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Evaluation of the dao1 gene as a selectable marker in transformation of apple rootstock M26 as well as transformation of a vector containing GA20 oxidase gene into *Agrobacterium*

Utvärdering av *dao1* genen som en selektionsmarkör vid transformering av äpplegrundstammen M26 samt transformering av en vektor innehållande GA20 oxidasgen till *Agrobacterium*

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Sammanfatting

Syftet med detta projekt var att undersöka möjligheten att vid transformering av äpplegrundstammen M26 genomföra selektion med hjälp av D-aminosyra (D-alanin) istället för andra vanligt förekommande selektionsämnen, så som antibiotika och herbicider.

Transformeringar med D-alanin som selektionsämne genomfördes, med *Agrobacterium tumefaciens* stammen C58C1(pGV3101) innehållande vektorn pPCV70:dao1. Vektorn innehöll även genen *nptII*, som ger de transformerade explantaten resistans mot antibiotika, kanamycin. *dao1* genen kodar för enzymet D-amino-acid oxidase (DAAO). Enzymet bryter ner det giftiga ämnet D-alanin till ofarliga ämnen för växten. De transformerade explantaten odlades på medium innehållande ökande koncentration av D-alanin, med utgångskoncentrationen 1.2 mM. Åtta putativa transformerade kloner selekterades på selektionsmediet. Ytterligare molekylära analyser måste dock genomföras för att bekräfta resultaten.

Försök genomfördes även för att transformera en vektor innehållande *GA20 oxidas* genen i *A. tumefaciens,* för att få fram en vektor färdig för användning vid transformering av växter. Vektorn innehållande *GA20 oxidas* genen och *nptII* genen transformerades in i *A. tumefaciens* stammen C58C1(pGV3850). DNA från *A. tumefaciens* var sedan isolerad och fragmenterad. Resultatet visualiserades på en agarose gel under UV ljus. Slutsatsen av försöket är att vektorn transformerats in i *A. tumefaciens* stammen C58C1.

Abstract

The aim of this project was to evaluate the possibility to use D-alanine as a selective marker instead of commonly used antibiotics and herbicides in transformation of apple rootstock M26.

The transformation was carried out using *Agrobacterium tumefaciens* strain C58C1(pGV3101) containing the vector pPCV70:dao1. The vector contained the *dao1* gene resistant to D-amino acids and the *nptII* gene resistant to antibiotic, kanamycin. The *dao1* encodes for the enzyme D-amino-acid oxidase (DAAO). The enzyme detoxifies D-amino acids, such as, D-alanine, which are harmful to plants.
The transformed explants were grown on medium containing increased concentrations of D-alanine, with a starting concentration of 1.2 mM. Eight putative transgenic clones were obtained from the selection medium. However, further molecular analyses have to be carried out to confirm the result.

A second experiment was performed to transform the *GA20 oxidase* gene into *A. tumefaciens* to get a ready-to-use transformation vector. The vector containing the *GA20 oxidase* gene was transformed into the *A. tumefaciens* strain C58C1(pGV3850). DNA from the strain was isolated and digested. The result was visualized on an agarose gel under UV light. The conclusion of the experiment is that the vector containing *GA20 oxidase* gene has been transformed into the strain C58C1.

1. Introduction

1.1 Background

Since long time ago people have tried to improve plants with help of breeding and selection to develop useful varieties with enhanced agronomical traits. The limitation of traditional breeding has been the restriction to the existing gene pool within each species or sexually compatible groups of species. To break this limitation it has been necessary to develop technologies for gene transfer into plants. In 1981, the introduction of foreign DNA into a plant was first demonstrated using genetic engineering. Since then, foreign genes have been introduced into more than 100 different plant species either though *Agrobacterium*-mediated transformation or by methods of direct DNA transfer (Primrose and Twyman, 2006).

The selection of transgenic cells or tissues is a critical step in plant transformation, since it is the most important factor affecting the transformation efficiency (Zhang et al, 2005). Antibiotic or herbicide resistant genes are often used for selection of transgenic plants. However, the presence of these marker genes may be undesirable in plants for commercial production due to the concerns of their potential negative effects on the environment and human health. One major concern from the public is the possibility of a herbicide resistant transgene moving from a cultivated plant to its wild relatives, and result in the creation of a 'super weed', which will be hard to control with the herbicides allowed at the market today. Another concern is the risk of antibiotic resistant genes moving into bacteria in the environment and the bacterial population in digestion system of animals and humans and reduces the effect of antibiotics used in medical purposes today (Gadaleta et al, 2006).

Dao 1 (D-amino acid oxidase) gene is a non-antibiotic and non-herbicide resistant gene and would therefore be a better alternative as selectable marker gene. The use of *dao1* gene in plant transformation would provide a safer selection compared with antibiotic and herbicide resistant marker genes. Another advantage of using the dao1 gene is to increase the transformation efficiency for those species sensitive to the antibiotic kanamycin.

1.1.1 Tissue culture

DNA can be introduced into most types of explants, such as, gametes, seeds, zygotes, embryos, organs and whole plants. The most critical part in plant transformation is not the DNA transfer itself, but the ability to recover transgenic plants after the transformation itself (Primrose and Twyman, 2006).

Plant tissue culture is required for almost all gene transformation methods. It is a process where explants are excised from a plant and grown on a nutrient medium. Since nutrient media also support the growth of microorganisms, it is important to wash and sterilize the explants in a disinfectant such as sodium hypochlorite or hydrogen peroxide. A nutrient medium has to contain the correct balance of plant hormones to obtain organogenesis. Most important is the auxin:cytokinin ratio. A low auxin:cytokinin ratio leads to shoot formation whereas a high ratio promotes root formation. Most frequently used media

consist of inorganic salts and metals, essential vitamins, an organic nitrogen source and sucrose as a carbon source. Culture media often include a gelling agent, such as agar, to solidify the media and provide support for the cultures (Primrose and Twyman, 2006).

1.1.2 Gene transfer strategies for plants

There are two strategies for gene transfer into plant cells, direct and indirect gene transfer. *Agrobacterium*-mediated transformation is an indirect gene transfer method. It is the most commonly used method for the reason that it works effectively with a wide range of plant species furthermore, it is a relatively inexpensive method. Direct gene transfer includes physical and chemical methods, of which the physical method, particle bombardment, is most commonly used method, especially for monocotyledonous plants. Chemical transformation methods are mainly used for protoplast transformation (Primrose and Twyman, 2006).

1.1.2.1 Indirect gene transfer through *Agrobacterium*

Gene transfer naturally occurs from soil bacteria to plants and causes crown gall disease. It has been proved that crown gall tissues represent true oncogenic transformation, since the undifferentiated callus can be cultivated *in vitro* and yet retain its tumorous properties. This can e observed even after the bacteria have been killed with antibiotics. The crown gall formation is caused by integration of a bacterial plasmid DNA into the plant genome. Such plasmids are accordingly called tumor-inducing (Ti) plasmids. Ti-plasmids contain oncogenes which cause crown gall formation and opine genes which synthysize opines, the nitrogen and carbon sources for the bacteria to grow in the host plant. The Ti-plasmid specifies the types of opines that are synthesized in the transformed plant tissues. Nevertheless, the presence of a plasmid in *Agrobacterium tumefaciens* does not mean that the strain is virulent. A number of strains contain plasmids that do not confer virulence, and in a few natural isolates, these plasmids are present together with a Ti-plasmid (Primrose and Twyman, 2006).

Ti-plasmid inserts its small specific segment, about 23 kbp, into the plant genome at a random site. This specific DNA segment is called transferred DNA (T-DNA), which contains oncogenes and opine genes. The T-DNA is flanked by 25-bp border sequences. The border sequences are involved in the transfer process, but are not transferred into the plant genome. Genes that have the responsibility for T-DNA transfer are located in a specific part of the Ti-plasmid, the *vir* (virulence) region. The enzymes VirA and VirG are constantly expressed at a low level, which control the plant-induced activation of the other *vir* genes. VirA, which is a kinase and spans the inner membrane of the bacteria, acts as a receptor for specific phenolic molecules that are released when the plant cells are wounded. The phenolic compounds do not attract bacteria to the wounded cells of the plant. Instead, the bacteria respond to sugar and amino acids, and after the bacteria attack the plant cell the *vir* gene is induced. Activated VirA triggers the transphoshorylation of the VirG protein, a transcriptional activator of other *vir* genes. It is not only VirG that encodes the transcription factors that regulate *vir* gene expression. Other genes on the bacterial chromosome might be also involved, but it is unknown at this moment. The expression of *vir* genes results in the synthesis of proteins that form a pilus, through which the T-DNA is transferred into the plant cell. The components of the pilus are

encoded by genes in the virB operon. The DNA transfer is activated by a product produced by the *virD1* and *virD2* genes. The *virC12* and *virC2* start a process where either single-strand nicks or a double-strand breaks at the T-DNA borders. The VirD2 protein remains covalent attached to the processed T-DNA. The T-strands are then coated with VirE2, which is a single-stranded DNA binding protein. The whole complex is transferred through the pilus and into the plant cell. The VirD2 protein functions as a protection for the T-DNA against nucleases, to target the DNA into the plant cell nucleus, and to integrate it into the plant genome (Primrose and Twyman, 2006).

In order to be used for plant transformation, plasmids have to be disarmed by removing the oncogenes and opine genes in order to avoid tumor formation and opine production. Meanwhile, a selectable marker is often inserted into a transformation vector to identify transformed plant cells.

1.1.2.1.1 Binary vectors

The problems with disarmed wild-type Ti-plasmids are the large size, which makes them difficult to manipulate *in vitro*, and there is no unique restriction sites in the T-DNA. This problem was initially addressed by constructing co-intergrate vectors. The T-DNA isolated from a parent Ti plasmid is subcloned into a conventional *E. coli* plasmid vector for easy manipulation, producing an intermediate vector. However, such a vector is not capable to replicate in *A. tumefaciens.* The transfer has to be done with triparental mating, namely, three bacterial strains mixed together: i) an *E.coli* strain carrying a helper plasmid, which is able to mobilize the intermediate vector in *trans* ii) an *E. coli* strain carrying the recombinant intermediate vector, iii) *A. tumefaciens* carrying the Ti-plasmid, these results in a conjugation between the two *E. coli* strains. The helper plasmid is transferred to the carrier of the intermediate vector and mobilized and transferred into the recipient *Agrobacterium*. Although intermediate vectors have been widely used, co-integrates are not needed for transformation. Reason for that is that the *vir* genes of the Ti-plasmid function in *trans* and can act on any T-DNA sequence that is present in the cell. Due to this function, it is possible to supply *vir* genes and disarmed T-DNA containing the transgene on separate plasmids. This is the principle of binary vector system where the T-DNA resides on one plasmid and the virulence genes on another plasmid (Primrose and Twyman, 2006). The binary vector system has almost completely replaced cointegrate vectors in plant transformation.

1.1.2.2 Direct gene transfer

1.1.2.2.1 Microinjection

Microinjection is a technique that guarantees to generate successful hits on the target cell. Except the method can only be applied to few cells in any one experiment. Commonly foreign DNAs are introduced into the plant cells by injections with microneedles (Primrose and Twyman, 2006).

1.1.2.2.2 Particle Bombardment

Particle bombardment involves coating small metal particles with DNA and then accelerating them into target tissues using a powerful force, such as, a blast of high-pressure gas or an electric discharge through a water droplet (Primrose and Twyman, 2006).

1.1.2.2.3 Electroporation

This technique is based on the cells ability to take up exogenous DNA from suspending solution when it is subjected to electric shock. Different factors affecting the efficiency of electroporation, such as temperature, electric-field parameters, the DNA concentration and host-cell factors (Primrose and Twyman, 2006).

1.1.3 Selectable markers

Today are approximately 50 marker genes (Miki and McHugh, 2003) used in transformation protocols to identify transformed cells and plants from the non-transformed (Zhang et al, 2005). Despite the large number of existing marker genes only a low number are used for plant research and development. The reason is that many of the marker genes have some limitations and have not been tested sufficiently to merit their widespread use (Miki and McHugh, 2003). The most commonly used selectable marker gene is neomycin phosphotransferase II (*nptII*). This marker gene is resistant to aminoglycoside antibiotics. Another widely used selectable marker is herbicide resistant gene *bar* resistance (bialaphos) (Zhang et al, 2005).

The selectable marker gene is co-transformed into the plant together with the gene of interest. The function of the marker gene is to encode an enzyme that can detoxify selective agents, such as, an antibiotic, a herbicicde, a drug or metabolite analogue or a carbon supply (Miki and McHugh, 2003). It is preferable to place the selectable marker on the left-border of T-DNA, since it will be transferred to the plant at last. This placement reduces the risk that the selected plant only contains the marker gene and not the transgene of interest (Primrose and Twyman, 2006).

The public concern that antibiotic and herbicide resistance markers could be harmful to environment and human has forced researchers to find out alternative marker system (Primrose and Twyman, 2006). One alternative for this could be the use of a marker gene that confers either mannose (Zhang et al, 2005) or D-alanine resistance. Another solution is to eliminate the markers by sexual crossing, transposition or site-specific recombination (Primrose and Twyman, 2006).

1.1.3.1 Selection with antibiotics

Selectable marker genes for transgenic plants are usually derived from bacterial sources. These genes have chimeric structures, since the regulatory sequences have to function in plants. They can act as selectable markers either in the nuclear or plastid genomes (Cheung et al, 1988). In the nucleus, the insertions are random and subject to position effects (Miki and McHugh, 2003). Whereas in the plastids the genes are targeted to favorable sites within the plastid genome by homologous recombination (Svab and Maliga, 1993).

1.1.3.1.1 Aminoglycoside-modifying enzymes

There are three major classes of aminoglyside-modyfing enzymes in selection systems for plants, based on their detoxification mechanisms: 1) ATP dependent O-phosphorylation by phosphotransferases, 2) acetyl coA-dependent N-acetylation by acetyltransferases, 3) ATP-dependent O-adenylation by nucleotidyltransferases.

Aminoglycoside antibiotics are molecules, such as, kanamycin, neomycin, getamicin derivate G418 and paromomycin. These compounds are very toxic to plant, animal and fungal cells. The frequently used kanamycin is produced by soil actinomycete *Streptomyces kankmyceticus* as a trisaccharide composed of a deoxystreptamine and two glucosamines. Neomycin is a tetrasaccharide produced by the actinomycete, *Streptomyces fragdiae*. Both antibiotics inhibit protein synthesis in bacteria by binding to the ribosomal subunit (Miki and McHugh, 2003).

1.1.3.1.2 Aminoglyside-O-phosphotransferases

The *nptII* gene has been shown to be effective as a selectable marker in mammalian and yeast cells, therefore it was the first marker gene tested in plants. It is today the most widely used selectable marker in plant transformation. The enzyme NPTII catalyses the APT-dependent phosphorylation of the 3´-hydroxylgroup of the amino-hexose portion of aminoglycosides, such as, neomycin, kanamycin, geneticin (G418) and paramomycin. Strong promoters such as cauliflower mosaic virus 35S promoter raise the level of NPTII enzyme activity and the tolerance to kanamycin without making the expression of the *nptII* instable. International regulatory agencies have approved the commercial release of genetically modified canola, maize, potato, tomato, flax, chicory and cotton containing the *nptII* gene (Miki and McHugh, 2003).

Hygromycin B is the second commonly used antibiotic for selection in plant transformation. It is an aminocyclitol antibiotic inhibitor of protein synthesis. The antibiotic is very toxic to plants. The gene *aphIV* (*hph*, *hpt*) coding for mygromycin B phosphotrandferase (HPT) confers resistance to hygromycin B (Miki and McHugh, 2003).

1.1.3.1.3 Aminoglycoside-N-acetyltransferases

The aminoglycoside-N-acetyltransferases (AAC) are a class of aminoglycoside-modifying enzymes that act as plant selectable marker genes. The enzymes AAC(3)-III and AAC(3)-IV detoxify gentamicin, kanamycin, tobramycin, neomycin and paromomycin by acetylation. AAC(3)-IV also modifies the compounds aparmycin and G418 (Miki and McHugh, 2003).

1.1.3.1.4 Aminoglycoside-O-nucleotidyltransferases

The bacterial *aadA* gene codes for the enzyme aminoglycside -3´´-adenyltransferase and can be used as a plant selectable marker (Miki and McHugh, 2003). Under the control of the 35S promoter, this gene confers the resistance to spectinomycin and streptomycin in tobocco (Svab et al, 1990), white clover (Larkin et al, 1996) and maize (Lowe et al, 1995). The selection is for contrasts between green tissues and chlorotic tissues, instead for survival and growth. The gene has not been broadly adopted as a nuclear selectable

marker gene for plant transformation, but it is a widely used selectable marker for plastid transformation (Miki and McHugh, 2003).

1.1.3.2 Selection with herbicides

Herbicides act on a variety of specific target sites within plants. Genes used for selection with herbicides origin from both bacterial and plants. At least two mechanisms are involved in herbicide resistance. One uses the resistance found in natural isozymes or generated by enzyme mutagenesis. Another involves detoxification of the herbicide by metabolic processes. In selection with herbicides, the agents are toxic to non-transformed plant cells and transformed plant cells are provided with mechanisms that allow them to escape the toxicity (Miki B and McHugh S, 2003).

1.1.3.2.1 Phosphinothicin

The L-isomer of phosphinothricin, PPT is the active agent in many commercial herbicides, such as, Basta, Ignite and Liberty. PPT inhibits the glutamine synthetase. This is the only enzyme that is able to catalyze the assimilation of ammonia into glutamic acid in plants. The accumulation of ammonia results in plant cell death.

Bacterial acetyltransferases that confer resistance to bialaphos have been used in plant transformation to create resistance to herbicides containing PPT. The *bar* gene from *S. hygroscopicus* (Thompson et al, 1987) and the *pat* gene from *S. viridochromogenes* (Wohlleben et al, 1988) that encode the enzyme PAT are used to confer tolerance to L-isomers in transgenic plants. PAT uses acetyl CoA as a cofactor to catalyze the acetylation of the free amino group of L-PPT. This makes it impossible for the acetylated form of L-PPT to bind to an inactivate glutamine synthetase. The *bar* gene is especially useful in plants, such as, orchids that are tolerant to antibiotics by nature (Knapp et al, 2000). The *bar* gene driven by plant promoters has also been proved to be an effective selectable marker gene in *Brassica napus* and *Brassica oleraccea* (DeBlock et al, 1989) and an excellent selectable marker for maize (Fromm et al, 1990; Gordon-Kamm et al, 1990), wheat (Vasil et al, 1992), rice (Rathore et al, 1993), legumes (Larkin et al, 1996) and conifers (Brukhin et al, 2000).

1.1.3.2.2 Glyphosate

Glyphosate (N-[phosphonomertyl]glycine) is an active ingredient in the commercial herbicide Roundup. Glyphosate inhibits the plastid enzyme 5-enolpyruvylshikimate-3-phosphate synthase (ESPS synthease), which is essential in the shikimate pathway for the biosynthesis of aromatic amino acids. However, according to Shah et al (1986) the mutant form of EPSP synthase confers glyphosate resistance in petunia. Glyphosate resistance has been used in crops including cotton, wheat, soybean and sugar beet (Miki B and McHugh S, 2003).

1.1.3.2.3 Oxynil herbicides

Oxynil herbicides inhibit the photosystem II electron transport and are active in most plants except monocots (Miki B and McHugh S, 2003). The *bnx* gene from *Klebsiella pneumoniae* subspecies *ozanaenae* encodes the enzyme nitrilase which hydrolyzes bromoxynil into 3,5-dibromo-4-dihydrixybenzoic acid and ammonia. Experiments have

proved that the *bnx* gene results in resistance to bromoxynil in tobacco (Stalker et al, 1988) and *Brassica napus* (Freyssinet et al, 1996). Transgenic lines with the *bnx* gene have been commercialized in canola and cotton (Miki B and McHugh S, 2003).

1.1.3.2.4 Gabaculine

The glutamate-1-semialdehyde aminotransferase (GSA-AT) enzyme is sensitive to gabaculie, an inhibitor of a wide range of pyridoxal-5-phospate-linked aminotransferases (Miki B and McHuag S, 2003). Gough et al. (2001) showed that the *hemL* gene, encoding DSA-AT, under the double 35S promoter was highly expressed in tobacco mutant GSA-AT. The product from the *hemL* gene was targeted to the chloroplasts and then yielding green transformed tissue that could be distinguished from chlorotic untransformed tissues.

1.1.3.2.5 Cyanamide

Cydanamide can be used as fertilizer either as calciumsalt of its aqueous form or by acting as a non-persistent herbicide. It can be applied to the field before sowing, since it is converted to ammonia in the soil due to the ability of the cyanamide hydratase to catalyze the hydration of the nitrile group of the cyanmide to form urea (Miki B and McHugh S, 2003). Cyanamide hydratase has been used as a selectable marker in tobacco (Maier-Greiner et al, 1991), wheat (Weeks et al, 2000), potato, tomato rice and *Arabidopsis* (Miki B and McHugh S, 2003).

1.1.3.3 Selection using inhibitors in metabolic pathways

Selection systems which are based on inhibiting the metabolic pathways have not been evalutaded and used in the same range as antibiotic and herbicide selection systems. The reason is that this kind of inhibitors sometimes interferes with the metabolism and thereby changes the composition of the transgenic plants (Miki B and McHugh S, 2003).

1.1.3.3.1 2-Deoxyglucose

The compound 2-deoxyglucose (2-dog) is phosphorylated to 2-DOG-6-phosphate by the enzyme hexokinase, while 2-DOG-6-phosphate competes with glucose-6-phosphate in plants and causes cell death since it inhibits the glycolysis (Miki B and McHugh S, 2003). Kunze et al (2001) have used the $DOG^{R}1$ gene controlled by 35S promoter as a selectable marker gene. Nevertheless the transformation efficiency was lower then the *nptII* is used as selectable marker in tobacco.

1.1.3.3.2 Inhibitors of the aspartate pathway

The pathway of the aspartate family, which is regulated by a range of feedback loops, leads to the biosynthesis of lysine, threonine, methionine and isoleucine. For example aspartate kinase, one of the key enzymes is feedback-inhibited by lysine and threonine (LT). Because lysine inhibits the dihydrodipicolinate in the protein synthesis. The inhibition of the pathway causes methionine starvation which results in inhibition of growth. Experiments have shown that enzymes from *E. coli* are less sensitive to the feedback inhibition. Transformed potato plants with the *E. coli* enzyme controlled by the 35S promoter showed a low number of escapes on selection with LT for aspartate kinase and AEC for dihydrodipicoliate synthase. One of the problems with this selection system

is that overproduction of lysine or threonine, resulting from the modifications of the metabolism, which can cause abnormalities in the plants (Perl et al, 1993).

1.1.3.3.3 Methotrexate and trimethoprim

Drugs, such as, methotrexate (Mtx) and trimethoprim (Tmp) target to the active site of the enzyme dihydrofolate reductase (DHFR). This results in cell death due to impaired proteins, RNA and DNA biosynthesis (Miki B and McHugh S, 2003).
Test preformed by Irdani et al. (1998) in transgenic tobacco and petunia has showed that genes resistant to DHFR found in *E. coli*, the fungus *Candida albicans* and mutant mammalian cells can be used for selection of transgenic plants grown on Mtx.

1.1.3.3.4 4-methyltryptophan (4-mT)

The compound 4-methyltryptophan (4-mT) is toxic to plants that do not have the enzyme tryphophan decarboxylase (TDS) that converts L-tryptophan into tryptamine. The *Catharanthus roseus* gene coding for TDC and driven by 35S promoter showed a similar efficiency in tobacco when selected with 4-mT compared with kanamycin (Goddijn et al, 1993).

1.1.3.4 Selection using non-toxic metabolic intermediates

Conditional-positive selection system using non-toxic metabolic intermediates is relatively new. It is based on external non-toxic substrates, which can be converted by a bacterial gene into molecules that provide growth advantage to the transformed cells. The system appears to yield high transformation frequencies in some plant species (Miki and McHugh, 2003).

1.1.3.4.1 Xylose and mannose

D-xylose can not be used as a sole carbon source by plant cells in species, such as, tobacco, potato and tomato. However, through isomerization, xylose can be catalyzed by the xylose isomerase enzyme to d-xylulose, which can be used as a carbon source. The xylA gene, isolated from *Streptomyces rubiniosus* and *Thermoanaerobacterium thermosulfurogenes*, expresses xylose isomerase and has been used as a selectable marker in potato (Haldrup et al, 1998).

Mannose is, like xylose, non-toxic to plants (Privalle et al, 2000). The mannose selection system dose not cause any risk to the safety of animal, human or environment (He et al, 2004). The enzyme phosphomannose isomerase (PMI) commonly found in all kingdoms in nature, but less in plants, although it has been observed in soybean and other legumes (Gadaleta et al, 2006). The *pmi* gene is used as a selectable marker together with mannose as a selective agent. The mannose is converted to mannose-6-phosphate, which prevents cell growth, since the accumulated mannose-6-phosphate inhibits glycolysis. In the presence of the enzyme PMI, mannose-6-phosphate can be converted to fructose-6-phophate, which can be metabolized in the plant cells. Because of this, transformed cells expressing the *pmi* gene can survive on the medium where mannose is a sole carbon, while the non-transformed cells will stop grow and finally die due to starvation (Zhang et al, 2005). The selection system has shown 10-folder greater transformation frequencies compared with the frequencies obtained using the *nptII* gene and kanamycin in sugar beet

(Joersbo et al, 1998). Similar results have been observed in maize, wheat, barley, watermelon (Reed et al, 2001), rice (Lucca et al, 2001) and cassava (He et al, 2004).

1.1.3.4.2 Glucuronide derivate of benzyladenine

The β-glucuroidase gene (*gus*) can be used both as a marker gene and as a reporter gene. When used as a marker gene the selective agent is a glucuronide derivative of cytokinin benzyladenine. Upon hydralization of the enzyme, GUS benzyladenine is released, which stimulate shoot regeneration. Joersbo and Okkels (1996) have proved that this process is an effective selection system in tobacco.

1.1.3.5 Selection using gene expression for enhanced cytokinin levels

For shoot formation, high cytokinin:auxin ratios are required. Therefore, it is possible to use genes that promote this condition endogenously and enhance regeneration of shoots as a non-conditional-positive selection system. The *A. tumefaciens ipt* (isopentyl transferase) gene is located on the T-DNA and induces cytokinin synthesis (Primrose and Twyman, 2006). The enzyme catalyzes the synthesis of isopentyl-adenosine-5-monophospate. Endo et al (2001) have shown that the *ipt* gene regulated by the 35S promoter gave a transformation efficiency of 2.7-fold greater than that of the 35S-*nptII* gene in tobacco. However, there is a problem with this selection system. The high levels of endogenous cytokinin result in abnormal plant growth due to the loss of apical dominance and lack of roots. In addition, it is suggested that cytokinin is transferred from transformed cells to other cells, which can cause enhanced regeneration in non-transformed shoots (Kunkel et al, 1999). The MAT vector system has been developed to solve this problem. The *ipt* gene is first used as a selectable marker gene, and then be removed after the selection by using the maize transposable element Ac (Ebinuma et al, 1997).

The cytokinin-independent 1 (*CKI1*) gene targets a potential cytokinin receptor (Kakimoto, 1999). When *CKI1* is expressed in transgenic plants, regulated by the 35S promoter, cytokinin responses, such as, shoot formation and lack of roots, can be observed. If expressing the gene under control of the β-estradiol-induced promoter in *Arabidopsis*, the calli produced shoots on β-estradiol containing medium, but no shoot regeneration was observed on non-inductive medium without exogenous cytokinin. Unlike the *ipt* gene, no escape was generated with the *CKI1* gene, (Zuo et al, 2002).

1.1.3.6 Selection using D-amino acids

The enzyme D-amino-acid oxidase (DAAO) is a flavoprotein (Alonso et al, 1999), located in the peroxisome, and is recognized for its function of detoxification of amino acids in animals (Erikson et al, 2004). DAAO catalyzes the oxidative deamination of D-amino acids, which leads to the production of 2-oxoaxid and ammonia. DAAO is considered to be a marker enzyme for peroxisomes. The enzyme was first detected 60 years ago in pig kidney (Alonso et al, 1998).

Eriksson et al (2004) has reported that genes encoding DAAO can be used as selection markers. One of them is *dao1*, which encodes the D-amino oxidase. The selection is based on the differences in the toxicity of different D-amino acids in plants, since the

enzyme catalyses the oxidative deamination of a number of D-amino acids (Gadaleta et al, 2006). This marker gene is of eukaryotic origin and encodes one of the most well-known enzymes (Scheid, 2004), and both its crystal structure and the catalytic mechanism have been determined by high-resolution X-ray spectroscopy (Erikson et al, 2004). The growth of non-transgenic plants that are lacking DAAO activity is inhibited by D-amino acids, such as, D-alanine, but is not affected by others, such as, D-isolencine. When using D-alanine as a selective agent, transgenic plants containing the *daol* gene detoxifying D-alanine and will survive, and this can be used as a positive selection system. However, transgenic plants will die on the D-isolencine medium and consequently it is also a negative selection system (Scheid, 2004). Therefore, amino acids can be used as either negative or positive selective agents in conjunction with a marker gene.

1.2 Objective

The aim of this project was to transform the apple rootstock M26 with the *dao1* gene using D-alanine as the selection agent in order to optimize the selection pressure. Second objective was to transform the vector containing *GA20 oxidase* gene into *Agrobacterium tumefaciens* in order to get a ready-to-use transformation vector.

2. Material and Methods

2.1 Transformation of M26 with dao1 gene

2.1.1 Plant material

For the transformation, the youngest unfolded leaves from four weeks old *in vitro* grown shoots of the apple rootstock M26 were used as explants.

2.1.2 *Agrobacterium* strain and binary vector

The *Agrobacterium tumefaciens* strain C58C1 (PGV3101) harbouring the binary vector pPCV702:*dao1* was used for transformation. The binary vector contained the *dao1* gene controlled by the CaMV 35S promoter and the *nptII* gene driven by the *nos* promoter (Erikson et al, 2004)

2.1.3 Transformation

Frozen bacteria, stored in liquid LB medium (appendix 1) with 15% glycerol at -85°C, were activated on solid LB medium containing 50 mg/l kanamycin and 200 mg/l carbenicillin at 28°C overnight. The Perti dishes were placed upside down to avoid condensation on the bacteria. Before transformation, the bacteria were scarped from the Petri dishes and put into an Erlenmeyer flask containing 25 ml liquid LB medium containing the same concentration of antibiotics as mentioned above. The bacteria were then incubated at 28 °C with shaking (150-200 rpm) for 18h. The bacterial solution was centrifuged at 3500 rpm for 20 min and the pellet was dissolved in liquid MS20 medium (appendix 2) to a concentration of 0.5-1 at OD_{420}.

Leaf explants were wounded perpendicularly to the midrib with a scalpel and immersed into bacterial solution for 20 min. Leaves were co-cultivated with the bacteria on CIM

medium (appendix 3) without addition of antibiotics in dark for three days. The leaf explants were then rinsed in liquid MS20 with 500 mg/l cefotaxime, blotted dry on sterile filter paper and transferred to CIM medium with 500 mg/l cefotaxime and 1.2 mM D-alanine. The Petri dishes were kept in dark until new shoots appeared. After two weeks the explants were transferred to fresh CIM medium with an addition of 1.6 mM D-alanine. After callus formation the explants were moved to a SPM medium (appendix 4) with 200 mg/l cefotaxime and 2.5 mM D-alanine. After another two weeks, the medium was changed to SMM (appendix 5) with same concentration of antibiotic but 3.5 mM D-alanine. For the next exchange, the identical medium was used except for an increase of D-alanine to 4 mM. Because of heavy bacterial infection, the concentration of D-alanine was decreased to 2.5 mM two weeks after being cultured on the 4 mM medium and finally completely removed from the medium.

2.1.4 DNA isolation and PCR analysis

Extraction of DNA was preformed using a CTAB protocol. Leaves from transformed plants and control were collected in sterile microfuge tubes and homogenized for 10 sec with sterile pestles. After adding 500 µl prewarmed (65°C) 2% CTAB buffer and additional grinding, the tubes were incubated at 65 °C for 10 min. 500 µl of a mixture of chloroform and isoamylalcohol in proportion 24:1 was added in order to separate proteins from the DNA. Tubes were vortexed and spun (12000 rpm) for 3 min at room temperature. Top phase was transferred to a fresh microfuge tube and 400 µl of isopropanol was added and spun for 5 min. Supernatant was discarded, and 500 µl wash buffer was added. Tubes were spun for 3 min and the supernatant was discarded with a pipette. Pellets were dried in laminar flow cabinet and later resuspendet in 30 µl TE buffer and stored in the freezer.

The PCR program for the *dao1* gene consisted of an initial denutration at 95 °C (5 min), 30 cycles of denaturation at 95 °C (15 sec), annealing at 52 °C (15 sec), elongation at 72°C (39 sec), and a final elongation at 72 °C for 5 min. The PCR was carried out with a 10 µl reaction volume. The PCR products were run on a 1% agarrose gel and visualized with ethedium bromide under UV light.

2.2 Transformation of GA20 oxidase *to* Agrobacterium

2.2.1 Competent cell

Frozen *Agrobacterium* strain C58C1(pGV3850) was cultured on solid LB medium with 100 mg/l carbencillin and 100 mg/l rifampicin at 28°C overnight. A portion of bacteria were cultured in 5 ml LB medium at 28 °C at 200 rpm overnight. The overnight culture of 0.5 ml was transferred into 50 ml liquid LB with antibiotics with the same concentrations as above, and cultured at 28°C with powerful shaking, 220 rpm, for a couple of hours until OD_{600} reached 0.5-1.0. The culture was cooled down on ice for 10 min, and centrifuged at 4 °C at 4000 rpm for 10 min. Supernatant was discarded and cells were re-suspended in 2.5 ml ice-cold and sterile 20 mM $CaCl_2$. The solution was portioned in small vials containing 200 µl bacterial solution each and 42 µl 86% glycerol was added into each aliquot. The vials were frozen in liquid N_2 and stored in -80°C, until use.

2.2.2 Transformation

The competent cells were thawn on ice and transferred into a 13 ml sterile tube. 30-40 ng or 1µg plasmid DNA, containing the *GA20 oxidase* gene, were added to the tube and mixed well by flicking the tube, and incubated on ice for 40 min. The cells were heat shocked at 37°C for 5 min and then incubated on ice for 20 min. 0.5 ml liquid LB, without antibiotics, was added and then incubated at 30°C with shaking, 200 rpm, for 3h. The cultured solution was pipetted on to solid LB with 50 mg/l kanamycin and 100 mg/l rifampicin and gently pasted evenly. The Petri dish was air-dried for a short while and cultured in 28°C until single colonies appeared, approximately 2-3 days.

2.2.3 PCR analysis and DNA digestion

PCR was preformed without any good results showing the presence of the *GA20 oxidase* gene. In order to confirm the presence of this gene in the plasmid, DNA was digested with restriction enzyme *Sac*I. The following solutions were added to eppendorf tubes in the following order: 6 µl H_2O, 2 µl buffer A, 10 µl DNA and 2 µl *Sac*I. The enzyme *Sac*I will give the *GA20 oxidase* a band of 1186 bp. DNA was digested in waterbath at 37°C for 2 h. Digested samples were run on a 1 % agarose gel and visualized under UV light after being stained with ethedium bromide.

3. Results

3.1 Transformation of M26 with the dao1 gene

Since many of the transformed explants or regenerated shoots died or were dying, probably mainly due to stress and bacterial infection, only the most vigorous and well-developed ones were chosen for PCR analysis. A total of 8 putative shoots were analyzed with PCR. The PCR results were not good, probably due to the low concentration of DNA or non-optimised PCR conditions (Data not shown). Unfortunately, there was no time to perform more PCR analyses on both the *dao1* gene and *nptII* gene within the project period. Obviously, further molecular analyses on the putative transgenic clones are needed to verify the results.

3.2 Transformation of GA20 oxidase *to* Agrobacterium

DNA was isolated from different colonies. PCR was preformed, but no positive results could be observed, probably due to non-optimized PCR conditions, lacking of suitable primers and the annealing temperature.

To prove the presence of the *GA20 oxidase* gene in the the *Agrobacterium*, DNA was digested with restriction enzyme *Sac*I. The result provides evidence that the vector containing the gene (1186 kb) has been transferred into the *Agrobacterium* strain (Fig. 1).

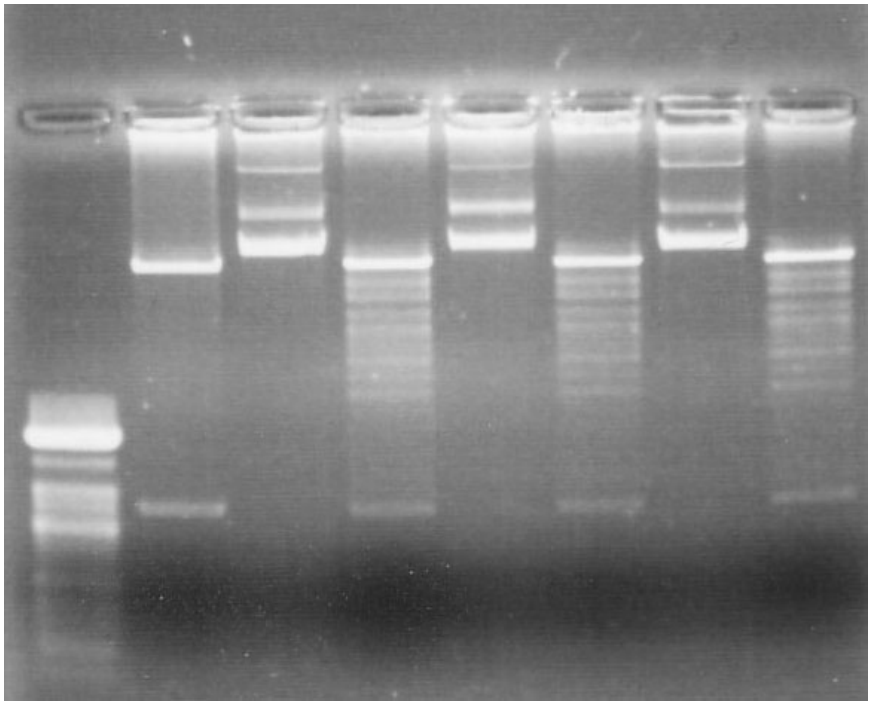

Figure 1. The fragment of the *GA20 oxidase* gene (1186 bp), digested from the plasmid DNA isolated from the strain C58C1. DNA molecular weight markers (lane 1), positive control (lane 2), digested DNA from 3 colonies (lane 4, 6,8), unfragmentized DNA of the same colonies (lane 3, 5, 7)

4. Discussion

There are many factors affecting the transformation efficiency. For example, plant species, age of the plant material, *Agrobacterium* strain and selectable marker gene, etcetera. These factors often interact with each other.

Many plant species are sensitive to kanamycin (Zhu et al, 2004). Due to this, it is important to develop alternative selection systems in order to increase the transformation efficiency to a sufficient level. A large number of selectable marker genes are available today, but only a few have been adopted in the production of transgenic plants. To increase the number of adopted marker genes research has to be done on effectiveness and safety. The cost of the selection system will more or less decide if the system is applicable in commercial production of transgenic plants (Miki and McHugh, 2003).

Selection with D-amino acids is a new selection system that may someday replace the selection with antibiotics or herbicides. The natural levels of D-amino acids and other DAO substrates in plants have never been proved to cause a problem. Even plants with high DAAO expression are indistinguishable from transgenic-free plants under nonselective conditions. The selection efficiency is not a problem either, since the selection rates when using *dao1*gene and D-alanine selection are efficient those obtained with *nptII* gene and kanamycin selection in *Arabidopsis* (Scheid, 2004).

Another positive advantage of using DAAO system is that it is possible to combined with site-specific excision technique. It means that after the positive selection of the transgenic plants, an initiation of recombination will be followed where it switches to negative selection which verifies the removal of the now redundant marker (Scheid, 2004). The biggest disadvantages for the DAAO system is that little is known about the endogenous enzyme activity in plants and the distribution of the substrates among plants and plant eaters (Scheid, 2004).

The highest concentration used in this study of the D-alanine was 4 mM. The reason for decreasing the concentration later on during plant regeneration, was a heavy bacterial infection and the plants were severely stressed. To keep the plants alive, the concentration had to be decreased. Although, according to Erikson et al (2004), no negative effects were observed on the transformed *Arabidopsis* plants even at concentrations of 30 mM D-alanine.

The conclusion of this study is that the *dao1*can replace the *nptII* gene as a selectable marker gene in transformation of the apple rootstock M26. However, more studies have to be done to optimize selection pressure in order to increase transformation frequency. *dao1* gene may replace antibiotic or herbicide selection genes in the future.

6. References

Alonso J, Berredo JL, Armisén P, Diez B, Salto F, Guisán JM, Garcia JL and Cortéz E (1999) *Engineering the D-amino acid oxidase from Trigonopsis variabilis to facilitate its overproduction in Escherichia coli and its downstream processing by tailor-made metal chelate support.* Enzyme and Microbial Technology **25,** 88-95

Alonso J, Barredo JL, Diez B, Mellado E, Salto F, Gracia JL and Cortés E (1998) *D-amino-acid oxidase gene from Rhodotorula gracilis (Rhodosporidium toruloides) ATCC 26217.* Microbiology **144,** 1095-1101

Ahah DM, Horsch RB, Klee HJ, Kishore GM, Winter JA,, Tumer NE, Hironaka CM, Sanders PR, Gasser CS, Aykent S, Siegel NR, Rogers SG and Fraley RT (1986) *Engineering herbicide tolerance in transgenic plants.* Science **233,** 478-481

Brukhin V, Clapham D, Elfstrand M and von Arnold S (2000) *Basta tolerance as a selectable and screening marker for transgenic plants of Norway spruce.* Plant Cell **19,** 899-903

Cheung AY, Bogorad L, Van Montagu M and Schell J (1988) *Relocating a gene for herbicide tolerance: a chloroplast gene is converted into a nuclear gene.* Proceedings of the national academy od sciences of the United States of America **85,** 391-395

DeBlock M, De Brower D and Tenning P (1989) *Transformation of Brassica napus and Brassica oleracea using Agrobacterium tumefaciens and the expression of the bar and neo genes in the transgenic plants.* Plant Physiology **91,** 694-701

Ebinuma H, Sugita K, Matsunaga E and Ymakado M (1997) *Selection of marker-free transgenic plants using the isopentenyl transferase gene.* Proceeding of the national academy of sciences of the United states of America **94,** 2117-2121

Endo S, Kasahara T, Sugita K, Matsunaga E and Ebinuma H (2001) *The isopentyl transferase gene is effective as a selectable marker gene for plant transformation in tobacco (Nicotiana tabacum cv. Petite Havana SR1).* Plant Cell reports **20,** 60-66

Erikson O, Hertzberg M and Näsholm T (2004) *A conditional marker gene allowing both positive and negative selection in plants.* Nature biotechnology **22,** 455-458 (Published online 21 March)

Freyssinet G, Perlissier B, Freyssinet M and Delon R (1996) *Crops resistant to oxynils: from the laboratory to the market.* Field Crops research **45,** 125-133

Fromm ME, Morrish F, Armstrong C, Williams R, Thomas J and Klein TM (1990) *Inheritance and expression of chimeric gene in the progeny of transgenic maize plants.* Biotechnology **8,** 833-839

Gadaleta A, Giancaspo A, Blechl A and Blanco A (2006) *Phoshphomannose isomerase, pmi, as a selectable marker gene for durum wheat transformation.* Journal of Cereal Science **43,** 31-37

Goddijn OJM, van der Duyn Schouten PM, Schilperoort RA and Hoge JHC (1993) *A chimeric tryptophan decarboxylase gene as a novel selectable marker in plant cells.* Plant Molecular Biology **22,** 907-912

Gordon-Kamm WJ, Spencer TM, Mangano ML, Adams TR, Daines RJ, Start WG, O'Brien JV, Chambers SA, Adams Jr. WR, Willetts NG, Riche TB, Mackey CJ, Krueger RW, Kausch AP and Lemaux PG (1990) *Transformation of maize cells and regeneration of fertile transgenic plants.* Plant Cell **2,** 603-618

Gough KC, Hawes WS, Kilpatrick J and Whitelam GC (2001) *Cyanobacterial GR6 glutamate-1-semialdehyde aminotransferase: a novel enzyme-based selectable marker for plant transformation.* Plant Cell reports **20,** 296-300

Haldrup A, Petersen SG and Okkels FT (1998) *Positive selection: a plant selection principle based on xylose isomerase, an enzyme used in the food industry.* Plant Cell reports **18,** 76-81

He Z, Yeping F, Huamin S, Guocheng H, Shihong Z, Yonghong Y and Zongxiu S (2004) *Phosphomannose isomerase (pmi) gene as a selectable marker for rice transformation via Agrobacterium.* Plant Science **166,** 17-22

Irdani T, Bogani P, Mengoni A, Mastromei G and Buiatti M (1998) *Construction of a new vector conferring methotrexate resistance in Nicotiana tabacum plants.* Plant Molecular Biology **37,** 1079-1084

Joersbo M and Okkels FT (1996) *A novel principle for selection of transgenic plant cell: positive selection.* Plant Cell reports **16,** 219-221

Joersbo M, Donaldson I, Kreiberg J, Petersen SG and Brunstedt J (1998) *Ananlysis of mannose selection used for transformation of sugar beet.* Molecular Breeding **4,** 111-117

Kakaimoto T (1996) *CKI, a histine kinase homolog implicated in cytokinin sgnal transduction.* Science **274,** 982-985

Knapp JE, Kausch AP and Chandlee JM (2000) *Transformation of three genera of orchids using the bar gene as a selectable marker.* Plant Cell **19,** 893-989

Kunkel T, Chan YS and Chua NH (1999) *Inducible isopentenyl transferase as a high-efficiency marker for plant transformation.* Nature Biotechnology **17,** 916-919

Kunze I, Ebneth M, Heim U, Geiger M, Sonnewald U and Herbers K (2001) *2-Deoxyglucose resistance: a novel selection marker for plant transformation.* Molecular Breeding **7,** 221-227

Larkin PJ, Gibson JM, Mathesius U, Weinmann JJ, Gartner E, Hall E, Tanner GJ, Rolfe BG and Djordjevic MM (1996) *Transgentic white clover. Studies with the auxin.responsive promoter, GH3, in root gravitropism and lateral root development.* Trans. Res. **5,** 325-335

Lowe K, Bowen B, Hoester G, Ross M, Bond D, Pierce D and Gordan-Kamm B (1995) *Gerline transformation of maize following manipulation of chimeric shoot meristems.* Biotechnology **13,** 677-682
Lucca P, Ye X and Potrykus I (2001) *Effective selection and regeneration of transgenic rice plants with mannose as selective agent.* Molecular Breeding **7,** 43-49

Maier-Greiner UH, Obermaier-Skrobranek BMM, Estermaier LM, Kammerloher W, Freund C, Wulfing C, Burkert U, Matern D, Breuer M, Eulits M, Kufrevioglu OI and Hartmann GR (1991) *Isolation and properties of a nitrile hydratase from the soil fungus Myrthecium verrucaria that is higky specific for the fertilizer cyanamide and cloning of its gene.* Proceedings of the national academy of science of the United States of America **88,** 4260-4264

Miki B and McHugh S (2003) *Selectable marker genes in transgenic plants: applications, alternatives and biosafety.* Journal of Biotechnology **107,** 193-232

Perl A, Galili S, Shaul O, Ben-Tzvi I and Galili G (1993) *Bacterial dihydrodipicolinate synthase and desensitized aspartic kinase: two selectable markers for plant transformation.* Biotechnology **11,** 715-718

Primrose & Twyman, 2006. Principles of Gene Manipulation and Genomics. Seventh Edition. Blackwell Publishing.

Privalle LS, Wright M, Reed J, Hansen G, Dawson J, Dunder EM, Chand YF, Powell ML and Meghji M (2000) *Phosphomannose isomerase – a novel system for plant selection: mode of action and safety assessment.* In: Fairburn C, Scoles G, McHughen A (Eds.), Proceedings of the 6th International Symposium on The Biosafety of Genetically Modified Organisms. 171-178

Rathore KS, Chowdhury VK and Hodges TK (1993) *Use of bar as a selectable marker gene for the production of herbicide-resistant rice plants from protoplasts.* Plant Molecular Biology **21,** 871-884

Reed J, PrivalleL, Powell ML, Meghji M, Dawson J, Dunder E, Sittie J, Wenck A, Launis K, Kramer C, Chang YF, Hansen G and Wright M (2001) *Phosphomannose isomerase: an efficient selectable marker for plant transformation.* In Vitro Cell Dev. Biol.-Plant **37,** 127-132

Scheid OM (2004) *Either/or selection markers for plant transformation.* Nature Biotechnology **22,** 398-399

Stalker DM, McBirde KE and Malyj L (1988) *Herbicide resistance in transgenic plants expressing a bacterial detoxification gene.* Science **242,** 419-423

Svab Z, Harper EC, Jones JDG and Maliga P (1990) *Aminoglycoside-3''-adenyltransferase confers resistance to spectinomucin and streptomycin in Nicotiana tabacum.* Plant Molecular Biology **14,** 197-205

Svab Z and Maliga P (1993) *High-frequency plastid transformation in tobacco by selection for a chimeric aadA gene.* Proceedings of the national academy of science ot the United States of America **90,** 913-917

Vasil V, Castillo AM, Fromm ME and Vasil IK (1992) *Herbicide resistant fertile transgenic wheat plants obtained by microprojectile bombardment of regenerable embryogenic callus.* Biotechnology **10,** 667-674

Weeks JT, Koshiyama KY, Maier-Greiner U, Schäeffner T and Anderson OD (2000) *Wheat transformation using cyanamide as a new selective agent.* Crop Science **40,** 1749-1754

Zhang S, Zhu L-H, Li X-Y, Ahlman A and Welander M (2005) *Infection by Agrobacterium tumefaciens increased the resistance of leaf explants to selective agents in Carnation (Dianthus caryophyllus L. and D. chenensis).* Plant Science **168:1,** 137-144

Zhu LH, Li XY, Ahlman A, Xue ZT and Welander M (2004) *The use of Mannose as a Selection Agent in Transformation of the Apple Rootstock M26 via Agrobacterium tumefaciens.* Acta Hort **663,**503-506

Zuo J, Niu QW, Ikeda Y and Chua NH (2002) *Marker-free transformation: increasing transformation frequency by the use of regeneration-promoting genes.* Current opinion in biotechnology **13,** 173-180

Appendix

Appendix 1
LB medium for 1 liter:

Tryptone	10 g
Yeast extract	5 g
NaCl	10 g
pH	7.0
agar	15 g

Appendix 2
Liquid MS20 medium for 1 liter (volumes listed are from stock solutions):

MS Macro nutrients	100 ml
MS Micro nutrients	10 ml
MS Vitamins	10 ml
Sucrose	20 g
pH	5.2

Appendix 3
Callus induction medium (CIM) for 1 liter

MS Macro nutrients	100 ml
MS Micro nutrients	10 ml
MS Vitamins	10 ml
Sucrose	30 g
TDZ	10 μM
NAA	2.7 μM
pH	5.2
Gelrite	2.5 g

Appendix 4
Shoot production medium (SPM) for 1 liter

MS Macro nutrients	100 ml
MS Micro nutrients	10 ml
MS Vitamins	10 ml
Sucrose	30 g
BAP	22 μM
NAA	1.1 μM
pH	5.5
Gelrite	2.5 g

Appendix 5

Shoot multiplication medium (SMM) for 1 liter

MS Macro nutrients	100 ml
MS Micro nutrients	10 ml
MS Vitamins	10 ml
Sucrose	30 g
BAP	4.4 µM
IBA	0.5 µM
pH	5.5
agar	7 g

Appendix 6

Composition of macro-, micronutrients and vitamins in the MS stock solution

Macro-nutrients	g/l	Micro-nutrients	mg/l	Vitamins	mg/l
NH_4NO_3	16.5	Fe-Na-EDTA	4000	Myo-inositol	10 000
KNO_3	19.0	$ZnSO_4 \cdot 7H_2O$	860	Nicotinic acid	50
$CaCl_2 \cdot 2H_2O$	4.4	H_3BO_3	620	Pyridoxine Hydrochloride	50
$MgSO_4 \cdot 7H_2O$	3.7	$MnSO_4 \cdot 4H_2O$	2230	Thiamine Hydrochloride	10
KH_2PO_4	1.7	$CuSO_4 \cdot 5H_2O$	2.5	Glycine	200
		KJ	83		
		$Na_2MoO_4 \cdot 2H_2O$	25		
		$CoCl_2 \cdot 6H_2O$	2.5		