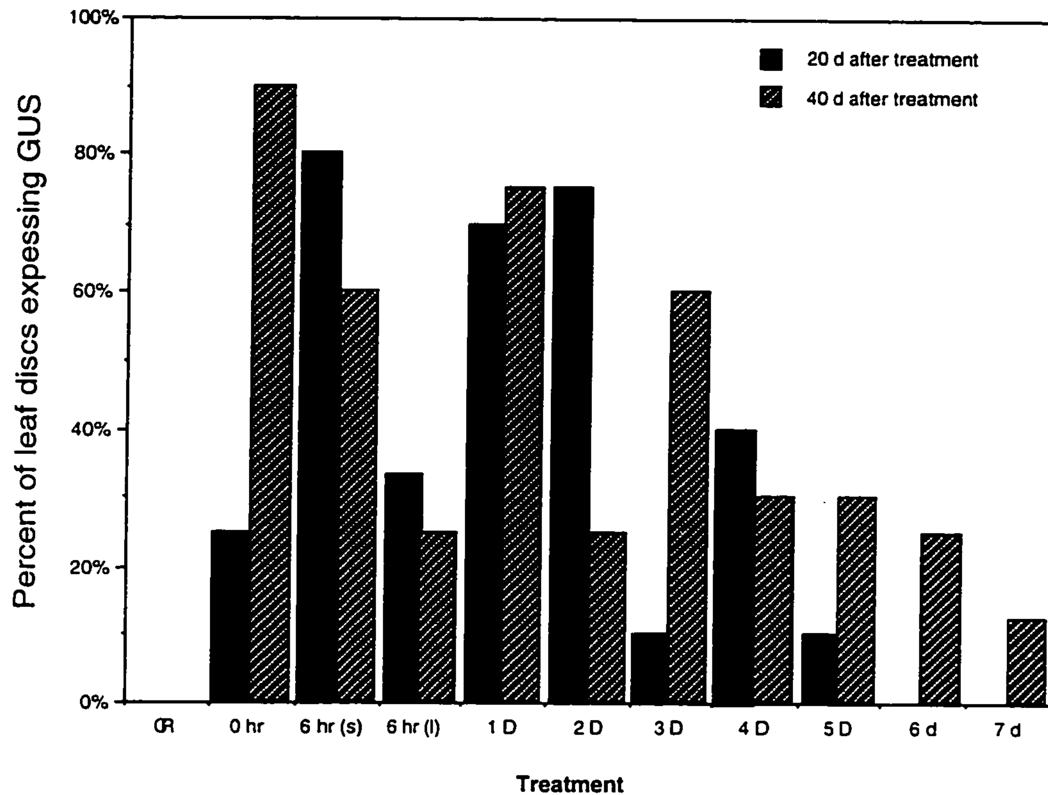


Figure 4.1 Influence of IBA pretreatment on the percentage of transformed apple leaf discs. Leaf discs from 'Fuji' apple cultivar were excised from one month old *in vitro* rooted plants and cultured on media containing 3 mg/l IBA for various times prior to *Agrobacterium* infection. Mean percentages of leaf discs sampled for GUS expression 20 and 40 days after infection are presented.

Abbreviations: CR - non infected regeneration control

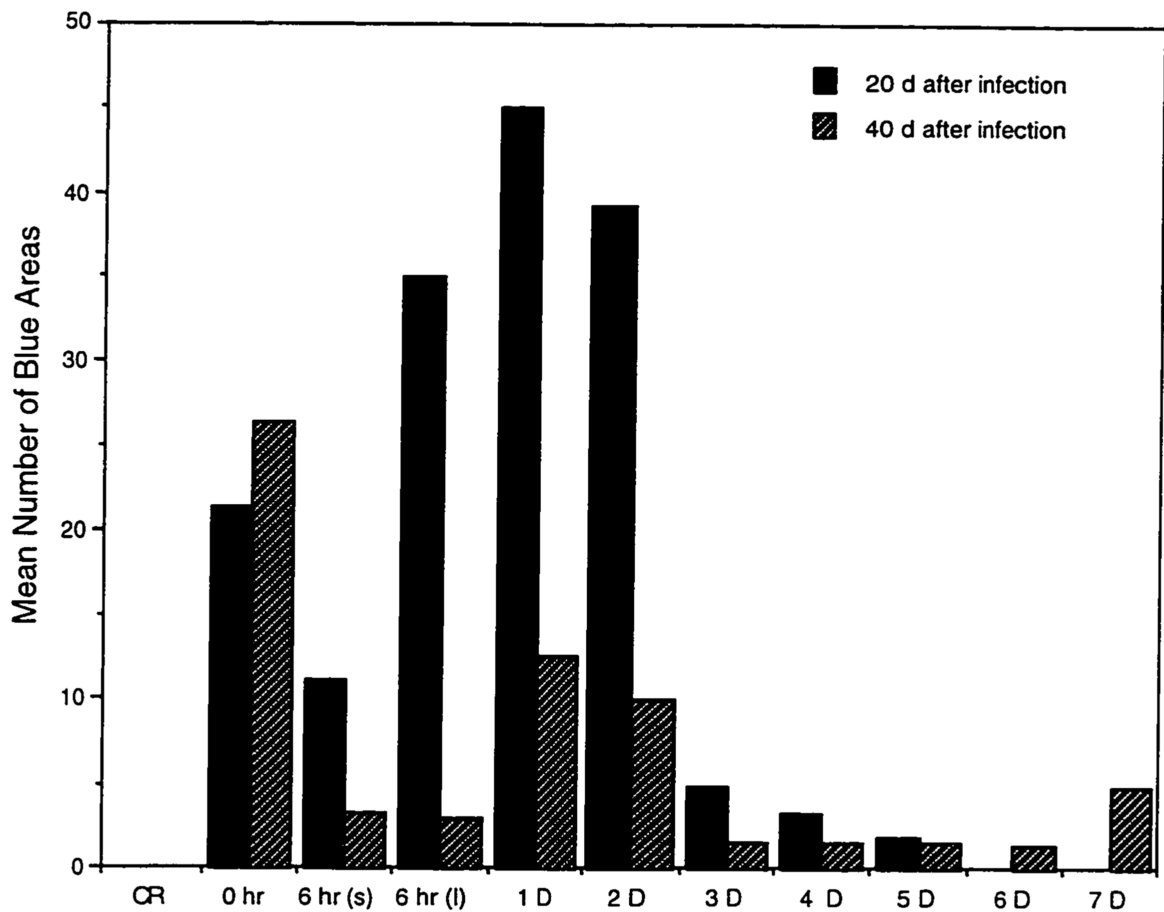


Figure 4.2 Influence of IBA pretreatment on the mean number of blue areas per leaf disc. Leaf discs from 'Fuji' apple cultivar were excised from one month old *in vitro* rooted plants and cultured on media containing 3 mg/l IBA for various times prior to Agrobacterial infection. GUS histochemical analysis was performed 20 and 40 days after infection. Mean number of blue areas per total number of leaf discs showing GUS expression are presented.

Abbreviations: CR - non infected regeneration control

4.4 *Discussion*

The preculture of leaf discs with 3 mg/l IBA prior to bacterial infection did not improve the transformation efficiency or the regeneration of transformed calli and shoots. Twenty days after infection, the percentage of transformed discs increased after treatments of 6 h on solid medium, 1 day, and 2 day, but after 40 days on selection 0 hour pretreatment resulted in the highest percentage of transformed discs. Similar results were recorded for the mean number of blue areas. Twenty days post infection, the mean number of blue areas peaked at the 1 day treatment, but the 0 hour treatment had the highest number after 40 days. The decrease in mean number of blue areas after 40 days could be explained by the occurrence of gene silencing in the later stages of calli development after the initial cell divisions. The results from this study are consistent with previous reports showing that preculture of apple explants on regeneration media did not increase the number of stably transformed calli however these results are unique in that the previous studies did not evaluate the effects of auxin alone (De Bondt et al., 1994; Yao et al., 1995). The number of escapes regenerated from this experiment can be attributed to the two day delayed selection and/or to the low concentration of kanamycin (25 mg/l) in the selection media. Yao et al. (1995) stated that 'Royal Gala' leaf explants pretreated for 3 days with 5 mg/l BAP and 0.2 mg/l NAA showed no improvement in transformation and that a 5 day pretreatment inhibited transformation. The results from my experiment support these observations. Three day pretreatment resulted in a 30% lower transformation than no pretreatment and the transformation for the 5 day treatment was 63% lower than the no pretreatment control.

In conclusion, efforts to increase transformation and regeneration efficiency of *Agrobacterium* transformation of 'Fuji' apple by applying IBA to the leaf disc prior to

bacterial infection were unsuccessful. Although such treatments do increase transformation rates for *Arabidopsis*, apparently apple tissues respond differently. However, it is still possible that similar treatments with different phytohormones, for example the strong synthetic cytokinin thidiazuron, or combinations of several hormones, could increase transformation rates. But, as it is indicated in the next chapter, interaction with *Agrobacterium* is not a rate limiting step in this process, and perhaps other steps in the process should be the topics of future studies.

Chapter 5

EARLY EVENTS OF *AGROBACTERIUM*-MEDIATED GENETIC TRANSFORMATION OF APPLE REVEALED BY THE EXPRESSION OF THE GREEN FLUORESCENT PROTEIN GENE

5.1 Introduction

Genetic engineering offers an exciting opportunity for improvement of plants with long generation and breeding cycles such as apple. Transformation of apple plants expressing marker genes such as nopaline synthase (*nos*), β-glucuronidase (GUS), and neomycin phosphotransferase (*nptII*) have been achieved, but the transformation frequencies reported are relatively low (James et al., 1990; James et al., 1989). Regardless of the high shoot regeneration frequencies achieved for many apple cultivars with leaf explants cultured on non-selective media (Ancherani et al., 1990; Antonelli & Druart, 1990; Fasolo & Predieri, 1990; James et al., 1990; James et al., 1989; James et al., 1988a; James & Thurbon, 1981b; Maheswaran et al., 1992; Pua & Chong, 1985; Sriskandarjah et al., 1994; Theiler-Hadtrich & Theiler-Hedtrich, 1990; Welander & Maheswaran, 1991), the regeneration of transformed plants remains difficult and is genotype dependent. Recent reports with *Agrobacterium*-mediated transformation of the commercial cultivars 'Delicious' (Sriskandarjah et al., 1994), 'Royal Gala' (Yao et al., 1995) Gala, 'Golden Delicious' and 'Elastar' (Puite & Schaart, 1996), indicated that the regeneration of GUS and *nptII* positive plants per total number of explants varied from 1.5 to 8.7%.

Green fluorescent protein (GFP) from the jellyfish *Aequorea victoria* is as a novel genetic reporter system (Kitts et al., 1995) and has become an important *in vivo* reporter in plants (Haseloff & Amos, 1995; Hu & Cheng, 1995; Niedz *et al.*, 1995; Pang et al., 1996; Sheen *et al.*, 1995). When expressed in either eukaryotic or prokaryotic cells and illuminated with blue light (l=395 nm), GFP yields bright green fluorescence. This fluorescence is stable, species-independent, and can be monitored non-destructively. Light stimulated GFP fluorescence does not require any co-factors, substrates, or additional gene products. GFP has now been expressed and has displayed fluorescence in a wide number of organisms including *Escherichia coli*, yeast, the nematode *Caenorhabditis elegant* (Chalfie et al., 1994), *Drosophila melanogaster* (Wang, 1994), *Arabidopsis thaliana* (Haseloff & Amos, 1995), and *Nicotiana tabacum* (Pang et al., 1996). In these organisms, GFP accumulation did not appear to have a toxic effect.

The SGFP-TYG gene is a synthetic GFP gene with codon usage optimized for eukaryotes and with the major restriction sites and cryptic intron site found in wild type GFP removed (Haseloff & Amos, 1995). The serine at position 65 was mutated to threonine, which has previously been shown to result in a single excitation peak by blue light and to emit green light (Heim *et al.*, 1995). The combination of all the modifications of the gene resulted in 120-fold brighter green light emission than the original jellyfish gene (personal communication with Dr. Jen Sheen).

The purpose of this study was to investigate the early events in the transformation and regeneration of various apple cultivars, in order to identify the rate-limiting steps which dramatically lower the regeneration of transgenic tissues when compared to significantly higher regeneration rates obtained from nontransgenic tissues. The GFP gene was used as a sensitive and non-invasive marker to visualize transformed cells during these early events. The results indicated that individual cells in leaf explants from all apple cultivars

tested can be efficiently transformed by *Agrobacterium* and that GFP expression can be detected in single cells within 48 hours after infection. No correlation between transformation rates and recovery of transgenic plants was observed, suggesting that factors other than *Agrobacterium* interaction and T-DNA transfer are the rate limiting steps in apple transformation.

5.2 Materials and Methods

5.2.1 Bacterial strain and vectors

The synthetic SGFP-TYG gene used in this study was provided in plasmid pUC18-HBT-SGFP-*nos* plasmid by Dr. Jen Sheen (Sheen et al., 1995). The SGFP-TYG gene was PCR amplified from pUC18-HBT-SGFP-TYG-*nos* using the following PCR primers - CAG ATC TTT ACT TTG ACA GCT CGT CCA TGC CGT and GGG ATC CAT GGT GAG CAA GGG CGA GGA GCT GTT. The primers contain an additional 5'BamHI and 3'BglI restriction sites, to facilitate cloning. The PCR fragment was then purified and cloned into plasmid pCR^{tm} II (Invitrogen, San Diego, CA) resulting in plasmid pDM96.0417.

Binary plasmid pDM96.0501 was constructed by the ligation of SGFP-TYG as a BamHI-BglI fragment (excised from pDM96.0417) into the BamHI cloning site of binary plant transformation vector pDU92.3103 (Tao *et al.*, 1995) placing SGFP-TYG under the

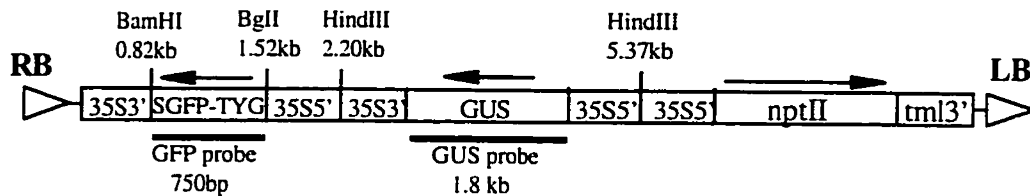


Figure 5.1 Schematic representation of the T-DNA region of the binary plant transformation vector pDM96.0501. Abbreviations: RB, right T-DNA border; 35S5', 35S promoter of the cauliflower mosaic virus; SGFP, coding region of the synthetic green fluorescent protein gene; 35S3', termination signal of the cauliflower mosaic virus; GUS, coding region of the β-glucuronidase gene; *nptII*, coding region for the neomycin phosphotransferase gene; tml3', tml termination signal

control of CaMV35S promoter and terminator (Fig. 5.1). Plasmid pDU92.3103 is a derivative of pCGN1559 (McBride & Summerfelt, 1990) and contains CaMV35S5'-*nptII*-tml3' expression cassette, GUS reporter gene with CaMV35S promoter and terminator, and a gentamycin bacterial selection marker. The CaMV35S promoter-GFP fusion junction was verified by DNA sequencing.

Introduction of pDM96.0501 in the disarmed *Agrobacterium* strain EHA101 was performed by electroporation (Lin, 1994). Plasmid integrity in *Agrobacterium* was verified by Southern blot analysis (not shown).

5.2.2 Growth and virulence induction of Agrobacterium

Agrobacterium tumefaciens EHA101/pDM96.0501 was maintained on a selection plate with 50 mg/l kanamycin and 20 mg/l gentamycin at 4°C. One full loop of bacteria was used to inoculate 10 ml of 523 bacterial media (Dandekar *et al.*, 1989) in a 50 ml screw top centrifuge tube. The culture was incubated for 18-20 hours at 25°C in the dark with rotary agitation (200 rpm). The 10 ml overnight bacterial culture was centrifuged for 20 minutes at 1600xg, resuspended in MS20 induction medium (James et al., 1993b) to 0.5 OD 420 nm, and incubated for 5 hours at 25°C in the dark with shaking at 100 rpm as described by James et al. (1993b).

5.2.3 Plant transformation and regeneration

In vitro clonally propagated and rooted shoots of 'Delicious', 'Golden Delicious', 'Royal Gala', and 'Greensleeves' were used as explant sources (see Chapter 2). The top three fully unfolded leaves of rooted apple shoots were cut with a scalpel blade perpendicular to the midvein into 2 to 3 mm explants and infected with 5 ml solution of induced *Agrobacterium* for 10-20 minutes in a Petri dish. After infection, the leaf discs were blotted dry and placed on top of sterile filter paper overlaid on modified BNZ511 medium (MBNZ511 - Table 5.1). The explants were co-cultured for three days in the dark at 25°C. After co-cultivation, the explants were transferred to MBNZ511 selection medium with 75 mg/l kanamycin and 400 mg/l cefotaxime.

Forty days after culture on MBNZ511 selection medium, all explants were transferred to secondary regeneration medium BN505 (Table 5.1). Once a week the explants were observed by fluorescent microscopy (see below). Leaf explants with green fluorescing leaf primordia were transferred to light with a 16/8 hour light/dark period under cool-white fluorescent light (110 $\mu mol/m^2/sec$). The remaining explants were cultured in the dark until GFP expressing primordia were developed. Every 20 days all explants were transferred to fresh selection media.

The numbers of GFP expressing calli were counted 40 days after bacterial infection and the results were analyzed to determine the percent of explants with one or more GFP expressing calli and the mean number of expressing calli per explant. The numbers of leaf explants with one or more GFP expressing shoots were calculated as a percentage of the total explants infected. Two different *Agrobacterium* infections of 200 to 300 explants were performed for each cultivar. Fifteen explants were cultured in individual Petri dishes

Table 5.1 Tissue culture media formula

	MBNZ511	BN505	RI	RE
MS basal media	4.4 g/l	4.4 g/l	-	2.2 g/l
BA	5 mg/l	5 mg/l	-	-
NAA	1 mg/l	0.5 mg/l	-	-
TDZ	1 mg/l	-	-	-
IBA	-	-	3 mg/l	-
Sucrose	-	-	15 g/l	20 g/l
Sorbitol	30 g/l	30 g/l	-	-
Agar Type A	-	6 g/l	-	6 g/l
PhytaGel™	3 g/l	-	-	-
pH	5.5	5.5	5.2	5.5

Abbreviations: BNZ511 medium (James & Dandekar, 1991); MBNZ511 - modified medium; RI - root induction medium; RE - root elongation medium; BA - 6-benzylaminopurine; NAA - α-naphthaleneacetic acid; TDZ - thidiazuron; IBA - indole-3-butyric acid; All chemicals were purchased from Sigma Chemical Company, St Louis, MO.

and each Petri dish was considered as a replicate. The results from the two separate infections were combined. Variation among cultivars was analyzed with analysis of variance techniques and means were separated using Duncan New Multiple Range Test at $p \leq 0.05$. Control regeneration and selection efficiencies were obtained by culturing 30 non-infected leaf explants from each cultivar on MBNZ511 regeneration medium with and without selection. The leaf explants with one or more shoots were counted and the results are presented as percentages of explants with one or more shoots from the total number of explants and mean number of shoots per regenerating leaf explant.

5.2.4 Rooting and acclimatization of plants to greenhouse conditions

Putatively transformed shoots were transferred to A17 propagation media with 50 mg/l kanamycin and 400 mg/l cefotaxime. After one or two subcultures, 2-3 cm long shoots were transferred to liquid root initiation media (RI-Table 5.1) and incubated in the dark for 48 hours at 25°C. The shoots were then transferred to root elongation media (RE-Table 5.1) and cultured with a 16/8 hour per day photoperiod at 25°C. After 10 to 15 days, plants with well developed root systems (3-4 roots, 3-5 cm long) were transferred to sterile soil in magenta boxes (Sigma Chemical Company, St. Louis, MO), and gradually exposed to the air in a growth chamber as the magenta lids were opened gradually for 5-7 days. The acclimatized plants were transferred to 20 cm pots with a soil-less growing mix (Pro-mix BX, Premier Horticulture Inc., Red Hill, PA) and transferred to a greenhouse.

5.2.5 Fluorescent microscopy

The fluorescent images were taken with a Nikon SMZ-4 dissecting microscope equipped with an epi-fluorescence attachment, a 100W mercury light source, and a Nikon N6006 35 mm camera. Two different emission filters were used: 515 nm long pass emission filter (transmitting red and green light) and 520-560 nm emission filter (transmitting only green light). A 450-490 nm excitation filter was used with each of the emission filters.

5.2.6 GUS histochemical assay

GUS histochemical assays were performed weekly for the first four weeks after co-cultivation. Random samples of 10 explants per cultivar were placed in microtiter plates with X-Gluc solution and tested for GUS activity. X-Gluc solution was prepared by dissolving 10.4 mg X-Gluc in 240 μl dimethyl formamide and adding 10 ml GUS incubation buffer to a final concentration of 2 mM (Draper et al., 1988; Gallagher, 1992b). The explants were incubated overnight at 37°C in the dark. Afterwards, the explants were washed with 70% ethanol to bleach chlorophyll from the tissue.

5.2.7 Southern blot analysis

Apple genomic DNA was isolated from 'Royal Gala', 'Greensleeves', and 'Delicious' transgenic and non-transgenic *in vitro* propagated shoots as previously described (Dellaporta *et al.*, 1983). One additional phenol:chloroform extraction and chloroform:isomyl alcohol extractions were performed. Genomic DNA was dissolved in 100 µl TE buffer and digested with RNase A (50 µg/ml) at 37°C for 1 hour. Ten µg of DNA form each line were digested with HindIII restriction enzyme, fractionated on a 0.8% agarose gel, and transferred to $HybondN^+$ (Amersham International) membrane by the alkaline blotting method (Sambrook *et al.*, 1989). Twenty-five ng of each probe (700 bp BamHI-GFP-PstI DNA (pUC18-HBT-SGFP-TYG-*nos*) and 1.8 kb BamHI-GUS-SacI DNA (pBI426)) were ^{32}P-labeled using the Random primer DNA labeling kit (Boehringer Mannheim GmbH, Germany). The membrane was hybridized sequentially at 65°C for 20 hours in hybridization solution (7% SDS, 0.5 M $NaHPO_4$), washed 2 times under low stringency conditions in 100 ml of 5% SEN (5% SDS, 400 mM $NaHPO_4$, 10 mM EDTA) for 20 minutes at 65°C, followed by wash under high stringency conditions in 100 ml of 1% SEN (1% SDS, 400 mM $NaHPO_4$, 10 mM EDTA) at 65°C, and exposed to a phosphor screen (Molecular Dynamics, Sunnyvale, CA) for 48 hours. The images were scanned using a Phosphor Imager and Image Quant software (Molecular Dynamics).

5.3 Results

5.3.1 Construction of a SGFP-TYG/GUS/nptII co-expression vector

The Ser65Thr mutated synthetic GFP (SGFP) is a reporter gene that potentially allows rapid *in vivo* detection of DNA transfer events in plant tissues. Unlike the GUS reporter gene without a plant intron, the SGFP gene under the control of 35SCaMV promoter reproducibly showed no background expression in *Agrobacterium tumefaciens* (data not shown), thus eliminating the problem of false positive reporter gene detection in plant tissue containing *Agrobacterium*.

The binary vector pDM96.0501 (Fig. 5.1) was constructed to investigate early events of transformation using the SGFP gene. The parental binary vector has high stability in *Agrobacterium*, and both high stability and copy number in *E. coli* (McBride & Summerfelt, 1990). This is due to the presence of two different origins of replication, ColE1 from *E. coli*, and pRiHRI from *Agrobacterium*. The GUS reporter gene and the *nptII* selectable marker gene also present in this plasmid provide additional alternatives for detection of DNA transformation. The presence of the bacterial gentamycin resistance gene allows for efficient selection of this vector in the highly virulent and kanamycin resistant *Agrobacterium tumefaciens* strain EHA101 (Hood et al, 1986).

5.3.2 Transient GFP expression reveals a high gene transfer rate

Leaf explants from *in vitro* propagated and rooted apple plants were inoculated with *Agrobacterium tumefaciens* EHA101 harboring plasmid pDM96.0501. After the initial infection, the explants co-cultivated on MBNZ511 medium for three days and GFP expression was monitored daily with a fluorescence stereo microscope. The first fluorescence was detected 48 hours after the end of the co-cultivation. Fluorescence was first observed along the cut edges and wounded sites on the leaf surface (Fig. 5.2 a, b, c, d). The highest number of fluorescent cells were seen at cut vascular tissues (Fig 5.2 d). Between 95 and 100% of the explants from all cultivars had a large number of infection sites, most of which lost their fluorescence within the following few weeks.

5.3.3 GFP expression in apple calli

Only a few of the large number of cells that were fluorescing immediately after transformation continued to express GFP after 30 days and formed calli on selection media. The 'Golden Delicious' fluorescing calli displayed on Fig. 5.2, panels m, n, q and r were grown on selection media for 34 days. The non-transformed tissue from the original explant was still green, but cell division was suppressed by the selective antibiotic (Fig. 5.2 e). Upon illumination with blue light only the GFP expressing calli emitted green light and were visible (Fig. 5.2 f). A 34 day old culture of transformed 'Delicious' tissue is presented in Figure 5.2 g, taken with 450-490 nm excitation and 515 nm emission filters. This filter setting allowed imaging of the red fluorescence from the chlorophyll and some endogenous background fluorescence from the non-transformed tissue. The formation

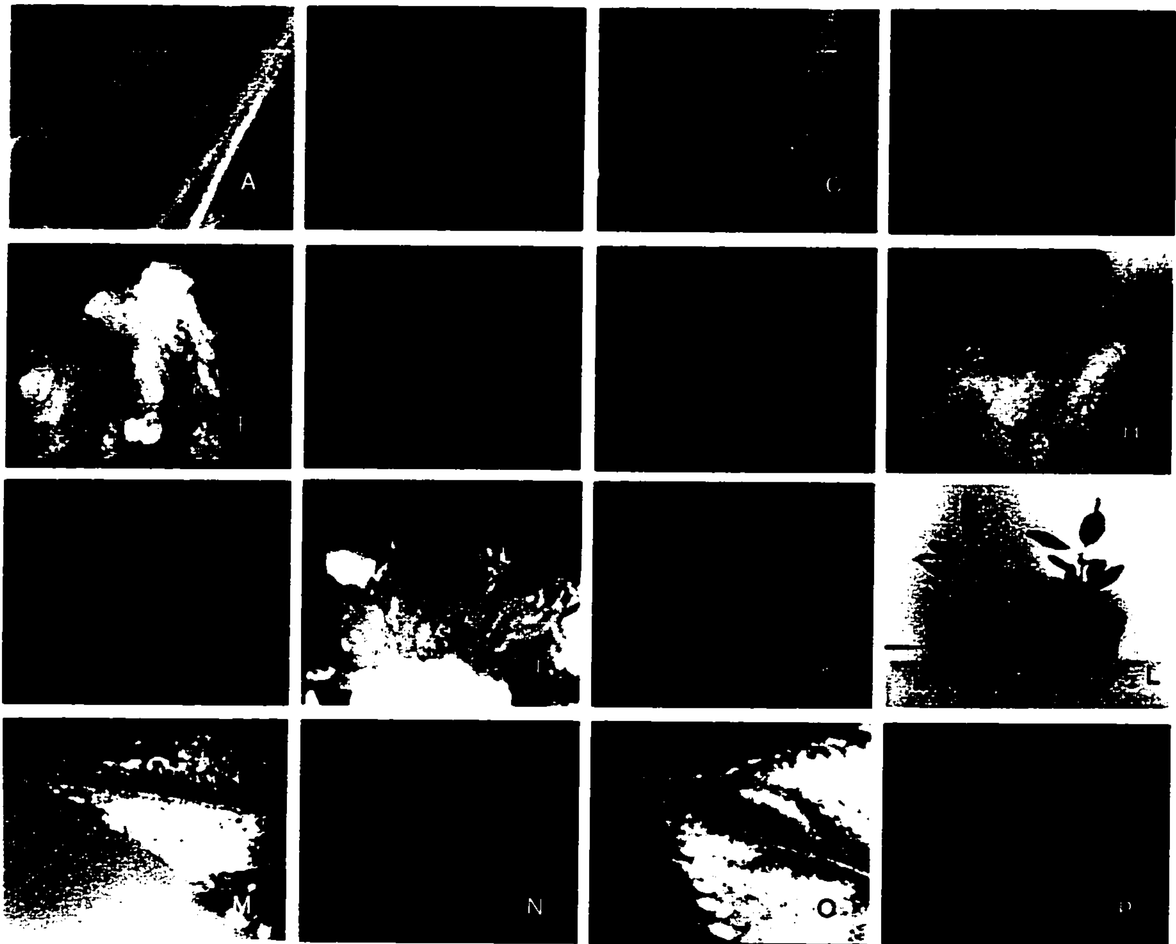


Figure 5.2 Green Fluorescent Protein expression in apple leaf explants and transgenic apple plants visualized by fluorescent microscopy. Panels A, C, E, H, J, M and O - light micrographs; Panel G - micrographs taken with 515 emission filter and 450-490 nm excitation filter; Panels B, D, F, I, K, N and P - micrographs taken with 520-560 nm emission filter and 450-490 nm excitation filter. A. Leaf explant 48 hours after *Agrobacterium* infection. B. Fluorescent images of the explants from panel "A". C. The cut edge of leaf explant 48 hours after *Agrobacterium* infection. D. Fluorescent images of the explants form panel "C". E. Agrobacterium infected leaf explant from 'Golden Delicious' cultured on selection media, 30 days after infection. F. Fluorescent images of the leaf explants from panel "E". G. Fluorescent image of 'Delicious' leaf explant with developed calli, 30 days after bacterial infection. H. Leaf primordia formed on *Agrobacterium* infected leaf explant after 60 days culturing on kanamycin media. I. Fluorescent images of the shoot primordia from panel "H". J. Leaf primordia developed on non-infected leaf explant grown without selection for 30 days. K. Fluorescent image of shoot primordia form panel "J" L. Control and transgenic 'Royal Gala' plants grown in greenhouse conditions. M. Light image of a green leaf from transgenic 'Greensleeves' apple plant. N. Fluorescent image of the leaves from panels "M". O. Light image of a leaf from non-transgenic 'Greensleeves' plant. P. Fluorescent image of the leaf explant from panel "O".

non-transgenic calli was closely associated with the GFP expressing cells. After an extended period of culture on selection media, the development of the non-transgenic calli stopped.

Forty days after *Agrobacterium* infection, the numbers of developing calli expressing GFP were counted (Table 5.2). 'Delicious' and 'Golden Delicious' had the highest percentage of transformed calli and mean number of GFP expressing calli per leaf explant, followed by 'Royal Gala'. 'Greensleeves' produced the lowest percentage of transformed calli and mean number of GFP expressing calli.

5.3.4 Detection of GFP fluorescence in etiolated and green shoots

Sixty days after bacterial infection some of the GFP expressing calli developed leaf primordia (Fig. 5.2 h). Compared to nontransformed leaf primordia regenerated without selection which showed no fluorescence (Fig. 5.2 j and k), the GFP expressing leaf primordia fluoresced brightly (Fig. 5.2 i).

After the first few leaves on the newly regenerated shoots were formed, the explants were transferred to light. The shoots developed chlorophyll and were transferred to micropropagation media with selective antibiotics. The green leaves of the transformed plants also expressed GFP and the green fluorescence was easily detectable (Fig. 5.2 m and n). However, the intensity of the fluorescence in green leaves was less than the etiolated shoots (Fig. 5.2 i) possibly due to absorption of the fluorescence from some of the newly developed leaf pigments, and/or the enlargement of the cell vacuole and dilution of the GFP protein in the cell.

Table 5.2 GFP expression in apple calli and regeneration of transgenic shoots

Apple cultivar	Control regeneration/ Mean # of shoots per leaf explant	Total number of infected explants	Percent GFP expressing explants	Mean # calli ± SE per leaf explant	Number of GFP plants/ percent regeneration
GD	71%/5.6	251	95.7%	4.12 ± 0.23^{a}	0/0.0%
RD	72%/8.2	423	89.6%	4.85 ± 0.59^{a}	1/0.2%
RG	87%/4.5	300	63.6%	2.37 ± 0.36^{b}	9/3.0%
GS	90%/7.9	159	37.1%	0.87 ± 0.13^{c}	9/5.8%

The results are presented as percentages of explants with one or more shoots from the total number of explants and mean number of shoots per regenerating leaf explant. The GFP expressing calli were counted 40 days after bacterial infection and the percent of explants with one or more GFP expressing calli and the mean number of expressing calli per explant were calculated. The leaf explants with one or more GFP expressing shoots were are presented as a percentage of the total explants infected. Means of all replicates for each cultivar were averaged to obtain the mean number of GFP expressing calli per explant and were compared by Duncan New Multiple Range test. Mean values followed by the same letter (a, b, c) are not significantly different at the 0.05 probability level.
Abbreviations: GD - 'Golden Delicious', RD - 'Delicious', RG-'Royal Gala', GS- 'Greensleeves'.

All cultivars exhibited high shoot regeneration potential on non-selective media, however genotypic variation was observed under selection pressure (Table 5.2). The cultivar with the highest transformation percentage ('Golden Delicious', 96.7%) did not produce any transgenic shoots. Surprisingly, 'Greensleeves', which had the lowest transformation percentage produced the highest number of transgenic shoots. This finding is consistent with previous studies that showed a higher frequency of production of transgenic plants from 'Greensleeves' compared to other apple cultivars (James, 1991; James et al., 1989; James et al., 1988a). All of the cultivars regenerated a number of non-transformed shoots that were usually associated with GFP expressing calli. Those shoots did not exhibit green fluorescence and, when transferred to light, did not develop chlorophyll and died.

5.3.5 Rooting and acclimatization of GFP transgenic apple plants to greenhouse conditions

Twenty-six plants from transgenic lines RGGFP2 ('Royal Gala'), GSGFP1, 4, 5, 6, 8, 9 ('Greensleeves') were cultured on RI and RE media (Table 5.1) with 50 mg/l kanamycin (Table 5.3). Eighteen of these plants (69%) developed two to five roots and were transferred to soil. The development of the transgenic plants was compared to control plants from the same cultivars regenerated without selection. All transgenic and control plants grew 10-15 cm within the first two weeks and developed 5-9 new leaves. No toxic or negative effects of the GFP protein on the transgenic plants were noticed (Fig. 5.2 I).

Table 5.3 Rooting of transgenic apple shoots

Transgenic line	Total number of plants cultured	Number of plants rooted
RG-CR	5	3
RGGFP2	5	3
GS-CR	5	3
GSGFP1	10	8
GSGFP4	1	1
GSGFP5	3	1
GSGFP6	1	1
GSGFP8	3	1
GSGFP9	3	3

Abbreviations: RGCR - 'Royal Gala', control regeneration; GSCR - 'Greesleeves', control regeneration; RGGFP2 - 'Royal Gala'-GFP transgenic line #2; GSGFP1- 'Greensleeves'-GFP transgenic lines #1, 4, 5, 6, 8, and 9.

5.3.6 Southern blot analysis

To confirm the integration of T-DNA in transgenic apple plants, Southern blot analysis of three independent transgenic clones and two nontransformed clones was performed (Fig. 5.3). Genomic DNA was isolated from non-transformed 'Royal Gala' and 'Greensleeves' *in vitro* propagated plants (lane 2 and 4) and transformed 'Delicious' - clone RDGFP1 (lane 1), 'Royal Gala' - clone RGGFP2 (lane 3), and 'Greensleeves' - clone GSGFP1 (lane 5). DNA was digested with HindIII and hybridized sequentially with ^{32}P-labeled GFP (Fig. 5.3a) and GUS probes (Fig. 5.3b). As expected the GFP probe hybridized to fragments longer than 2.2 kb with DNA isolated from transgenic plants and did not hybridize to digested DNA from non-transformed plants (Fig 5.3a). The sizes of the bands (5.09, 3.05, and 3.50 kb) corresponded to the distance between the HindIII site at position 2.20 kb in the T-DNA region and the nearest HindIII site in the genomic DNA flanking the insertion sites. The different fragment sizes indicated that each plant arose from separate transformation event. When the intact Ti plasmid pDM96.0501 was cut with HindIII, a single band of 16.5 kb was produced (data not shown), indicating that the various bands in the apple DNA digests were not result of contaminant from *Agrobacterium* DNA. The presence of single bands indicate one T-DNA insertion per apple genome. When hybridized with a GUS probe, DNA from all of the transgenic plants analyzed produced an expected 3.17 kb band, corresponding to the intact internal HindIII fragment of the transformation construct (Fig. 5.3b).

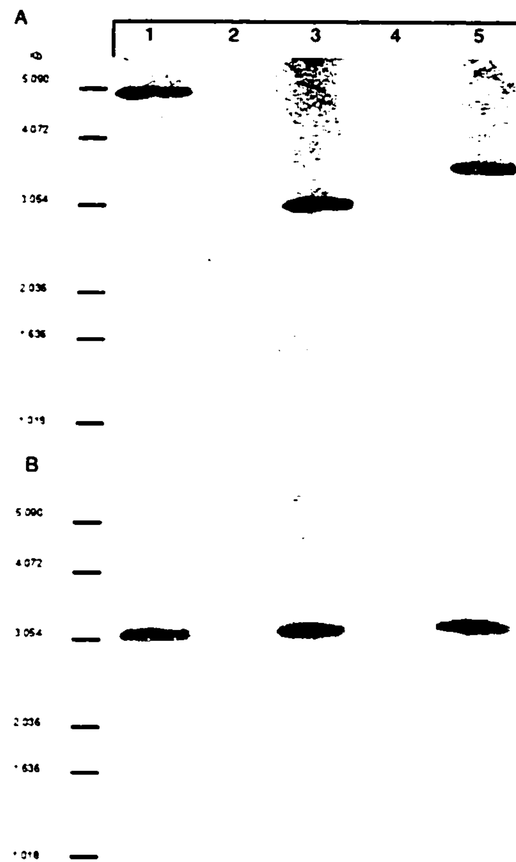


Figure 5.3 Southern blot analysis of genomic DNA from transgenic apple plants containing the T-region of pDM96.0501. Genomic DNA was isolated from non-transformed 'Royal Gala' and 'Greensleeves' *in vitro* grown plants (lane 2 and 4) and transformed 'Delicious' - clone RDGFP1 (lane 1), 'Royal Gala' - clone RGGFP2 (lane 3), and 'Greensleeves' - clone GSGFP1 (lane 5). 10 µg of genomic DNA digested with HindIII from each sample were electrophoresed on a 0.8% agarose gel, transferred to HybondN+ membrane and hybridized sequentially with 32P-labeled GFP (panel A) and GUS probes (panel B).

5.4 Discussion

Four different apple cultivars were genetically engineered to express the green fluorescent protein. A synthetic, codon optimized GFP gene was demonstrated to be an efficient reporter gene in transgenic apple plants and to have a number of advantages over other commonly used reporter genes. Using a stereo-microscope with epifluorescent attachment, the green fluorescence can be detected within 48 hours following co-cultivation without the use of a lethal assay system as for GUS. This provides an opportunity for monitoring early events of transformation over time and rapidly testing the influence of different factors on the efficiency of DNA transfer.

The results from this study confirmed that the procedure used for inducing virulence in *Agrobacterium* for the infection of apple leaf tissue provided a high number of transformation events in all four cultivars tested. The highest number of GFP expressing cells were associated with the cut vascular tissues. This could be explained by the higher cell density and the larger number of cells in the vascular tissue of the apple leaf, compared to the lower cell density (due to large air spaces) and cell number in the leaf blade. Another possibility is that the vascular cells have specific morphological or biochemical characteristics, perhaps wound induced, which enables them to become highly susceptible to *Agrobacterium*. The type and the metabolic state of cells competent for *Agrobacterium* transformation appear to vary among plant species and different tissue explants and to a certain extent depend upon the phytohormone content in the culture media (Colby *et al.*, 1991; De Bondt et al., 1992; De Bondt et al., 1994; Monacelli *et al.*, 1988; Sangwan et al., 1992). For example, when *Arabidopsis thaliana* cotyledon explants were cultured on callus-induction media and infected with *Agrobacterium*, competent cells were found

among the mesophyl cells located both around vascular traces and beneath the epidermal layer in contact with the medium (Sangwan et al., 1992).

The localization of the GFP protein/fluorescence allows a precise identification of individually transformed cells. GFP does not appear to move inter-cellularly or to be secreted out of the cell. A key feature of GFP as a reporter gene for these experiments was the absence of endogenous background fluorescence in apple tissue. However, in some plant species, for example *Nicotiana tabacum*, the use of GFP as a genetic reporter gene could be restricted due to the high autofluorescence of some tissues, particularly roots (data not shown). The *Agrobacterium* strains examined did not have significant endogenous fluorescence at the wavelengths used for GFP detection. Additionally when pDN96.0501 was introduced into EHA101, the GFP gene did not produce any bacterial expression.

The *in vivo* GFP assay permits for monitoring the growth of individually transformed cells and their development into calli and/or shoots. The results from this experiment indicated that significant numbers of individually transformed cells underwent cell division and formed GFP expressing calli (up to 4.58 calli per leaf disc), but only a few developed leaf primordia. This was independent of the regeneration potential of the leaf tissue or the frequency of the DNA transfer, and seemed to be due to the continuous selection pressure applied. Although all cultivars expressed very high regeneration potential on non-selection media, when infected with *Agrobacterium* and cultured on selection media, the percent regeneration decreased dramatically regardless of the high transformation efficiency in some cultivars. Surprisingly, the cultivars that produce highest percentage of transformed calli did not regenerate, or regenerated a very low number of transgenic shoots (Table 5.2).

The results also indicated that there were differences in the response to the selection pressure among the cultivars. Two of the cultivars tested, 'Greensleeves' and 'Royal

Gala', regenerated a number of transgenic shoots, which suggested a higher tolerance of the tissue to the antibiotic or perhaps a different mechanism of adventitious shoot regeneration. In all cultivars it was observed that most of the shoots formed on non-selection media occurred within 30-40 days after culture initiation and regenerated without an intermediate callus phase. Perhaps these shoots had a multicellular origin and arose from adjacent healthy unwounded cells. Similar shoots that regenerated from infected leaves under antibiotic selection were non-transgenic escapes. On non-transformed explants, a very small number of shoots, primarily associated with the cut edges, developed with an intermediate calli phase (data not shown). This kind of shoot regeneration was slower than direct shoot regeneration. Similar shoot development was observed after *Agrobacterium* infection, where the cut edges of the explants were the main sites of DNA transfer. The regeneration process in these explants was also inhibited by the presence of the selective antibiotic in the media. The fact that transgenic cells from some of the cultivars cannot overcome the selection pressure, and failed to regenerate shoots, indicates that the original regeneration media, although inducing a very high number of primordia with multicellular origins from leaf explants without selection, even over an extended period of time, did not effectively induce morphogenesis from a single cell or unorganized calli cells. It is possible that the content of the regeneration media needs to be adjusted for the individual cultivars considering the extended time of culture and the tissue requirement changes in relation to *Agrobacterium* infection.

One of the potential concerns about expressing GFP in plants under the control of a constitutive promoter has been its toxicity at the cellular levels and on general plant development under direct light. Regardless of the very high fluorescence in etiolated tissues, the regenerated plants developed a normal phenotype when transferred to light. Eighteen plants from different transgenic lines were rooted on selection media and

successfully acclimatized to greenhouse conditions. These plants continued to grow and did not display developmental abnormalities or differences from the control plants.

These observations raise a number of possibilities as to the rate limiting step in apple *Agrobacterium*-mediated transformation. The proposed model of the stages of transformation is depicted in Figure 5.4. The fact that large numbers of fluorescing cells per leaf explant were observed 48 hours after co-cultivation, suggests that *Agrobacterium* infection and the DNA transfer is not the rate limiting step. However, two weeks following infection only a few of these cells developed calli and showed stable expression, indicating that either the DNA was not efficiently integrated into the genome, or if integrated, gene silencing occurred at high frequencies. Additionally, regeneration of transgenic apple shoots appears to be a second low efficiency process, possibly because selection with kanamycin forces a single cell origin. Together, both steps reduced the efficiency of the transformation and regeneration process approximately 10,000 fold, suggesting that factors other than *Agrobacterium* interaction and T-DNA transfer are rate limiting steps in *Agrobacterium*-mediated transformation of apple. In the case of regeneration in the absence of selection, which occurs at very high frequencies, regeneration is evident with no calli intermediate, perhaps from multiple cells in the leaf tissue (Colby et al., 1991; D'Amato, 1985; Monacelli et al., 1988). In the case of regeneration with selection, the new shoots must arise from a single cell, which first forms a disorganized callus tissue. It is possible that positional information in the different cell layers involved is a factor in generation of new meristems with high efficiency. In the case of single cell origin, disorganized calli must organize into a meristematic structure. A similar process has been observed during shoot formation from 5 days old tomato cotyledon cultures, where groups of 2-4 initial cells differentiated within the callus and led to formation of zones of intensive meristematic activity (Monacelli et al., 1988). Detailed

histological analysis of tissues undergoing regeneration is necessary to resolve this question.

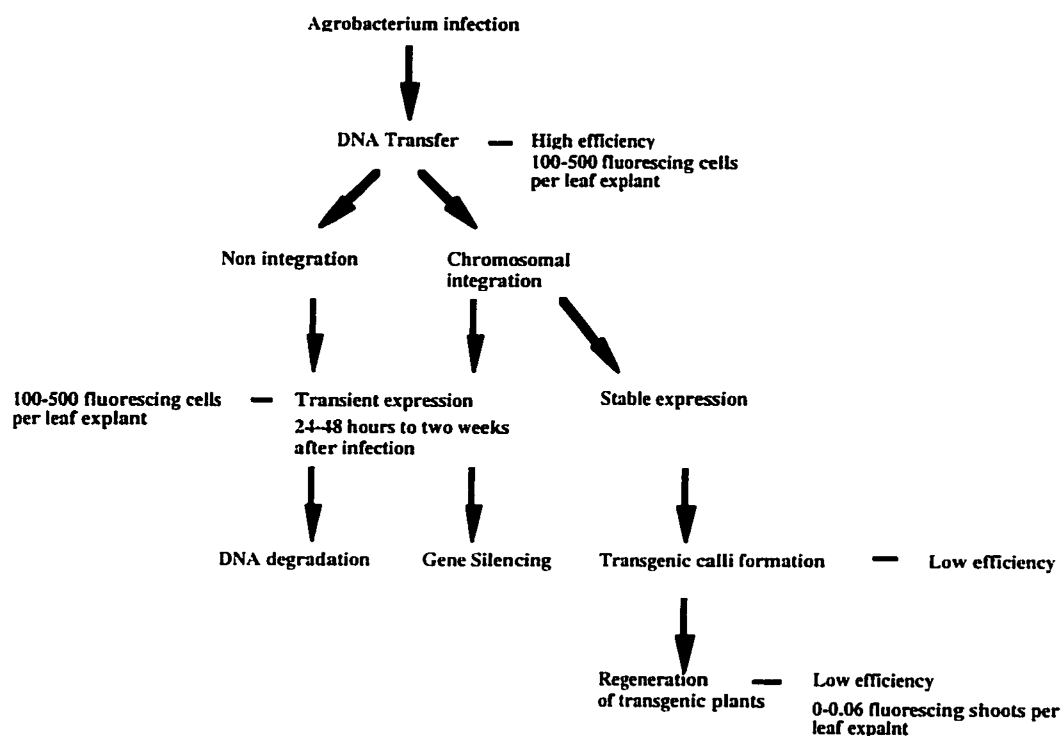


Figure 5.4 Proposed model for the efficiency levels of different stages of *Agrobacterium*-mediated transformation of apple leaf explants.

Chapter 6
FUTURE RESEARCH

6.1 Further investigation of Agrobacterium-mediated transformation of apple: Identification of the rate limiting steps.

A synthetic, codon optimized GFP gene was shown to be an efficient reporter gene in transgenic apple plants. The *in vivo* assays revealed that frequency of DNA transformation is not the limiting step of the transformation process. For more detailed study of the early events of transformation and transient GFP expression, an *in vivo* time course experiment following the progression of the expression could be performed. This experiment is necessary to support the previous observation that GFP expression levels increase during the first week after bacterial infection and co-cultivation, followed by decrease in expression levels due to cessation of transient expression or gene silencing.

One possible explanation for the high levels of transient expression, followed by low frequencies of transgenic plant regeneration was offered in a recent paper by Nam et. al. (1997). According to this study there is a considerable variation among ecotypes of *Arabidopsis* in their susceptibility to crown gall disease. One ecotype showed efficient transient GUS expression, but was deficient in crown gall tumorigenesis and stable transformation. This ecotype was also more sensitive to γ radiation compared to ecotypes more susceptible to transformation. The authors speculated that the radiation sensitivity may reflect a defect in single- and/or double-strand DNA break repair and that such repair processes may play role in T-DNA integration via illegitimate recombination. It is possible that the inability of apple to stably integrate T-DNA is due to similar defect in DNA repair

mechanism. Investigating this possibility may provide additional information about the rate limiting step/steps of the *Agrobacterium*-mediated transformation of apple.

6.2 *Optimization of the regeneration media for individual apple cultivars*

For all of the cultivars tested, most of the adventitious shoots formed on non-selective media within 30-40 days after culture initiation without an intermediate callus phase. After *Agrobacterium* infection and culture on selective medium, adventitious shoots were primarily associated with the cut edges and developed with an intermediate callus phase within a period of 2 to 12 months. These results suggest that two different mechanisms of regeneration are operative in these cases. The fact that transgenic cells from some of the cultivars cannot overcome selective pressure and failed to regenerate shoots, indicates that the original regeneration media, although inducing a very high number of primordia from leaf explants without selection, did not effectively induce morphogenesis after *Agrobacterium* infection. Additional experiments on the content of the regeneration media are needed to adjust for individual cultivars.

6.3 Introduction Bt genes into apple cultivars for insect resistance

One exciting possibility is the introduction of Bt toxin genes into apple. Some of the most important pests of apple are Bt susceptible, including apple maggot, codling moth, gypsy moth, oblique-banded leaf roller, apple pandemis, omnivorous leafroller, fruit tree leaf roller, western tussock moth and green fruitworm. Currently, most of the apple biotechnology work is being performed in California, New York, Europe and Australia. In the long run our research program will be focused on traits and cultivars important to Pennsylvania. For example, Empire is used extensively in Pennsylvania for its improved storage, heat resistance and color compared to McIntosh. However, to our knowledge, we are the only laboratory working to engineer this cultivar. Having such a research program in place at Penn State will insure that the Pennsylvania apple growers will have access to the technologies of the future.

DNA constructs containing Bt toxin genes or other genes will be provided to Penn State. Transformed and regenerated plants will be rooted and placed in the greenhouse environment. Various economically important cultivars will be used. Proof of transformation will include: GFP marker gene detection when applicable, Southern blot analysis of plant genome-Ti plasmid border fragments for each transformed line, gene and protein expression data where applicable.

6.4 Field testing of transgenic trees

Transgenic plants will be grafted to dwarfing rootstock and planted at Penn State field stations to obtain maximal growth rate and to allow large numbers of plants to be

grown and observed. USDA APHIS and Penn State clearance will be first obtained. Eventually around 1,000 trees will be planted on about 2 acres of land. Additionally, plants containing insecticidal protein genes will be able to be tested for resistance against foliar insects. This will require collaborative arrangements with Dept. of Entomology Scientists.

Once fruit set is achieved, testing of the fruit for the targeted traits will be performed with collaborations between Penn State Scientists in The Biotechnology Institute, Horticulture, Food Sciences and Entomology as well as with food processing and apple production companies in Pennsylvania.

Appendix A

IDENTIFICATION OF APPLE LEAF CELLS COMPETENT FOR *AGROBACTERIUM* TRANSFORMATION

Introduction

Arabidopsis cotyledon and leaf cells competent for *Agrobacterium* transformation have been characterized in a study by Sangwan et al. (Sangwan et al., 1992). Competency was induced in dedifferentiating mesophyll cells by wounding and/or phytohormone treatment (callus-induction medium for 3-7 days). The induced cells were small, isodiametric, with thin primary cell walls, small and multiple vacuoles, prominent nuclei and dense cytoplasm. Additionally, cells induced by wounding had thin, primary cell walls with gaps in them and no plasmodesmata. These cells were observed only at cut surfaces. All competent cells were metabolically active and dividing. The highest transformation frequency was recorded after 5-6 days of explant preculture. At this stage 2C nuclei had their highest protein level, were the principal metabolic sinks, and were being recruited into S phase. Other cells types such as epidermal and vascular-bundle cells did not undergo dedifferentiation and were not transformed. Mesophyll cells not treated with phytohormones did not differentiate.

Presented here are preliminary results from microscopic studies of cultured apple tissues. The objectives of this study were to assess if apple cells cultured on regeneration medium MBNZ511 display dedifferentiation and morphology similar to the *Arabidopsis* competent cells characterized by Sangwan et al. (1992).

Materials and Methods

Leaf discs (7 mm) were excised from *in vitro* propagated and rooted plants of 'Delicious' apple (see Chapter 2). The discs were cultured on MBNZ511 regeneration medium and samples were collected at 2 hours (0 days), 1, 3, 5, 6, and 7 days after culture initiation.

The samples were fixed in 1.5% gluteraldehyde and 2.5% paraformadlehyde in 0.1M phosphate buffer (pH7.4) for 2 h, then washed 3X for 5 min each in 0.1M phosphate buffer (pH7.4), post-fixed in 1% osmium tetroxide in 0.1M phosphate buffer (pH7.4) for 1.5 h, and washed 3X for 5 min each in 0.1M phosphate buffer (pH7.4) for 1.5 h. After dehydration in an ethanol series, the samples were stained overnight with 2% Uranyl acetate in 100% ethanol at 4C, washed 3X for 10 min each with 100% ethanol and 3X for 10 min. each with 100% propylene oxide acetone, and were embedded in Spurrs resin (Electron Microscopy Science, Washington, PA). Thin sectioning (60-90 nm) was performed with an Ultramicrotome (Model LKBIII-8800, LKB Instruments, Rockville, MD) and the section plane was from the center of the leaf to the peripheral edge of the leaf. Specimens were observed with TEM (Model 1200EXII, JEOL Com., Peabody, MA). The sample preparation and sectioning were performed by the staff of the Electron Microscopy Facility at Penn State.

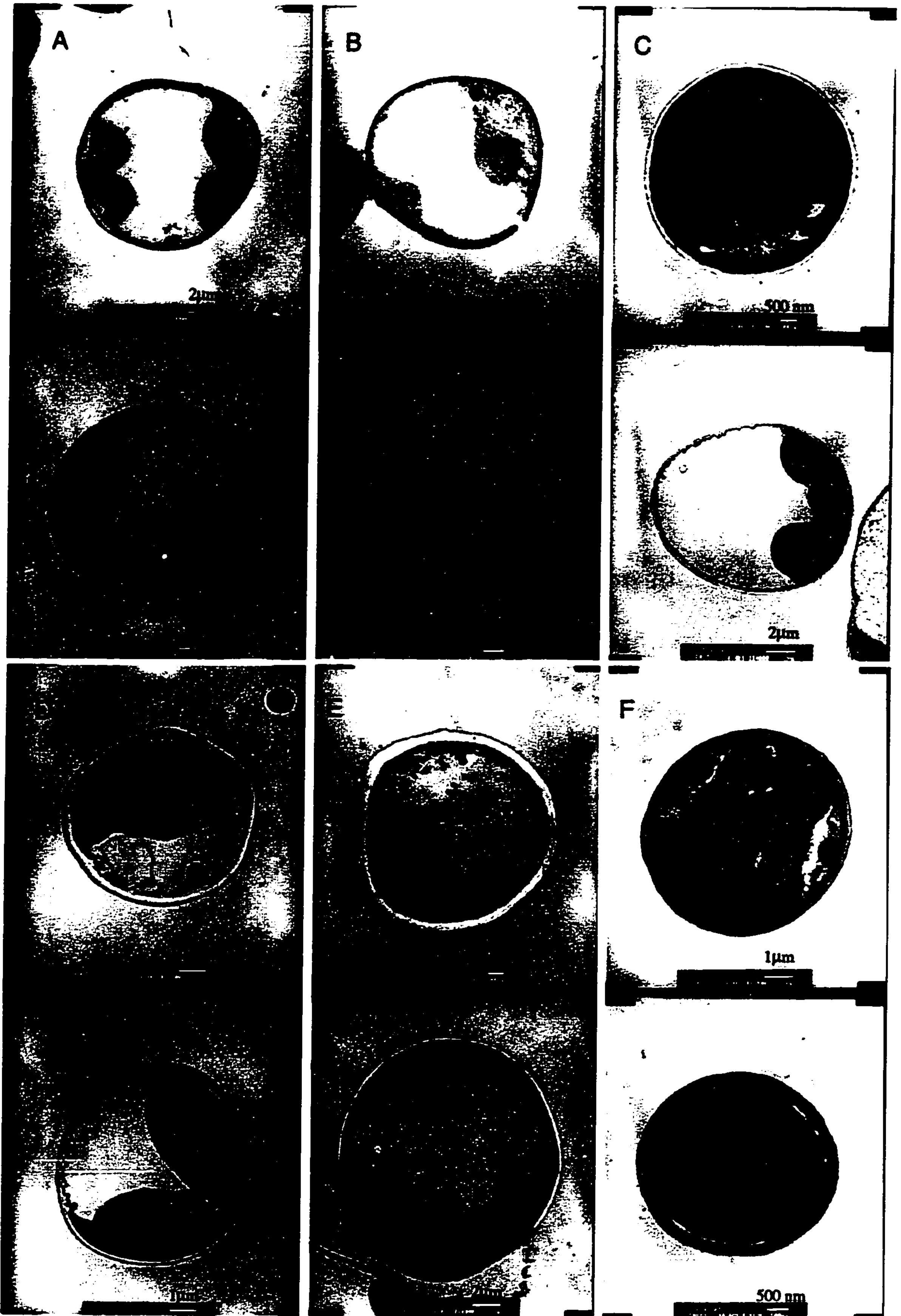
Preliminary results

An even distribution of mesophyll cells resembling Sangwan's description was observed across the entire plane of each section for treatments 0 to 3 days on regeneration

medium (Fig A.1 a, b and c). Cells without phytohormone treatment (0 days, Fig A.1 a) that originate from actively growing leaf tissues, displayed some of the characteristics of wound induction without phytohormone treatment. They are actively dividing with isodiametric form, a peripheral layer of cytoplasm and thin cell walls. Cells observed after 1 day treatment on regeneration medium had similar characteristics as the 0 day treatment cells, but their cell walls were thinner and wall-less areas were observed (Fig. A.1 b). Thus the cells we observed in 0 to 3 days treatments resemble the description of competent cells induced by wounding and not as a result of phytohormone treatment. Multi-vacuolate cells (as a main characteristic of the phytohormone induced cells) were not observed after these treatments. All the cells had a one large vacuole. Some multi-vacuolate cells were observed after 6 days treatment (Fig. A.1 e). In general, after 5 to 7 days culture, while some of the characteristic morphology was still present, the cell walls were thicker. This could be a disadvantage for *Agrobacterium* transformation (Fig. A.1 e, d, f).

Further microscopic observations are necessary for confirmation of cell competency. The preliminary results suggested that 1 to 3 days of explant culturing on regeneration media may be advantageous for transformation, but only experiments with cells infected with *Agrobacterium* could prove this hypothesis.

Figure A.1 TEM images of cells from 'Delicious' leaf discs cultured on regeneration medium MBNZ511 for 0 (A), 1(B), 3 (C), 5 (D), 6 (E), and 7 (F) days. Each image is representative of 25-30 cells.


A
B
C
F
2μm
500 nm
2μm
1μm
500 nm

Appendix B

AGROBACTERIUM-MEDIATED TRANSFORMATION OF THE *LEAFY* GENE FROM *ARABIDOPSIS* INTO 'GREENSLEEVES' APPLE CULTIVAR

Introduction

LEAFY (LFY) is one of several floral-meristem identity genes in *Arabidopsis* that are required to determine the fate of newly organized meristems (Mandel *et al.*, 1992). It has been demonstrated that *LEAFY*, constitutively expressed in transgenic *Arabidopsis* plants, encodes a developmental switch the activity of which is sufficient to convert all lateral shoots into precociously induced solitary flowers (Weigel & Nilsson, 1995). In the same study, *LEAFY* was also constitutively expressed in hybrid aspen that naturally flowers only after 8-20 years. The regenerated transgenic aspen shoots also produced solitary flowers in the axis of normal leaves. This floral development was specific for these transgenic lines and has not been observed in more than 1,500 other transgenic lines previously generated after transformation with different constructs. Thus the flowering time has been reduced from years to months. However the flowers formed had abnormal morphology and were sterile (personal communications with Dr. Meeks-Wagner)

A long juvenile period without a reproductive phase is one of the characteristics of apple that inhibits breeding efforts. One hypothesis is that introducing *LEAFY* into apple and expressing the gene in shoot meristems where is not normally active will induce early flowering and fruit production.

Transformation Experiments

Two hundred leaf explants from *in vitro* propagated and rooted plants from cultivar 'Greensleeves' were infected with *Agrobacterium* EHA101 harboring plasmid pDU96.0409 (see Chapter 5 for the procedure). Plasmid pDU96.0409 is a derivative of pDU93.3103 (Tao et al., 1995) and contains *LEAFY* gene under control of 35S prompter and terminator signals, 35S5'-GUS-35S3' reporter gene, and 35S5'-*nptII*-tml3' kanamycin resistance gene. The explants were co-cultivated on MBNZ511 medium for three days and transferred to MBNZ511 selection medium with 75 mg/l kanamycin and 400 mg/l cefotaxime. The explants were subcultured on fresh selection medium every 20 days. Sixty days after co-cultivation two GUS positive shoots were isolated from the leaf explants, cultured on BN505, and transferred to light with a 16/8 hour light/dark photoperiod under cool white fluorescent light (110 $\mu mol/m^2/sec$). After the shoots elongated to 0.5 cm and developed chlorophyll green leaves, they were propagated on A17 maintenance medium. Five plants from each transgenic line were rooted, acclimatized and transferred to greenhouse condition. No flowering was observed during the tissue culture or greenhouse development of the transgenic plants. Further DNA analyses are necessary to verify the insertion of the transgenes into the genome. One possibility is that *LEAFY* is not sufficient to induce flower development in apple or tissue specific promoters are necessary to direct the expression.

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Abstracts:

Maximova, S. and Kolova, L. Anther culture of apple (*Malus domestica* Borhk.), Abstracts VIIIth International Congress of Plant Tissue and Cell Culture, Firenze, Italy, 1994