UNIVERSITY OF LJUBLJANA
BIOTECHNICAL FACULTY

Maja PETERNELJ

ARABIDOPSIS THALIANA TRANSFORMATION BY *AGROBACTERIUM TUMEFACIENS* - *ESCHERICHIA COLI* CO-INFECTION

M. SC. THESIS

Ljubljana, 2014

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M. SC. THESIS

TRANSFORMACIJA NAVADNEGA REPNJAKOVCA (*Arabidopsis thaliana* L.) S KOINFEKCIJO Z BAKTERIJAMA *Agrobacterium tumefaciens* IN *Escherichia coli*

MAGISTRSKO DELO

Ljubljana, 2014

Based on the Statute of the University of Ljubljana and by decision of the Senate of the Biotechnical Faculty and decision of the University Senate of 27.10.2010 it was confirmed that the candidate qualifies for a MSc Postgraduate Study of Biological and Biotechnical Sciences and the pursuit of a masters thesis in the field of biotechnology. Doc. Dr. Jernej Jakše was appointed as a supervisor and Prof. Dr. Katherine Pappas as a co-supervisor. The rasearch was conducted in University of Athens, Faculty of Biology at the Department of Biotechnology.

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Date of defence:

This M.Sc. thesisis is a result of an original research. The author allows it to be published on the web pages of the Digital Library of Biotechnical Faculty. Digital and printed version of this work are identical.

Maja Peternelj

Na podlagi statuta Univerze v Ljubljani ter po sklepu Senata biotehniške fakultete z dne 27.10.2010 je bilo potrjeno, da kandidatka izpolnjuje pogoje za magistrski podiplomski študij bioloških in biotehniških znanosti, področje biotehnologije. Za mentorja je bil imenovan doc. dr. Jernej Jakše, za somentorico pa prof. dr. Katherine Pappas.

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AB *Arabidopsis thaliana* is a widely used model plant and is frequently transformed by *Agrobacterium tumefaciens* using the floral dip technique, which involves vector and transgene construction steps in *Escherichia coli* and then the introduction of the desired constructs into *Agrobacterium tumefaciens* for further transfer to the plant. In this work *A. thaliana* plants were transformed by co-inoculation with *Escherichia coli* strain DH5α (pKP80)(pKM101) as a transgene donor and a disarmed *Agrobacterium tumefaciens* strain GV3101 as a transfer intermediate, using alterations of floral dip method. As a positive control of the experiments the *Agrobacterium tumefaciens* strain GV3101 (pKP80) was used. Different inoculation techniques, concentrations of bacterial cultures and preconjugated and non-preconjugated cultures were tested. The highest percentage of transgenic plants was 10.58, obtained in plants, inoculated by drops with a 10-fold concentrated non-preconjugated culture. The 10-fold concentrated drop-by-drop culture application (drops of bacterial inoculum directly onto the plant's buds) proved to be effective also among the positive controls which yielded 7.69 % of transgenic plants.

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AI *Arabidopsis thaliana* je pomembna modelna rastlina v rastlinski genetiki in pogosto jo transformiramo z uporabo tehnike potapljanja cvetov v kulturo bakterij *Agrobacterium tumefaciens*. Ta tehnika vključuje konstruiranje vektorja s transgenom v bakteriji *Escherichia coli* in nato vnos v *Agrobacterium tumefaciens*, ki je sposobna transformirati rastlino. V pričujočem delu smo rastline arabidopsisa transformirali z novo tehniko koinokulacije z bakterijo *Escherichia coli* DH5α (pKP80)(pKM101) kot donorjem transgena ter razoroženim sevom *Agrobacterium tumefaciens* GV3101, ki je deloval kot mediator transferja. Preizkusili smo več različnih metod inokulacije, različne koncentracije bakterijskih kultur ter predhodno konjugacijo bakterij. Najvišji odstotek transgenih rastlin smo dosegli pri kapljični inokulaciji inokulacijo (nanos kapljic bakterijskega inokuluma neposredno na popke rastlin) z nekonjugiranima 10x koncentriranima kulturama, in sicer 10,58 %. Deset odstotna koncentracija v kombinaciji s kapljično se je izkazala za učinkovito tudi pri rastlinah, inokuliranih z GV3101 (pKP80) kot pozitivno kontrolo, saj smo s to tehniko dobili 7,69 % transgenih rastlin.

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Annex B: Graphical representation of the percentage of vital seeds from control plants (A+) on the selective plates in regard to the part of the selection.

ABBREVATIONS AND SYMBOLS

Ap50 ampicillin 50µg/ml

***At*mt** *Agrobacterium tumefaciens*-mediated gene transfer

Cfu colony forming units

G418 antibiotic from *Micromonospora rhodorangea*

Gen40 gentamycin 40 µg/ml

IM infiltration medium

LB Luria-Bertani medium

MES 2-[*N*-morpholino] ethanesulfonic acid

MS Murashige-Skoog medium

OD_{600} optical density at 600 nm

Rif30 rifampicin 30 µg/ml

rpm rounds per minute (speed of centrifuge rotor)

Spc100 spectinomycin 100µg/ml

(A+) Infecting *Agrobacterium tumefaciens* strain GV3101 (pKP80) as a positive control

(A-) Disarmed *Agrobacterium tumefaciens* strain GV3101 as a negative control

(E-) E. coli strain DH5α (pKP80)(pKM101) as a conjugative donor or as a negative control

(AE) Disarmed *Agrobacterium tumefaciens* strain GV3101 and conjugative E. coli strain DH5α (pKP80)(pKM101) co-infection

(AEC) Disarmed *Agrobacterium tumefaciens* strain GV3101 and conjugative E. coli strain DH5α (pKP80)(pKM101) co-infection after conjugation

1 INTRODUCTION

Arabidopsis thaliana is a self-pollinating plant that belongs to the mustard or crucifer family (Brassicaceae) which comprises widely distributed genera and species of major economic importance. It is a widely used model plant in genetic research and is routinely transformed by using *Agrobacterium*-mediated transformation methods (Meyerowitz and Somerville, 1994).

Agrobacterium tumefaciens is a Gram-negative soil phytopatogenic bacterium which causes crown gall disease in dicot plants by transfer and integration of a segment, the so called T-DNA (transferred DNA) of its tumour inducing (Ti) plasmid into the host's genome. The tissue that receives the T-DNA is transformed, becomes malignant and forms tumours. Tumours, in turn, produce unusual molecules called opines, which are condensation products between keto-acids, amino acids, or sugars and serve as important carbon, nitrogen, and phosphorus sources for the infecting bacterium (Tzfira and Citovsky, 2008).

Escherichia coli is a Gram-negative bacterium which is widely used as a bacterial model organism because it has been extremely well studied for decades, and also as it is easily manipulated, grows rapidly, and is available in a wide collection of strains suitable for genetic engineering purposes. *E. coli* is one of many bacterial species that posses the ability to transfer DNA via bacterial conjugation, which allows genetic material to spread horizontally from a donor population of bacteria to a recipient one of the same or other species (Sussman, 1997).

Obtaining genetically transformed plants is made possible by the inoculation of plant tissues, cell cultures or protoplasts, or even whole plants, with *Agrobacterium tumefaciens* (*Agrobacterium tumefaciens*-mediated transformation – *At*mt). A second major route for creating transgenic plants involves direct gene transfer techniques (biolistic method, electrotransfer techniques, or techniques involving the use of DNA precipitants), which apply only to isolated tissues and not to the whole plants. *Arabidopsis thaliana* is routinely transformed by using *Agrobacterium*-mediated transformation methods, which involve vector and transgene construction steps in *Escherichia coli* and, at a second stage, the introduction of the desired constructs into *Agrobacterium tumefaciens* for further transfer to the plant. One of the problems associated with *Agrobacterium*-mediated transformation is the decreased stability of large DNA fragments in wild-type *A. tumefaciens* strains routinely used for transformation (Shibata and Liu, 2000).

The growth of plant cells in a cell culture can cause somaclonal variation (clonal proliferation of genotypically deviant cell lines and tissues), which is the consequence of growth in a non-natural environment. Another problem of cell cultures are fungal contaminations. Thus, many researchers have tried to avoid cell cultures: Feldmann and Marks (1987) successfully transformed the germinating seeds of *A. thaliana*. Later, Bechtold et al. (1993) introduced vacuum infiltration of *Arabidopsis* plants, which involves the application of vacuum in order to infiltrate the *Agrobacterium* transforming cells in the inflorescences of *Arabidopsis*. The method was simplified by Clough and Bent (1998) by

omitting the vacuum step and replacing it by dipping the whole flowering plants in an *Agrobacterium* cell suspension enriched in sucrose and a surfactant. This very simple technique is nowadays known as the 'floral dip method' and is now routinely used for the genetic transformation of *Arabidopsis*. As it was demonstrated by Labra et al. (2004), the floral dip technique reduces DNA changes which cause somaclonal variation to an undetectable level.

Nicotiana tabacum, an alternative model plant in transgenic technology, was successfully transformed in recent years with the application of a novel approach. This involves the use of a conjugative *E. coli* strain as the T-DNA and transgene carrying host and a disarmed (T-DNA-less) *A. tumefaciens* strain as an *in situ* conjugal mediator for the transfer of the transgene to the plant (Pappas and Winans, 2003). The novelty of this method lies in that it simplifies cloning and strain construction steps, since it circumvents the need to create a dedicated *Agrobacterium* strain for transgene transfer. Additionally, it is especially convenient for the transfer of large and unstable DNA fragments, which are better retained in the genetic background of the *E. coli* host. *N. tabacum* tissue cultures (leaf disks) were treated in this way and proved that a bacterium that is far from natural in the plant habitat (an enterobacterium like *E. coli*) can collaborate in the infection process without considerably compromising the plant tissue.

This same principle was recently tested for the transformation of *Arabidopsis thaliana* using the floral dip method (Koumpena et al., 2008). It proved, for the first time, that *E. coli* and *A. tumefaciens* can collaborate as infectious agents not only for the *in situ*, but also for the *in planta* transformation of a whole plant specimen. Transformation frequencies following this method were considerably low, which called for method improvement, pursued in the work herein.

1.1 SCOPE OF THE WORK

In the present work, the enhancement of the transformation efficiency and the simplification of the floral dip co-inoculation technique developed by Koumpena et al. (2008) were attempted. The most appropriate *Agrobacterium* strain for *Arabidopsis* transformation was used throughout the work, and pre-mating of this strain with an *E. coli* transgene donor was tested in order to increase transformant yields; various other modifications in plant inoculation frequency and mode were also applied. These efforts resulted in a several-fold increase of transformant yields and indicated ways for the further improvement and applicability of the *E. coli – A. tumefaciens* co-inoculation principle.

1.2 RESEARCH HYPOTHESIS

In our research we hypothesized that the a) application of different conditions in the bacterial growth stage and concentration upon inoculation, b) bacterial pre-mating prior to inoculation at various time intervals, c) an increased number of plant inoculation treatments to enhance plant-cell receptivity, and d) the use of different inoculation techniques *per se*, might increase transformant yields, as is usually the case with various bacteria-to-bacteria or bacteria-to-eukaryote gene transfer procedures. An increase in the

transformation ratios obtained by the biparental infection developed herein, to the levels obtained by standard *At*mt infections, would consequently render the method we propose suitable for general use in transgene technology.

2 REVIEW

2.1 *ARABIDOPSIS THALIANA*

Arabidopsis thaliana belongs to the superkingdom Eukaryota, kingdom Viridiplantae, phylum Streptophyta, unranked groups Streptophytina, Embryophyta, Tracheophyta, Euphyllophyta, Spermatophyta, Magnoliophyta, Eudicotyledons, core Eudicotyledons, subclass Rosids, unranked group malvids, order Brassicales, family Brassicaceae, tribe Camelineae, and genus *Arabidopsis* (UNIPROT – taxonomy *Arabidopsis thaliana*, 2013).

2.1.1 The family Brassicaceae

The genus *Arabidopsis* belongs to the mustard or crucifer family (Brassicaceae), a widely distributed family of approximately 340 genera and 3,350 species with the greatest abundance of species and genera in the temperate zone of the northern hemisphere. The members of the family are annual or perennial herbs and are characterized by their cross-shaped corolla and a capsular fruit known as the silique. Siliques usually consist of two valves separating from the central partition at maturity (Meyerowitz and Somerville, 1994).

The family is of major economic importance as a source of vegetable crops, oil crops, spices and ornamental plants. The most important genera of this family are *Brassica* (mustard, canola, cabbage, broccoli, cauliflower, turnip, and others), *Raphanus* (radish), and *Armoracia* (horseradish) (Meyerowitz and Somerville, 1994).

2.1.2 The genus *Arabidopsis*

Arabidopsis as a genus consists of nine species, all of which are indigenous to Europe, with the ranges of two species extending into the Northern and Eastern Asia and North America, specifically into the central United States (Al-Shehbaz and O' Kane, 2002). The only important species in this genus is *Arabidopsis thaliana.* It is widely used as a model organism and although it has no direct significance for agriculture, it is useful for the understanding of the genetic, cellular, and molecular biology of flowering plants in general, and of the economically important edible and oil-producing crops it relates to, in particular.

2.1.3 *Arabidopsis thaliana*

Arabidopsis thaliana plants are annual herbs that reach the height of 20–30 cm. The basal leaves are 1.5–5 cm long and 5–8 mm broad, oval-shaped and form a rosette. The leaves on the stem are smaller, subsessile or sessile, and are usually not very numerous. The stems are erect, they grow from the base, and are simple or branched. The flowers have a typical structure of the Brassicaceae family and have a diameter of approximately 3 mm. The siliques are 5–20 mm long, linear and smooth, while their seeds are ellipsoid, light to

reddish brown, and 0.3–0.5 mm long. The chromosome number of *Arabidopsis thaliana* is 2*n*=10 (Al-Shehbaz and O'Kane, 2002).

According to Gledhill (2002), the name of the genus *Arabidopsis* means *Arabis*-resembling because these plants resemble those from another closely related genus *Arabis*. The »*thaliana*« adjective originates from the surname of the German botanist Johann Thal from the 16th century.

A. thaliana has one of the smallest genomes among plants. It consists of 157 million base pairs and comprises of five chromosomes (Bennett et al., 2003). The smallness of the genome makes it very suitable for genome mapping or sequencing. Its life cycle is short; it takes about eight weeks from germination to a mature plant for laboratory varieties such as ‘Columbia 1’. The small size of the plant is convenient for cultivation in a limited space and a single plant can produce several hundred seeds. Due to all of these characteristics, *Arabidopsis thaliana* is very suitable as a model organism.

2.2 *ESCHERICHIA COLI*

Escherichia coli belongs to the superkingdom Bacteria, phylum Proteobacteria, class Gammaproteobacteria, order *Enterobacteriales*, family *Enterobacteriaceae*, and genus *Escherichia* (NCBI – taxonomy *Escherichia coli*, 2013).

Escherichia coli cells are short, straight Gram-negative bacilli that are non-sporulating, usually motile with peritrichous flagella, often fimbriate and occur singly or in pairs in rapidly growing liquid cultures. A capsule or microcapsule is often present and a few strains produce and profuse polysaccharide slime. *E. coli* is a facultative anaerobe, capable of a fermentative and respiratory metabolism. Its optimum growth temperature is 37° C and it grows readily on a wide range of simple culture media and on simple synthetic media (Sussman, 1997). It is widely used in biology as a model organism. As many other species of bacteria, *E. coli* possesses the ability to transfer DNA via bacterial conjugation, which may be due to chromosomal- or plasmid-borne properties, and allows genetic material to spread horizontally through the *E. coli* donor population to recipient populations of the same or other species of bacteria (Sussman, 1997).

2.3 *AGROBACTERIUM TUMEFACIENS*

2.3.1 Taxonomy

Agrobacterium tumefaciens belongs to the superkingdom Bacteria, phylum Proteobacteria, class Alphaproteobacteria, order *Rhizobiales*, family *Rhizobiaceae*, and group *Rhizobium*/*Agrobacterium* (NCBI – taxonomy browser *Agrobacterium tumefaciens*, 2013). The genus *Agrobacterium* is subdivided into three groups (biovars) based on various physiological characteristics (Keane et al., 1970; Kerr and Panagopoulos, 1977).

The taxonomy of the genus *Agrobacterium* is complicated and the classification has derived mostly from pathogenicity traits, i.e., the ability of bacteria to induce tumours in

plants. The genus *Agrobacterium* is closely related to *Rhizobium*, a genus of bacteria capable of nitrogen fixation and nodule formation at the roots of legume plants. For both genera, their distinctive generic characteristics are now thought to be merely the result of the presence or absence of interchangeable conjugative plasmids that confer specific oncogenic or nodule-forming capabilities (Young, 2008). Hooykaas et al. (1977) proved the transfer of the Ti plasmid from a mutant strain of *A. tumefaciens* to a strain of *Rhizobium*, which was then capable of causing tumours in plants. Conversely, Van Veen et al. (1989) proved that not all the members of *Rhizobiaceae* family become tumourigenic upon acquisition of a Ti plasmid. They introduced the Ti plasmid from *Agrobacterium tumefaciens* to *Sinorhizobium meliloti* but the latter failed to induce tumours (Van Veen et al., 1989).

Young et al. (2001) proposed to incorporate all members of the genus *Agrobacterium* into the genus *Rhizobium*, based on the resemblance of 16S rRNA sequences. Some authors opposed this idea: Farrand et al. (2003) argued that at least some biovars of *Agrobacteria* exhibit phenotypic characteristics and also differences in their genome structure and chromosomes that clearly set them apart from the other members of *Rhizobiaceae*. The biovar I agrobacteria seem to form a distinctive group, different from *Rhizobium* as well as from the other two *Agrobacterium* biovars (Holmes and Roberts, 1981; de Lajudie et al., 1994). The genome structure of biovar I agrobacteria and species *Agrobacterium rubi* consist of two chromosomes, one circular and one linear, whereas the other members of the *Rhizobiaceae* family carry one or two circular chromosomes (Jumas-Bilak et al., 1998). Furthermore, the chromosomes of *Rhizobium spp.* and *Sinorhizobium meliloti* contain characteristic genetic elements called rhizobium-specific intergenic mosaic elements - RIME not present in the prototypical biovar I *Agrobacterium tumefaciens* strain C58 (Østerås et al., 1995).

Despite the fact that there is no agreement about the taxonomy of *Agrobacterium* and *Rhizobium* species, the name *Agrobacterium* is widely used in on-going literature reporting on the biology or applications of this genus. The name *A. tumefaciens* is used for bacteria that cause crown gall disease, while *A. rhizogenes* for those causing hairy root disease. The name *A. radiobacter* is commonly used for non-pathogenic species (Young, 2008).

2.3.2 *Agrobacterium*-mediated transformation

Agrobacterium and related species are currently the only known organisms capable of interkingdom gene transfer. The ability of *Agrobacterium* to transport its own DNA to a foreign cell and integrate it into the host's genome is based on a complex secretion system and a diverse molecular integration system which enable *Agrobacterium* to transfer DNA to a broad group of organisms. It has been proven that *Agrobacterium* can transform cereals (Chan et al., 1992; Cheng et al., 1997; Tingay et al., 1997), members of the *Brassica* family (Curtis and Nam, 2001; Metz et al., 1995; Quing et al., 2000; Bartholmes et al., 2007), and other Angiosperms (Wroblewski et al., 2005; De Bondt et al., 1994.), different species of Gymnosperms (Humara et al., 1999; Leeve et al., 1997; Wenck et al., 1999), liverworts (Ishizaki et al., 2008), fungi (de Groot et al., 1998; Pardo et al., 2002; Kemppainen et al., 2005; Sugui et al., 2005), the Gram-positive bacterium *Streptomyces lividans* (Kelly and Kado, 2002) and even animal cells. Kunik et al. (2001) proved that *A.*

tumefaciens can lead to a stable genetic transformation of human HeLa cells and Bulgakov et al. (2006) used an *A. tumefaciens*-mediated transformation (*At*mt) approach to successfully transform sea urchin embryos.

2.3.3 The mechanism of *Agrobacterium*-mediated transformation

2.3.3.1 Molecular basis of plant cell transformation

The molecular basis of plant cell transformation by *Agrobacterium* is the transfer of T-DNA from the bacterium to the plant and its integration into the plant's nuclear genome (Chilton et al., 1977). The transferred DNA (T-DNA) is a part of the bacterium's Ti plasmid and is referred to as the T-region (Barker et al., 1983).

T-regions are flanked by highly homologous 25-bp long T-DNA border sequences (Jouanin et al., 1989; Yadav et al., 1982) which are the targets of the VirD1/VirD2 border-specific endonuclease which processes T-DNA from the Ti plasmid (De Vos and Zambryski, 1989; Filichkin and Gelvin, 1993). The right borders (RBs) are more important than the left ones (LBs) because they are the primary target for the VirD1/VirD2 endonuclease and also become covalently attached to VirD2 (Hepburn and White, 1985; Jen and Chilton, 1986; Wang et al., 1984). VirD2 nicks the double stranded T-DNA molecule at both the RB and LB sides and by doing so releases a single-stranded molecule termed T-strand, and attaches to its 5' end (Filichkin and Gelvin, 1993; Durrenberger et al., 1989; Herrera-Estella et al., 1988; Howard at al., 1989). In many cases, so-called 'overdrive' sequences appear near the right borders and enhance the transmission of T-strands to the cells (Van Haaren et al., 1989, 1987; Peralta et al., 1986).

Proteins encoded by *vir* genes play an essential role in the *Agrobacterium*-mediated transformation process (Zupan et al., 2000; Tzfira et al., 2000; Christie, 1997; Pitzschke and Hirt, 2010) (Figure 1). The two-component signal-receiving kinase, VirA, senses the presence of plant phenolic compounds released on wound sites, as well as monosaccharides and acidic pH (Turk et al., 1994; Stachel and Zambryski, 1986; Lee et al., 1995). VirA phosphorylates the response regulator VirG with the aid of the ChvE transporter or phenolics (Jin et al., 1990a; Jin et al., 1990b). Phospho-VirG binds itself to all *vir* operon promoters and activates the level of the transcription of the *vir* genes (Pazour and Das, 1990).

The type IV secretion system, which is necessary for the transfer of the T-DNA complex, consists of VirD4 and 11 VirB proteins (Christie, 1997; Vergunst et al., 2000). The VirB proteins form a membrane channel or serve as ATPases that provide energy for the export process (Figure 3), whereas VirD4 probably serves as a linker to promote the interaction of the processed T-DNA/VirD2 complex with the VirB-pore. VirB proteins are also important because they form a T-pilus (mainly comprised of VirB2), which probably provides a channel for the T-DNA complex transfer or docks the bacterium to the plant cell (Hamilton et al., 2000; Lai and Kado, 1998; Sagulenko et al., 2001; Eisenbrandt et al., 1999). VirE2 also plays an essential role in the transformation as it is a single-strand DNA binding protein that decorates the T-DNA strand, either in the bacterium or in the plant cytoplasm

(Anand et al., 2007; Gelvin, 2003). Both VirD2 and VirE2 protect the T-DNA strand from nucleolysis and guide it through the VirB-pore into the plant cytoplasm and then the nucleus. Nuclear targeting is aided by the lysine-rich nuclear localization signals that both VirD2 and VirE2 harbour, while plant proteins also play a role in the T-DNA integrity and transfer (karyopherins, cyclophilins) (Ballas and Citovsky, 1997; Lacroix et al., 2004; Deng et al., 1998; Tzfira and Citovsky, 2002). In the plant nucleus, the T-DNA complex is stripped from VirE2, and with VirD2 remaining attached to the very end, it engages in illegitimate recombination with the plant chromosome. Bacterial chromosomal genes are also essential for transformation. They include genes for polysaccharide production, modification, and excretion (attachment *att* genes), and may also play a minor role in the transformation process (Citovsky et al., 1994; Ziemienowicz et al., 2001; Zupan et al., 1996; Gelvin, 2003).

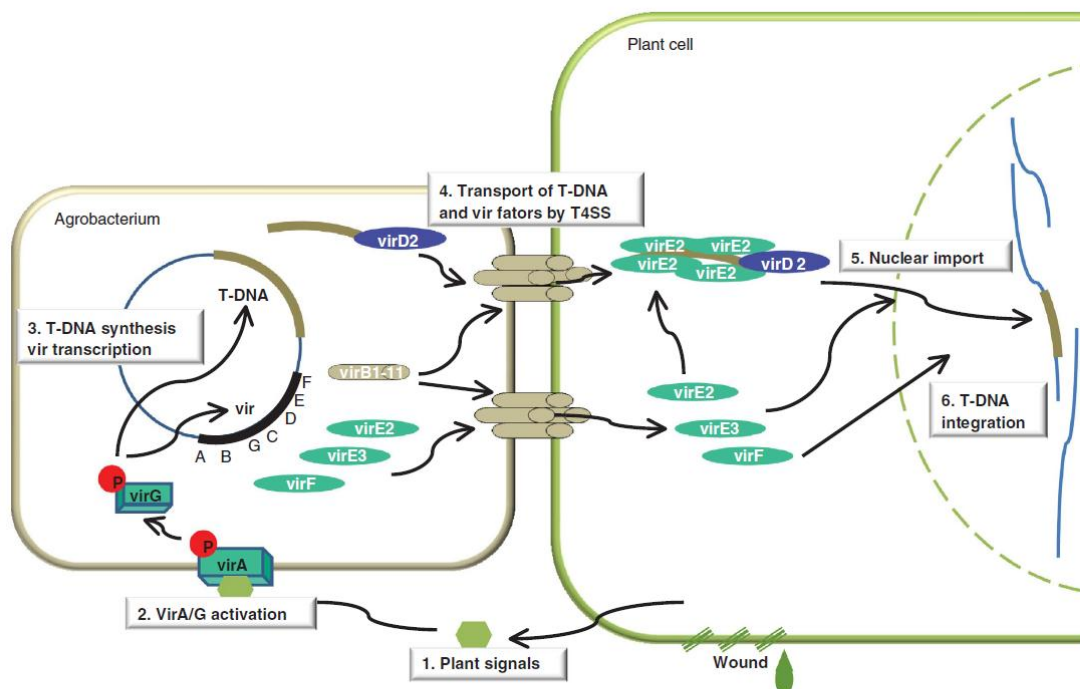

Figure 1: The schematic representation of interactions between the plant and the *Agrobacterium* cell. The wound of the plant cell induces signals (1) which activate the VirA/G proteins (2). The next step is T-DNA synthesis and *vir* gene expression in the *Agrobacterium* cell (3). Vir proteins and T-DNA are then transferred into the host cell trough a type IV secretion system (T4SS) (4). The T-DNA-vir protein complex then enters the cell nucleus (5) where it becomes integrated into the host's genome (6) (Pitzschke and Hirt 2010).

2.3.3.2 Bacterial secretion systems

Many bacterial species exploit specialized secretion systems to transfer macromolecules across membranes. These secretion systems are assembled into six major groups, named types I, II, III, IV, V, and VI (Thanassi and Hultgren, 2000; Henderson et al., 2004; Mougous et al., 2006). The secretion systems ancestrally related to the bacterial conjugation machinery are referred to as the type IV secretion systems (T4SSs) (Lawley et al., 2003; Christie et al., 2005) (Figure 2). The T4SSs are unique among other bacterial

secretion system types due to their ability to transfer both proteins and nucleoprotein complexes (Juhas et al., 2008).

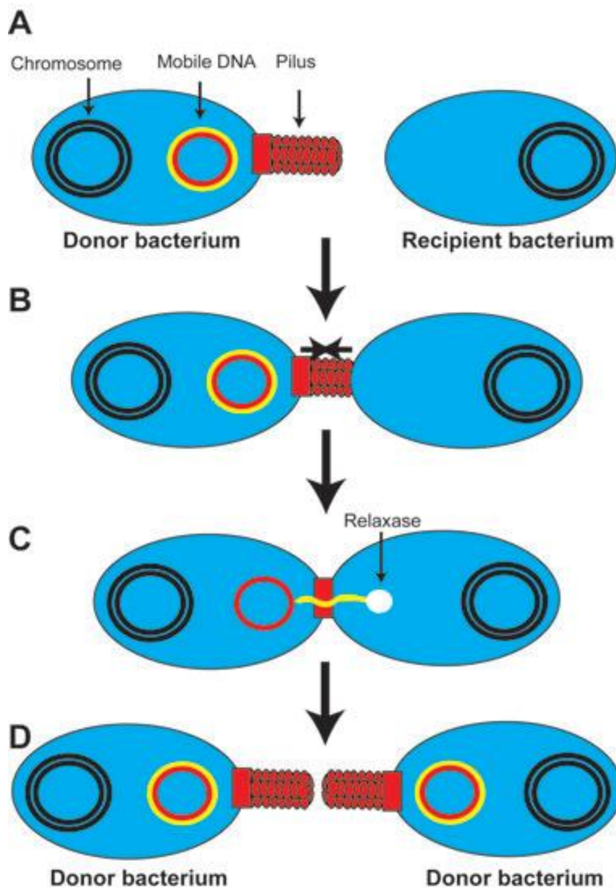

Figure 2: The schematic representation of the bacterial conjugation. The large subfamily of T4SS are conjugation systems which process the conjugative transfer of DNA from the donor to the recipient cell (A). The contact between the cells is usually mediated by a pilus-like structure (B). A single strand (ssDNA) of the mobile genetic element is transferred from the donor to the recipient bacteria with the help of the relaxase (C). The recipient and donor cells synthesise the complementary DNA strands and the former recipient bacterium becomes a potential donor of the mobile DNA (D) (Juhas et al., 2008).

2.3.3.3 Type IV secretion system (T4SS)

The mechanism of *Agrobacterium*-mediated insertion of DNA into the plant genome has been studied in detail and shows remarkable similarity to the bacterial conjugation process and the nucleoprotein transfer via the type IV secretion system (Lessl et al., 1992; Scheiffele et al., 1994; Schmidt-Eisenlohr et al., 1999; Christie, 1997; Zupan and Zambryski, 1995; Christie and Alvarez-Martinez, 2009; Juhas et al., 2008). Based on a number of features, T4SSs have been divided into several groups. Types F and P (type IVA) of T4SSs resembling the archetypal VirB/VirD4 system of *Agrobacterium tumefaciens* are considered to be the paradigm of type IV secretion (Juhas et al., 2008). Originally, the T4SSs were divided into three types, F, P, and I, named after the incompatibility group of the representative conjugative plasmids, IncF (plasmid F), IncP (plasmid RP4), and IncI (plasmid R64) respectively (Lawley et al., 2003). In the alternative

classification schemes, types F and P have been grouped together as type IVA systems, which resemble the archetypal VirB/VirD4 system of *Agrobacterium tumefaciens*. Type I, which varies significantly in its component modules from members of both F and P types, was named as the type IVB system. Genetic determinants of the type IVB systems are related to the archetypal Dot/Icm system of *Legionella pneumophila.* A third group in this classification scheme, composed of all the other known T4SS representatives that bear only limited or no homology to IVA and IVB system, has not been well characterized (Christie et al., 2005).

Type IV secretion systems are multisubunit molecular structures which span over the cell membranes. They have a secretion channel and often a pilus or other filamentous protein structure (Juhas et al., 2008). Conjugation systems, including the one encoded by the F plasmid, represent a large subfamily of the T4SSs and are used by bacteria in the process of the conjugative transfer of DNA from donor to recipient cells (Juhas et al., 2008). *A. tumefaciens* hosts two T4SSs (both encoded on the Ti plasmid): one determining inter-bacterial transfer of the Ti plasmid (*tra* system), and one determining bacterium-to-plant gene transfer (*vir* system). The mating-pair formation component of the *vir* T4SS is encoded by an approximately 10 kb *virB* operon comprising 11 open reading frames and a separate *virD4* gene, while other *vir* components acting on donor DNA processing or *vir* gene regulation lie also in close proximity on the Ti plasmid. Of the 11 VirB protein determinants, proteins VirB2 and VirB5 are pilus components, VirB3 and VirB7 are pilus-associated proteins, VirB4 and VirB11 are nucleoside triphosphatases that provide energy for transfer, while VirB6, VirB7, VirB8, VirB9 and VirB10 constitute components of the transmembrane channel (Figure 3) (Juhas et al., 2008).

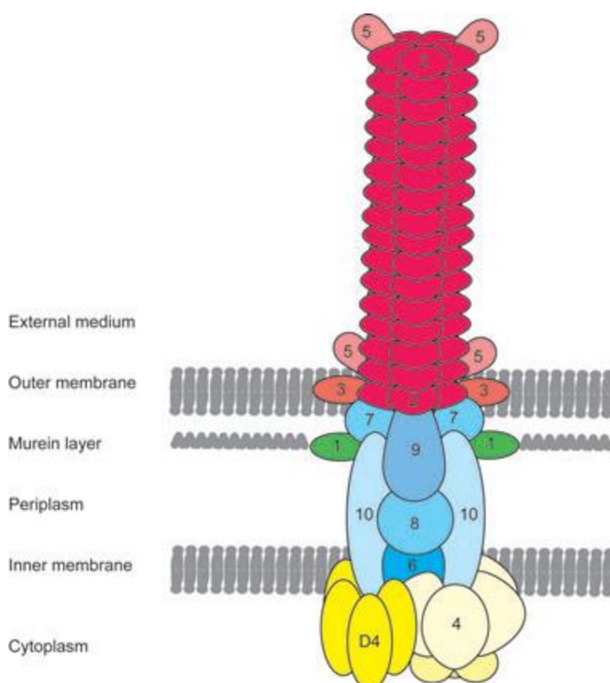

Figure 3: The schematic model of the *vir* T4SS in *A. tumefaciens*. The system is composed of several subunits which span over the cell membrane. The structure consists of 11 VirB proteins (VirB1–VirB11) and VirD4. The nucleoside triphosphatases that provide energy for the transfer are coded with yellow colour,

components of the transmembrane channel are shown in blue, pilus-forming components in red, and lytic transglycosylase responsible for the degradation of the murein (peptidoglycan) layer at the site of assembly in green (Juhas et al., 2008).

2.4 VECTORS FOR PLANT TRANSFORMATION

Agrobacterium-based plasmid vectors allow the transformation of a wide range of plant species by taking advantage of a natural bacterial system to introduce DNA into the nuclear genome of plants. As mentioned before, *Agrobacterium* inserts a part of its DNA, the T-DNA, into the nuclear genome of the host plant. The genes which are necessary for T-DNA transfer and the T-DNA itself lie on the tumour-inducing Ti plasmid. The T-DNA is bordered by 25 bp repeats (RB and LB) and its delivery into the host cell is mediated by the *vir* genes. The bacterial T-DNA contains the plant phytohormone synthesis genes that induce tumour formation, as well as opine synthesis genes, which, even though they are of bacterial origin, have evolved to function only in the eukaryotic nucleus. The removal of all the genes within the T-DNA does not impede the ability of *Agrobacterium* to transfer DNA, but it does prevent the formation of tumours. The Ti plasmids that are no longer oncogenic are termed ‘disarmed’. The two main components for a successful *Agrobacterium*-mediated gene transfer, T-DNA and the *vir* region, can reside on separate plasmids called binary Ti vector systems. The *vir* gene functions are provided by the disarmed resident Ti plasmid of the *Agrobacterium* strain. The T-DNA, where the genes to be transferred are located, is provided on a small shuttle vector that can replicate in both *E. coli* and *A. tumefaciens*. The shuttle vector must carry the selectable markers appropriate for selection in *E. coli* and *A. tumefaciens* (Hellens et al., 2000).

A recently proposed promising transient expression system in the plant cell relies on *Agrobacterium*-mediated gene transfer (*At*mt) technology and recruits viral replicons for gene expression (Marillonnet et al., 2004 and 2005). This technology combines the advantages of three biological systems – the transformation efficiency of *A. tumefaciens* used for viral replicon transfection, the high expression yields obtained with viral vectors and the post-translational capabilities of plants. The wider applicability of this method in stable transgenic technology remains to be proven.

2.5 TRANSFER OF GENES INTO PLANTS

There are two major ways to transfer genetic material into plant cells: via direct gene transfer and via *Agrobacterium*-mediated gene transfer (*At*mt). Recently, Broothaerts et al. (2005) reported success in plant transformation with the use of other members of the *Rhizobiales* and with the technology and vectors developed for *At*mt. However, despite offering proof-of-principle, this method is not yet routinely used.

2.5.1 Direct gene transfer

The most popular direct methods of gene transfer are electroporation (He et al., 2001; D'Halluin et al., 1992; Zhang HM et al., 1988; Laursen et al., 2004), the use of polyethylene glycol (PEG) as a DNA precipitant (Hayashimoto et al., 1990; O'Neill et al., 2005; Golds et al., 1993), the biolistic method (Wright et al., 2001; Li et al., 1992; Perl et

al., 1992; Hartman et al., 1994; Jain et al., 1996; Zhong et al., 1993) and ultrasonication (Zhang L-J. et al., 1991; Zhang H., et al. 1997). The use of ultrasound is sometimes combined with the *Agrobacterium*-mediated gene transfer in order to improve the transformation efficiency (Trick and Finer, 1997; Humara et al., 1999). Some other direct gene transfer methods were published, but have not become widely used are silicon carbide-mediated transformation (Kaeppler et al., 1991), electroporation of intact cells and tissues, electrophoresis, microinjection, the pollen-tube pathway method, and the use of liposomes (Rakoczy-Trojanowska, 2002). The downside of direct gene transfer techniques is that they can be used almost exclusively for transformation of plant cells or tissues. Additionally, the entry of unnaturally large numbers of DNA fragments into the cell nucleus (as is usually the case with direct gene transfer technology) often leads to host responses that involve gene silencing or inactivation (Vaucheret et al., 1998; Wassenegger et al., 2004; Stam et al., 1997a; Stam et al., 1997b). Lastly, not all plant species can be transformed by direct techniques.

2.5.2 *Agrobacterium*-mediated gene transfer methods

2.5.2.1 *Agrobacterium*-mediated transfer to cell lines, protoplasts, and tissue cultures

Agrobacterium-mediated transfer to cell lines, protoplasts, and tissue cultures is a standard technique for obtaining genetically modified plants. This technique is suitable for transformation of a wide variety of different plant species from garlic (*Allium sativum* L.) (Kondo et al., 2000), *Medicago varia* (Deak et al., 1986), citrus fruit (Vardi et al., 1990), plants of the *Poaceae* family like rice (Hiei et al., 1997; Kyozuka et al., 1987; Lin and Zhang, 2005), barley (Tingay et al., 1997), and tall fescue (*Festuca arundinacea* Schreb.; Gao et al., 2008), to lilies (Hoshi et al., 2003), poplar (Fillatti et al., 1987), and bananas (Ganapathi et al., 2001) and others.

Agrobacterium-mediated transfer has some advantages over the direct gene transfer. Travella et al. (2005) compared *Agrobacterium*-mediated and particle bombardment transfer in barley in terms of transformation efficiency, transgene copy number, expression, inheritance, and physical structure of the transgenic loci. The efficiency of *Agrobacterium*-mediated transformation was double compared with the particle bombardment and *Agrobacterium*-derived lines integrated a lower copy number of transgenes. The integrated T-DNA of *Agrobacterium*-mediated transformation was more stable and transgene silencing, which was frequently detected in the T_1 populations of the bombardment-derived lines, was not observed.

Later, Gao et al. (2008) performed a similar experiment with another member of the *Poaceae* family, the tall fescue (*Festuca arundinacea* Schreb.). They compared the transformation efficiency, the transgene integration, and the expression of the *bar* and GUS genes. The average transformation efficiency across the callus lines used in the experiments was almost the same for *Agrobacterium*-mediated transformation as for particle bombardment. However, while GUS activity was detected in leaves of 53 % of the *Agrobacterium*-transformed lines, only 20 % of the bombarded lines showed GUS activity.

2.5.2.2 *In planta Agrobacterium*-mediated gene transfer methods

In planta Agrobacterium-mediated gene transfer methods were developed to circumvent the need for isolation of plant cells and tissues and the need to prepare plant cell cultures. Apart from the fact that growing plants is much easier than growing cultures and the hands-on time needed is much shorter, *in planta* methods are much less susceptible to fungal contaminations and somaclonal variation. Except for seed-transformation processes that hold wider promise (see 2.5.2.2.1), whole-plant transformations are as yet mostly confined to *Arabidopsis* and its relatives, and have only recently been tested for other similarly easy-to-manipulate plants (i.e., cereals; 2.5.2.2.4).

2.5.2.2.1 Transformation of germinating seeds

The first report of *in planta* transformation was written by Feldmann and Marks in 1987. Germinating seeds of *A. thaliana* were co-cultivated with an *A. tumefaciens* strain carrying a Ti plasmid with a T-DNA gene encoding for kanamycin/G418 resistance.

The seeds were shaken in liquid BM medium and exposed to constant light. After this treatment, the overnight culture of *A. tumefaciens* was added and left for co-cultivation for 24 hours. The treated seeds were spread on vermiculite with a nutrition solution to help them grow. After T_1 plants reached maturity, T_2 seeds were collected and spread on selective plates containing kanamycin. Four out of six different treatments yielded observable transformants and the highest rate of transformants was 0.32 %.

The method was later used for the transformation of *Arabidopsis* (Feldmann, 2005; Castle et al., 2004) and other plant species such as soybean (Chee et al., 1989) and *Medicago truncatula* (Trieu et al., 2001).

2.5.2.2.2 *In planta* transformation of wounded plants

Katavic et al. (1994) reported another technique which does not require a tissue culture: the transformation of wounded *Arabidopsis* plants. *Arabidopsis* plants were grown in pots and when the primary inflorescence shoots reached the height of 1-2 cm, they were cut off together with some rosette leaves. The wounds were inoculated with overnight *Agrobacterium* culture. When secondary bolts appeared (7-10 days after the first cut), the procedure was repeated. Plants were then grown to maturity; their seeds were collected and spread on a selective medium to screen for transformants. The researchers inoculated 1440 T_0 plants, 95 of which yielded kanamycin-resistant T_1 seedlings.

2.5.2.2.3 Vacuum infiltration of plants

The next step in developing *in planta* transformation was the introduction of the vacuum infiltration of whole plants, developed by Bechtold, Ellis, and Pelletier (1993): the *Agrobacterium* culture was grown to the final OD_{600}=0.8 in liquid LB medium with appropriate antibiotics. The culture was then centrifuged and the pellet was re-suspended in the infiltration medium (MS macro and micro nutrients, 6-benzylaminopurine and 5 % sucrose) at one third of the initial culture volume. Three- to four-week-old plants were taken out of the soil, rinsed with water, immersed in the infiltration medium containing the

Agrobacterium culture carrying the bar resistance gene as a selective marker, and put in a vacuum chamber. These plants were in the vacuum for 20 minutes. After the vacuum treatment they were planted into new soil and covered with plastic wrap for two days. Mature seeds were harvested and the transformed seedlings were selected to be grown on sand in a greenhouse, irrigated with water containing the herbicide Basta. With this technique they managed to obtain up to 5 transgenic seeds per inoculated plant.

2.5.2.2.4 Floral dip method

Clough and Bent (1998) developed an even simpler method by replacing the vacuum step with a versatile dip of the whole plant into a bacterial solution containing surfactant. *Arabidopsis* plants were grown in pots in a greenhouse; primary inflorescences were clipped to encourage the emergence of secondary bolts. The *Agrobacterium tumefaciens* strain was grown to the stationary phase in liquid LB medium in a shaking incubator (250 rpm, 25 to 28° C). The cells were harvested by centrifugation (5,500 *g*, 20 min, at room temperature), and then re-suspended in an infiltration medium to a final OD_{600} of 0.8. Clough and Bent tested different concentrations of infiltration media components and then developed a protocol for the floral dip. The transformation frequency of around 1 % can be routinely obtained by this method. The primary target of *A. tumefaciens* in the floral-dip method is the female reproductive tissue of *A. thaliana* (Figure 4) (Desfeux et al., 2000). The T_1 transformants are typically hemizygotous, carrying T-DNA at one of the two alleles of the given locus (Bechtold et al., 1993).

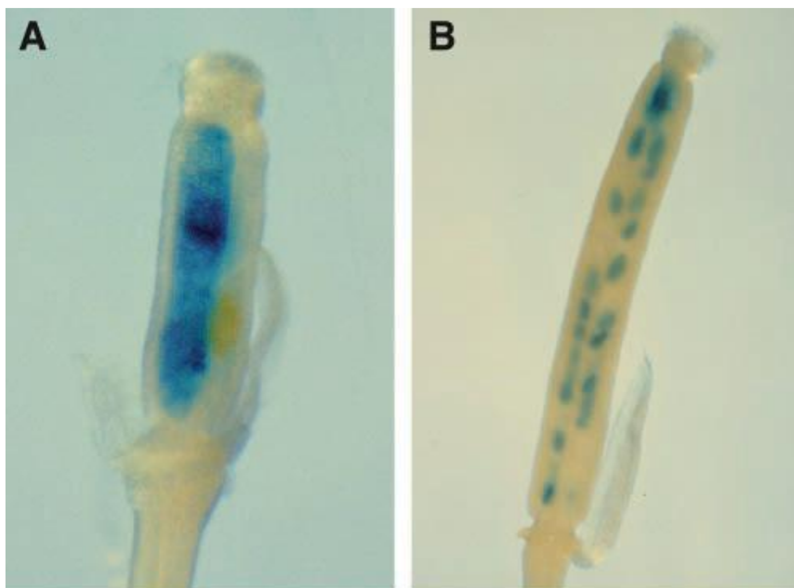

Figure 4: The GUS expression in ovules/developing seeds of *Arabidopsis thaliana*. The left photograph (A) shows a staining of an entire locule cavity, likely due to bacterial GUS expression from *Agrobacterium* colonizing the locule interior. The right photograph (B) shows an elongating seed pod from a fertilized flower (Desfeux et al., 2000).

The floral dip method was also successfully used for the transformation of shepherd's purse (*Capsella bursa-pastoris*) (Bartholmes et al., 2007), radish (*Raphanus sativus* L.

longipinnatus Bailey) (Curtis and Nam, 2001), and recently even for wheat (*Triticum aestivum*) (Zale et al., 2009).

2.5.2.2.5 Improvements and alterations of the floral dip method

Chung et al. (2000) developed an alteration of the floral dip method – the floral spray method, where bacterial culture is sprayed onto flowers. It was successfully used for generating transgenic *Arabidopsis* and could also be used for generating transgenic plants from plant species too big to be dipped (Chung et al., 2000). The transformation efficiency of the floral spray can reach up to 11 % (Ye, personal information, 20 October 2008). Martinez-Trujillo et al. (2004) reported a successful transformation and an improved transformation efficiency for *Arabidopsis*, reached by the drop-by-drop inoculation of dense *Agrobacterium* culture (OD_{600}<2.0). In 2006, Logemann et al. reported a simplified method for preparing *Agrobacterium* cells for the floral dip. Cells were grown on a solid YEB medium instead of the liquid medium. The storage of bacteria plates in a refrigerator for up to one week did not noticeably reduce the transformation efficiency, since the number of transformants obtained using such bacteria was nearly the same as in liquid-grown cultures. The method allows the storing of bacterial cultures for the transformation until plants reach an optimal growing stage. Davis et al. (2009) developed a direct dip protocol for *Arabidopsis*: *Agrobacterium* cells were grown in a specific medium (YEBS). When the culture reached the appropriate density, the surfactant Silwet® L-77 was added and the plants were dipped into this solution. This method reduces the need for preparing an infiltration medium and the need for the centrifugation of the cells. The direct dip method was also successfully used for simultaneous transformation with two transformation vectors (Davis et al., 2009).

2.5.2.2.6 Plant transformation by co-inoculation

Plant transformation by co-inoculation with a disarmed *A. tumefaciens* strain and an *E. coli* strain carrying mobilizable transgenes was first reported by Pappas and Winans in 2003. The molecular mechanism of T-DNA transfer in *Agrobacterium* exhibits a striking similarity to the conjugal transfer of broad-host range plasmids in-between bacterial species (Lessl et al., 1992; Scheiffele et al., 1994; Schmidt-Eisenlohr et al., 1999) and this fact encouraged the researchers to investigate the possibility of plant transformation with *E. coli* used as a transgene host.

Pappas and Winans (2003) first tried to transform tobacco leaf explants with *E. coli* strains harbouring *Inc*P-type and *Inc*N-type *tra* systems, but except for one rare instance of an *Inc*N- derived callus failing to differentiate, the majority of these efforts was without success. They then introduced another plasmid that expresses VirE proteins, which, together with all other T4SS components, are specifically required in the *Agrobacterium*-to-plant DNA transfer. In the case of the *Inc*P system, they detected some slow-growing calli, but again these calli proved to be abortive, since they failed to grow in subsequent transfers in the selective medium. They also inoculated two separate *E. coli* strains with *Inc*P *tra* genes, one expressing the VirE2 protein and the other the *npt*II (*kan*^{r}) transgene, and again they rarely detected the slow-growing, abortive calli. Because it seemed possible that the conjugation systems in *E. coli* are able to transfer T-DNA but not the VirE protein,

they co-inoculated leaf explants with an *E. coli* harbouring the *npt*II gene and a disarmed *A. tumefaciens* as a donor of VirE. The use of the *Inc*N system proved to be efficient and they detected an average of two calli per leaf strip, but the frequency of this transformation was 30 to 40 times lower than the control transfer of the *npt*II gene from *A. tumefaciens*. To determine whether the plant received the *npt*II gene directly from *E. coli* or via *Agrobacterium tumefaciens*, they used a mutated *virD*$^{-}$ *A. tumefaciens* strain, which lacks the ability to process T-DNA but retains all other *vir* functions. This experiment yielded no results, which indicates that *E. coli* itself is not able to successfully transform plant tissue. The shuttle vector that carries the kanamycin resistance transgene is conjugally transferred from *E. coli* to *A. tumefaciens* which acts as a recipient in the process, and then from *A. tumefaciens* to the plant cell via standard *vir*–mediated T-DNA transfer. This co-inoculation principle was in turn used for the transformation of *A. thaliana* by floral dip (Koumpena et al., 2008). Here, between *A. tumefaciens* strains EHA101 and GV3101, the latter proved to be optimal for *A. thaliana* transformation, and also proved an excellent recipient of the binary vector in matings with *E. coli* donors. The overall transformation of *A. thaliana* for the *npt*II marker by the bacterial co-infection technique described was relatively low (0.004 %). However, it improved markedly upon bacterial pre-mating prior to infection; this was tested only in the case of EHA101. The frequencies of *E. coli – A. tumefaciens* conjugal matings, directly affecting the subsequent transformation success, were amenable to enhancement via modifications in temperature, donor-recipient ratios, as well as the medium and the duration of incubation. This was the first report implicating *E. coli* as an *in planta* transformation vector in plant transgenic technology. Together with the previously mentioned leaf-explant transformation route, it is a method that it simplifies strain construction procedures and provides better maintenance of large and unstable DNA fragments in the course of their transfer to the plant.

2.5.3 Non-*Agrobacterium*-mediated gene transfer

The researchers of *Agrobacterium* face a serious problem of patent and intellectual property rights, which create an obstacle for the use of *Agrobacterium*-based techniques in plant biotechnology. In order to avoid the use of patented *Agrobacterium* strains, attempts were made to transform plants with other species of bacteria. Broothaerts et al. (2005) used *Sinorhizobium meliloti*, *Rhizobium sp*., and *Mesorhizobium loti* to transform tobacco leaf discs, rice, and *A. thaliana*. T-DNA based binary vectors, a disarmed Ti plasmid pEHA105 from a hypervirulent *Agrobacterium* strain, and a pCAMBIA1105 were introduced into the non-*Agrobacterium* strains. *S. meliloti* was proven to be competent to transfer genes into monocots and dicots, and into a range of tissues (leaf tissue, immature ovules, and undifferentiated calli). They obtained positive results also with *Rhizobium sp*. and *Mesorhizobium loti.* Although the transformation ratios were much lower than those obtained with *Agrobacterium* as a control, the use of alternative bacterial species is a promising approach in plant biotechnology.

2.6 SELECTION OF TRANSFORMANTS AND STERILIZATION TECHNIQUES

The crucial part of floral dip technique is sterilization and selection of the transgenic seeds. The mature seeds are collected when the inoculated plant is completely dry, that is 4–6 weeks after the dipping procedure. Dry seeds and plants are covered with agrobacteria

which remained from the floral dip and with normal bacterial and fungal flora of the plant, as well as air-borne bacteria and fungi from the environment. When placed onto selective plates these microorganisms may overgrow plantlets and destroy them.

Most of the authors use liquid sterilization of seeds. In general, seeds are first washed with ethanol, then with sodium hypochlorite (bleach), with the addition of the detergent Tween, and rinsed several times with sterile water. The concentrations of sterilization agents and the duration of exposure are very variable: Clough and Bent (1998) used 95 % (v/v) ethanol for 0.5–1 min, and then 50 % bleach in sterile water (v/v) + Tween 20 (0.05 %) (v/v) for 5 min. Zhang et al. (2006) used 70 % (v/v) ethanol for 1 min, 50 % (v/v) bleach + Tween 20 (0.05 %) (v/v) for 10 min. Martinez-Truillo et al. (2004) used 95 % (v/v) ethanol for 10 min, 6 % (v/v) chlorine solution + Tween 20 (0.1 %) (v/v) for 15 min. Desfeux et al. (2000) used isopropanol instead of ethanol for liquid sterilization. Another technique for sterilization of *Arabidopsis* seeds is vapour sterilization: seeds are sterilized in the vapour of a mixture consisting of 100 ml bleach and 3 ml of glacial HCl for 4–15 hours (Desfeux et al., 2000).

The selection of transformants is usually carried out on agar selective plates with appropriate selection agents in favour of transgenic seeds and against contaminating bacteria, and takes 7 to 10 days (Clough and Bent, 1998; Zhang et al., 2006; Desfeux at al., 2000). Harrison et al. (2006) developed a rapid method for identifying the transformed seedlings of *Arabidopsis*, shortening the selection time to less than 4 days. Sterilized seeds are placed on selective plates, left in the refrigerator at 4° C for 2 days for stratification, and then for 4–6 hours at 22° C in continuous light in order to stimulate germination. The plates are then wrapped in aluminium foil for two days and then transferred to continuous light (or 16 hours light / 8 hours dark regime) for 1 to 2 days.

Davis et al. (2009) developed a protocol for the selection of transgenic plants on quartz sand or on silicon dioxide sand instead of the solid growth medium. This protocol does not require the sterilization of seeds and lowers the possibility of fungal contamination.

3 MATERIALS AND METHODS

3.1 PLANT MATERIAL AND GROWING CONDITIONS

The seeds of *Arabidopsis thaliana* ecotype Columbia 1 were treated in water in a refrigerator (4° C) overnight for imbibition. The next day they were sown in small pots, in either sterilized or non-sterilized soil, and covered with a plastic dome to maintain humidity. The conditions in the growing chamber were as follows: temperature 21° C, relative humidity 60-70 %, 16 hours of light and 8 hours of darkness. After 5–7 days, when they reached the stage of two leaves (Figure 5), they were replanted into new pots, 4 plants per pot (Figures 6 and 7). The first generation (7 October 2008) was planted in commercial soil substrate Florrela green with 10 % (v/v) of added perlite. The 17 December 2008 and all later generations were planted in a substrate Potground P Klasmann. The substrate was not sterile and was without perlite. The 12 January 2009 and later generations were planted in Potground P Klasmann soil which was sterilized in an autoclave for 20 min. The sterilisation was employed to destroy insects and fungi present in the substrate which might be harmful to the plantlets.

3.2 INOCULATION EXPERIMENTS

The first inoculation of the plants took place when the bolts were 2–10 cm high, with few open flowers – that was approximately 30 days after planting (Figure 8 and Table 1). This is the most appropriate stage for the first floral dip. For higher efficiency, we repeated the dip after 5 or 6 days (Clough and Bent, 1998). The first generation (Experiment 1) and the sixth generation (Experiment 8) were reinoculated twice. The first reinoculation was 3 days after the initial inoculation and the second one four days after the first reinoculation in the first generation (Experiment 1) and in the sixth generation (Experiment 8) the first reinoculation was after five days and the second one after 10 days. The exact dates of the experiments are presented in the Table 1.

Figure 5: *A. thaliana* var. Columbia 1 plants in the stage of two leaves, just before replanting.
Slika 5: Rastline *A. thaliana* var. Columbia 1 v stadiju dveh listov, tik pred presajanjem.

Figure 6: *A. thaliana* var. Columbia 1 plants in the stage of two leaves, after replanting.
Slika 6: Rastline *A. thaliana* var. Columbia 1 v stadiju dveh listov, po presaditvi.

Figure 7: *A. thaliana* var. Columbia 1 plants in different growing stages. The plants at the front of the photo are 14 days old, those in the middle tray are one week older, and those at the rear are two weeks older.
Slika 7: Rastline arabidopsisa v različnih rastnih stadijih. Rastline v ospredju slike so stare približno 14 dni, rastline v sredini tri tedne, rastline v ozadju pa priblližno štiri tedne.

Figure 8: The plants in the most appropriate growth stage for the first inoculation (according to Clough and Bent, 1998) – primary bolts are 2–10 cm long, with a couple of open flowers.
Slika 8: Rastline v stadiju, ki je najbolj primeren za uspešno prvo inokulacijo (Clough in Bent, 1998): primarni poganjki so dolgi 2 do 10 cm, nekaj cvetov je že odprtih.

Table 1: The time schedule indicating plant cultivation and infection in experiments conducted in this work: planting dates, as well as dates, methods and intervals for the first or subsequent inoculations, dates of collection of siliques, and dates of transgenic seed assays.
Preglednica 1: Časovni potek poskusov: datum sejanja rastlin, datumi inokulacij, ponovnih inokulacij, intervali med inokulacijami, metode inokulacije, datumi pobiranja semena in datumi selekcije transgenih rastlin.

Experiment no.	Generation of T0 plants	Planted*	Date and mode of first inoculation	Period between inoculations	1st reinoculation	Period between inoculation	2nd reinoculation	Collection of siliques	Transgenic seeds assay
1	1st	7 October 2008	11 November 2008 (dip**)	3	14 November 2008 (drops)	4	18 November 2008 (dip)	14 January 2009	16 and 22 January 2009
2	2nd	17 December 2008	29 January 2009 (dip, conjugation)	5	3 February 2009 (dip, conjugation)			27 February 2009	17 and 27 March 2009
3	3rd	6 January 2009	5 February (dip, conjugation)	5	10 February (dip, conjugation)			24 April 2009	4 and 12 May 2009
4	3rd	6 January 2009	6 February (dip)	5	11 February 2009 (dip)			24 April 2009	22 June 2009
5	4th	12 January 2009	13 February (spray)	5	18 February 2009 (spray)			24 April 2009	22 June 2009
6	5th	26 January 2009	25 February 2009 (drops, different concentrations)	6	3 March 2009 (drops, different concentrations)			7 May 2009	22 June 2009
7	5th	26 January 2009	26 February 2009 (drops, different concentrations	5	3 March 2009 (drops, different concentrations			7 May 2009	22 June 2009
8	6th	9 February 2009	13 March 2009 (dip, conjugation)	5	18 March 2009 (dip, conjugation)	5	23 March 2009 (dip, conjugation)	13 May 2009	22 June 2009

*The 1st generation (7 October 2008) was planted in soil Florrela green with 10 % (v/v) of perlite. The 17 December 2008 and all later generations were planted in a new soil, Potground P Klasmann. The soil was not sterile and was without perlite. The 12 January 2009 and later generations were planted in Potground P Klasmann sterilized soil.

** dip, drop, or spray denotes the mode of infection; conjugation denotes bacterial pre-mating prior to infection.

3.3 GROWTH OF BACTERIAL STRAINS AND INOCULATION OF PLANTS

Bacterial strains were grown in liquid cultures as follows: *A. tumefaciens* was grown at 27° C in a shaking incubator (180 rpm) and *E. coli* at 37° C, left standing to reduce the speed of growth and to synchronize with the *A. tumefaciens* culture. Both bacteria were cultured in the LB medium (Bacto-tryptone 10 g/l, Bacto-yeast extract 5 g/l, NaCl 10 g/l, 1.5 % of agar for solid medium) with appropriate antibiotics (Table 2). Starting from an inoculum size of 10 ml, *A. tumefaciens* cultures reached the desired OD_{600} in approximately 24 hours. Four to six hours before *A. tumefaciens* reached the desired OD_{600}, the *E. coli* culture was placed into the shaking incubator to synchronize growth with the *A. tumefaciens* culture. Cultures of bacteria in solid media were left to grow for several days and then stored at 4° C for further use. We used the *E. coli* strain DH5α expressing the *Inc*N-type conjugation system encoded on plasmid pKM101 and plasmid pKP80 carrying the *npt*II transgene for resistance to kanamycin and the *Inc*N *ori*T region, which renders the plasmid mobilizable by the *Inc*N *tra* system (Pappas and Winans, 2003). The *A. tumefaciens* strains used were GV3101 and GV3101 with plasmid pKP80.

Table 2: The bacterial species, strains, and antibiotics used in the experiments.
Preglednica 2: Vrste in sevi bakterij ter uporabljeni antibiotiki.

Species	**Bacterial strain**	**Antibiotics***
E. coli	DH5α (pKM101)(pKP80)	Amp50, Spc100
A. tumefaciens	GV3101	Rif30, Gen40
A. tumefaciens	GV3101 (pKP 80)	Rif30, Gen40, Spc100

*Abbreviations: Amp50 – ampicillin 50 µg/ml, Spc100 – spectinomycin 100 µg/ml, Rif30 – rifampicin 30 µg/ml, Gen40 – gentamycin 40 µg/ml.

Bacterial cultures were used when they reached OD_{600} 0.5–0.8 for *E. coli* and 0.8–1.2 for *A. tumefaciens*, which is the most appropriate ratio for an efficient conjugation (Koumpena et al., 2008). They were centrifuged (5,000 rpm, 15 min) and resuspended in the infiltration medium – IM (MS salts 2.2 g/l, B5 vitamins 1ml/l, sucrose 50 g/l, MES 0.5 g/l, agar 4 g, pH adjusted to 5.7 with 1N KOH, the medium was sterilized in an autoclave), 200 µl/l of surfactant Silwet® L-77 was added afterwards (500 µl/l for the 9 February 2009 generation of plants). For the experiments with pre-conjugation, GV3101 and DH5α (pKM 101)(pKP 80) culture cells were centrifuged (5,000 rpm, 15 min), washed with sterile 0.9 % NaCl (w/v) (to wash away culture antibiotics), mixed together and recentrifuged (5,000 rpm, 15 min). The cell pellet was resuspended in a small quantity of the LB medium (0.5 ml) and deposited onto a nitrocellulose filter (45 µm pore size, 5 cm diameter, Millipore) lying on a pre-warmed LB plate and left for 4—5 hours at 28° C. After that period, the filter was removed and placed in an empty sterile petri plate, the cell paste was scraped from the filter and resuspended in the IM of the same volume as that of the agrobacterial culture, except for the 26 January 2009 generation experiments, where inocula for dipping were concentrated 10- and 100-fold (in addition to non-concentrated control), for the specific experiment purposes.

The plants were dipped in the prepared bacterial suspension for 2 minutes (Figure 9), or sprayed several times (Figure 11), or inoculated with drops from a pipette, according to the assay performed. Dipped plants were gently tilted into a horizontal position and left to blot

for a couple of minutes onto Whatman paper to remove the excess of cell suspension from all plant parts (Figure 10). They were then raised and inoculated with cell suspension drops directly onto the rosette of the plant using a pipette, so as to facilitate the inoculation of less protruding developing buds. After the inoculation, the plants were covered with plastic domes or plastic bags for 24 hours to prevent drying and were placed into the growing chamber (Figure 12). They were reinoculated 5 or 6 days after the first inoculation (Figure 13) in order to increase the transformation efficiency (Clough and Bent, 1998). The exact OD_{600} values, the modes of inoculation, and the number of inoculated plants are presented in Tables 3 and 4. The experiments are designated with abbreviations A+ for inoculation with GV3101 (pKP80) as a positive control, A- for inoculation with GV3101 as a negative control, E for inoculation with DH5α (pKM 101)(pKP 80) as a negative control, AE for inoculation with mixed GV3101 and DH5α (pKM 101)(pKP 80), and AEC inoculation with pre-conjugated mixed GV3101 and DH5α (pKM 101)(pKP 80) cultures (Tables 3 and 4). Detailed inoculation procedures for every experiment are described further on.

Figure 9: Grown *Arabidopsis* plants were inverted and their stems dipped for two minutes into the bacterial solution which fills a falcon tube (shown placed standing inside the beaker). This figure shows the second inoculation 5 days after the first one.
Slika 9: Nadzemne dele rastlin repnjakovca smo za dve minuti potopili v raztopino bakterij (na sliki se raztopina nahaja v epruveti, ki stoji v laboratorijski čaši). Slika prikazuje drugo inokulacijo raslin, ki smo jo izvedli pet dni po prvi.

Figure 10: Dipped plants were gently tilted horizontally and left to drip for a couple of minutes onto Whatman paper to remove the excess of cell suspension from all plant parts.
Slika 10: Rastline smo po potapljanju nežno položili na papirnato krpo, da je odvečna tekočina odtekla.

Figure 11: *Arabidopsis* plants were inoculated by spraying from a distance of 4–5 cm (the procedure was repeated several times). The picture shows the first inoculation of one-month-old plants.
Slika 11: Rastline smovečkrat popršili z razdalje 4-5 cm. Slika prikazuje prvo inokulacijo en mesec starih rastlin.

Figure 12: After the inoculation the plants were covered with plastic bags for 24 hours to keep the high humidity.
Slika 12: Po inokulaciji smo rastline za 24 ur prekrili s plastičnimi vrečkami, ki so zagotavljale primerno vlažnost.

Figure 13: The plants are prepared for the second inoculation, 5 days after the first inoculation. Primary bolts are 10 to 15 cm long, secondary bolts are just emerging from the rosette.
Slika 13: Rastline v stadiju, primernem za drugo inokulacijo, ki smo jo izvedli 5 dni po prvi. Primarni poganjki so dolgi 10 do 15 cm, sekundarni pa so tudi že vidni.

Table 3: The bacterial cultures and their ODs, the mode of first, second and third inoculation, and the number of inoculated plants used in recorded experiments 1, 2, 3, 4, 5, and 8.
Preglednica 3: Bakterijske kulture, njihova optična gostota, način prve, druge in tretje inokulacije, ter število uporabljenih rastlin v poskusih 1, 2, 3, 4, 5 in 8.

Experiment and generation of plants	**Bacterial cultures**	**Bacterial strains**	**Mode of 1st inoculation**	**OD_{600} of inoculum**	**Mode of 2nd inoculation**	**OD_{600} of inoculum**	**Mode of 3rd inoculation**	**OD_{600} of inoculum**	**No of inoculated plants (T_0 generation)**
1st experiment 7 October 2008 generation (1st generation)	Infective *Agrobacterium* as positive control (A+)	GV3101 (pKP 80)	Dip	0.81	Drops with pipette	1.66	Dip	0.87	8
	Disarmed *Agrobacterium* as negative control (A-)	GV3101	Dip	0.97	Drops with pipette	1.36	Dip	0.49	8
	Conjugative *E. coli* donor as a negative control (E)	DH5α (pKM 101)(pKP 80)	Dip	0.76	Drops with pipette	0.29	Dip	0.7	12
	Disarmed Agrobacterium and conjugative *E. coli* co-infection (AE)	DH5α (pKM 101) (pKP 80) + GV3101	Dip		Drops with pipette		Dip		24
2nd experiment 17 December 2008 Generation (2nd generation)	Infective *Agrobacterium* as positive control (A+)	GV3101 (pKP 80)	Dip*	1.19	Dip	1.55			5
	Disarmed Agrobacterium and conjugative *E. coli* co-infection after conjugation (AEC)	GV3101	Dip	1.18	Dip	1.73			8
		DH5α (pKM 101)(pKP 80)	Dip	0.55	Dip**	0.47 0.38			

continues

Continuation of Table 3

Experiment and generation of plants	**Bacterial cultures**	**Bacterial strains**	**Mode of 1st inoculation**	**OD_{600} of inoculum**	**Mode of 2nd inoculation**	**OD_{600} of inoculum**	**Mode of 3rd inoculation**	**OD_{600} of inoculum**	**No of inoculated plants (T_0 generation)**
3rd experiment 6 January 2009 generation (3rd generation)	Infective *Agrobacterium* as positive control (A+)	GV3101 (pKP 80)	Dip	1.24	Dip	1.10			8
	Disarmed Agrobacterium and conjugative *E. coli* co-infection after conjugation (AEC)	GV3101	Dip	1.05	Dip	1.11			32
		DH5α (pKM 101)(pKP 80)	Dip	0.41	Dip	0.59			
4th experiment 6 January 2009 generation (3rd generation)	Infective *Agrobacterium* as positive control (A+)	GV3101 (pKP 80)	Dip	1.0	Dip	1.10			8
	Disarmed Agrobacterium and conjugative *E. coli* co-infection (AE)	GV3101	Dip	1.19	Dip	1.15			32
		DH5α (pKM 101)(pKP 80)	Dip	0.57	Dip	0.65			
5th experiment 12 January 2009 generation (4th generation)	Infective *Agrobacterium* as positive control (A+) (spray)	GV3101 (pKP 80)	Spray	1.27	Spray	1.02			24
	Disarmed Agrobacterium and conjugative *E. coli* co-infection (AE) (spray)	GV3101	Spray	1.18	Spray	1.06			56
		DH5α (pKM 101)(pKP 80)	Spray	0.61	Spray	0.55			
8th experiment 9 February 2009, Silwet® l-77 0.05 %, OD_{600}=0.8*** (6th generation)	Disarmed *Agrobacterium* as negative control (A-)	GV3101	Dip	0.8	Dip	0.9	Dip	0.9	12
	Conjugative *E. coli* donor as a negative control (E)	DH5α (pKM 101)(pKP 80)	Dip	0.7	Dip	0.77	Dip	0.82	12
	Disarmed Agrobacterium and conjugative *E. coli* co-infection after conjugation (AEC)	DH5α (pKM 101)(pKP 80) + GV3101	Dip		Dip				24

* The temperature in the laboratory was 28° C, whereas at the other experiments it was between 20° to 25° C
** Two cultures were combined together
*** In this experiment we used both cultures grown to the OD_{600} of 0.8

Table 4: The concentrations of the control strain GV3101 (pKP 80) and experimental strains GV3101 and DH5α (pKM 101)(pKP 80), the OD_{600} and the mode of the first and second inoculation, the number of plants, and the concentrations used for experiments 6 and 7.
Preglednica 4: Koncentracije seva GV3101 (pKP 80) kot pozitivne kontrole in sevov GV3101 ter DH5α (pKM 101)(pKP 80), optična gostote, način prve in druge inokulacije, število rastlin ter koncentracije uporabljene pri poskusih 6 in 7.

Experiment and generation of plants	**Bacterial cultures**	**Strain**	**Mode of 1st inoculation**	**OD_{600} of inoculum**	**Mode of 2nd inoculation**	**OD_{600} of inoculum**	**No of plants T_0**	**Concentration**
6th experiment 26 January 2009 (5th generation)	Infective *Agrobacterium* as positive control (A+)	GV3101(pKP 80)	Drops	1.65	Drops	1.65	8	1x
							8	10x
							8	100x
	Disarmed Agrobacterium and conjugative *E. coli* co-infection (AE)	GV3101	Drops	1.66	Drops	1.65	8*	1x
		DH5α (pKM 101)(pKP 80)	Drops	0.89	Drops	0.80	8*	10x
							8*	100x
7th experiment 26 January 2009 (5th generation)	Infective *Agrobacterium* as positive control (A+)	GV3101(pKP 80)	Drops	1.66	Drops	1.66	4	1x
							4	10x
							4	100x
	Disarmed Agrobacterium and conjugative *E. coli* co-infection after conjugation (AEC)	GV3101	Drops	1.56	Drops	1.7	8*	1x
		DH5α (pKM 101)(pKP 80)	Drops	0.9	Drops	0.81	8*	10x
							8*	100x

* Every set of plants was inoculated with a combination of both cultures (GV3101 and DH5α (pKM 101)(pKP 80)), either directly mixed or preconjugated (AE or AEC experiments, respectively), at mentioned concentrations.

3.3.1 Detailed description of inoculation procedures of experiments

3.3.1.1 The first experiment - 7 October 2008

The first generation of plants was inoculated according to the inoculation procedure developed by Koumpena et al. (2008). Their technique is based on the standard inoculation procedure developed by Clough and Bent (1998), known as the floral dip, and is adapted for inoculation with two cultures, *A. tumefaciens* and *E. coli*. The Clough and Bent (1998) standard inoculation was performed as the positive and negative control (positive control GV3101 (pKP 80) marked as A+, negative control GV3101 marked as A-, and negative control *E. coli* strain DH5α (pKM 101)(pKP 80) marked as E) for the co-inoculation by *A. tumefaciens* and *E. coli* (AE).

Liquid bacterial cultures, grown to the appropriate OD_{600} (Table 3), were poured into the centrifuge tubes and centrifuged for 15 min at 5,000 rpm. The supernatant was poured off and the remaining pellet was resuspended in the infiltration medium in which the plants were dipped for two minutes. First we dipped the negative control plants – two pots of eight plants – in the *A. tumafeciens* strain GV3101 (A-) and three pots of total 12 plants in the *E. coli* strain DH5α (pKM 101)(pKP 80) marked as E. For the co-infection we combined A- and E solutions and dipped 24 plants as previously described. At the end, the

positive control plants were dipped in the GV3101 (pKP 80) marked as A+. After the dip the infiltration medium was applied with a pipette on the emerging buds in the rosettes of the plants. These buds were too small to be submerged in the infiltration medium by dipping, so they were also inoculated with drops from a pipette for a more effective inoculation. The comparison between the standard procedure and the conditions of this experiment is summarised in Table 5.

Table 5: The comparison between the standard procedure (Clough and Bent, 1998) and the modifications of the co-inoculation of *A. thaliana* with *A. tumefaciens* and *E. coli* for the first generation of plants from 7 October 2008. The optical density of *A. tumefaciens* GV3101 (pKP 80) inoculum is marked as OD_{600} A+, the optical density of *A. tumefaciens* GV3101 inoculum is marked as OD_{600} A, and optical density of *E. coli* DH5α (pKM 101)(pKP 80) inoculum as OD_{600} E.
Preglednica 5: Primerjava med standardnim načinom inokulacije (Clough in Bent, 1998) in modifikacijami pri sočasni inokulaciji z arabidopsisa z *A. tumefaciens* in *E. coli* pri prvi generaciji rastlin (7. oktober 2008). Optična gostota inokuluma *A. tumefaciens* GV3101 (pKP 80) je označena z OD_{600} A+, optična gostota inokuluma *A. tumefaciens* GV3101 z OD_{600} A, ter optična gostota inokuluma *E. coli* DH5α (pKM 101)(pKP 80) kot OD_{600} E.

The first experiment	**Standard**	**Modification**
OD_{600} A+	0.8 to 1.2	
OD_{600} A	0.8 to 1.2	
OD_{600} E	0.5 to 0.8	
Mode	Dip	Dip + drops
No of inoculations	2	3
Interval between inoculations	5 or 6 days	3 days * 4 days
Conjugation	No	
Concentration of surfractant	0.02 %	
Concentration of cultures	1x	

* The first re-inoculation was 3 days after the initial one, and the second re-inoculation was 4 days after the first one.

The developmental stage of the flowers is crucial for a successful transformation and since the *Arabidopsis* flowers do not emerge all at once, Clough and Bent (1998) recommended repeating the dipping after 5 or 6 days. In our experiment we used a slightly modified original procedure: instead of one re-dip after five or six days, we performed two re-dips, the second one three days and the third one seven days after the first one (Table 5). The transformation ratios obtained by dip repetition three days after the first dip are reported to be very low (0.5 %) compared to the repeated procedure after five or six days (3.0 %) and no data exists for repetition after seven days (Clough and Bent, 1998). To expose the plants to *A. tumefaciens* too frequently could be detrimental to their health (Clough and Bent, 1998), so we decided to reduce it by applying *A. tumefaciens* in the second inoculation only by drops with a pipette. By applying the bacterial cultures only to the blooms and emerging buds in the rosette, other plant parts were not exposed to pathogenic effects of the bacteria.

3.3.1.2 The second experiment – 17 December 2008

In the second experiment plants were inoculated with preconjugated cultures of *A. tumefaciens* strain GV310 and *E. coli* strain DH5α (pKM 101) (pKP 80). Bacterial conjugation prior to the inoculation increases transformation ratios at least 30-fold (Koumpena et al., 2008). The cultures grown to the appropriate OD_{600} (Table 3) were centrifuged down (15 min, 5,000 rpm), supernatant was poured off and the pellet was resuspended in sterile 0.9 % NaCl (w/v), mixed together in a 40 ml of plain LB medium (without antibiotics) and centrifuged down again. The supernatant was then poured away and the pellet was resuspended in a small quantity of plain liquid LB medium so that it formed a thick suspension. This cell suspension was then placed onto the nitrocellulose filter on the plain LB agar plates and left in the incubator at 28° C for 4.5 hours. The pellet was then scratched off the filter and resuspended in the infiltration medium. The further protocol was as in the first experiment. The temperature in the laboratory during the first inoculation was unusually high (28° C) and that might have influenced the results. The second inoculation was taken after five days with the same protocol as the first one. The comparison between the standard procedure and the conditions of this experiment is summarised in Table 6.

Table 6: The comparison between the standard procedure and the modifications in the second generation – 17 December 2008. The optical density of *A. tumefaciens* GV3101 (pKP 80) inoculum is marked as OD_{600} A+, the optical density of *A. tumefaciens* GV3101 inoculum is marked as OD_{600} A, and optical density of *E. coli* DH5α (pKM 101)(pKP 80) inoculum as OD_{600} E.
Preglednica 6: Primerjava med standardnim postopkom in modifikacijami v drugi generaciji (17. december 2008). Optična gostota inokuluma *A. tumefaciens* GV3101 (pKP 80) je označena z OD_{600} A+, optična gostota inokuluma *A. tumefaciens* GV3101 z OD_{600} A, ter optična gostota inokuluma *E. coli* DH5α (pKM 101)(pKP 80) kot OD_{600} E.

The second experiment	**Standard**	**Modification**
OD_{600} A+	0.8 to 1.2	
OD_{600} A	0.8 to 1.2	
OD_{600} E	0.5 to 0.8	
Mode	Dip	
No of inoculations	2	
Interval between inoculations	5 or 6 days	5 days
Conjugation	No	Yes
Concentration of surfractant	0.02 %	
Concentration of cultures	1x	

3.3.1.3 The third and fourth experiment – 6 January 2009

The third and fourth experiments were performed on the plants of the third generation, planted on 6 January 2009. Because of the huge loses of T_1 plants in the first experiment (AE) and no transformants in the second experiment (AEC), as well as the low transformation ratio in its positive control (A+), we decided to repeat both procedures with more plants.

The preparation of cultures, the conjugation process, and the dipping were performed just as in the first and the second experiment, but in both cases, the second inoculation was 5 days after the first one. The comparison between the standard procedure and the conditions of these experiments is summarised in Table 7.

Table 7: The comparison between the standard procedure and the modifications in the 6 January generation. One part of the plants was inoculated with the non-preconjugated culture, and the other part with a preconjugated culture. The optical density of *A. tumefaciens* GV3101 (pKP 80) inoculum is marked as OD_{600} A+, the optical density of *A. tumefaciens* GV3101 inoculum is marked as OD_{600} A, and optical density of *E. coli* DH5α (pKM 101)(pKP 80) inoculum as OD_{600} E.
Preglednica 7: Primerjava med standardnim postopkom in modifikacijami pri rastlinah generacije 6. januar 2009. En del rastlin je bil inokuliran z nekonjugiranima kulturama, drugi del pa s konjugiranima. Optična gostota inokuluma *A. tumefaciens* GV3101 (pKP 80) je označena z OD_{600} A+, optična gostota inokuluma *A. tumefaciens* GV3101 z OD_{600} A, ter optična gostota inokuluma *E. coli* DH5α (pKM 101)(pKP 80) kot OD_{600} E.

The third and fourth experiment	**Standard**	**Modification**
OD_{600} A+	0.8 to 1.2	
OD_{600} A	0.8 to 1.2	
OD_{600} E	0.5 to 0.8	
Mode	Dip	
No of inoculations	2	
Interval between inoculations	5 or 6 days	5
Conjugation	No	Yes, No
Concentration of surfractant	0.02 %	
Concentration of cultures	1x	

3.3.1.4 The fifth experiment – 12 January 2009

Chung et al. (2000) and Ye (2008, unpublished) reported that spraying the inflorescences with an *A. tumefaciens* culture generates transgenic plants in relatively high frequencies, so we adapted their technique for co-inoculation with *E. coli* and *A. tumefaciens*. The bacterial cultures were grown to the appropriate OD, centrifuged down and resuspended in IM as in the previous experiments. The liquid was then poured into a 100 ml spray bottle and sprayed from a distance of 5–10 cm directly into the rosettes and on the buds. Spraying forms aerosols which could cause contamination of plants with a positive control GV3101 (pKP 80) (A+), so we first sprayed the plants with AE, removed them from the laboratory, and placed them in the growing chamber, and then sprayed the control plants A+ (Table 8).

Because we wanted to test if the sterilization of the soil affects plant performance, half of the control plants as well as the plants co-inoculated with *E. coli* and *A. tumefaciens* were planted in sterile soil and the other half in non-sterile soil. Except for a slightly different colour of the plants, we did not observe any difference in plant fitness or growth that could be attributed to soil sterility.

Table 8: The comparison between the standard procedure and the modifications in the spray experiment. The optical density of *A. tumefaciens* GV3101 (pKP 80) inoculum is marked as OD_{600} A+, the optical density of *A. tumefaciens* GV3101 inoculum is marked as OD_{600} A, and optical density of *E. coli* DH5α (pKM 101)(pKP 80) inoculum as OD_{600} E.
Preglednica 8: Primerjava med standardnim postopkom in modifikacijami pri inokulaciji s pršenjem. Optična gostota inokuluma *A. tumefaciens* GV3101 (pKP 80) je označena z OD_{600} A+, optična gostota inokuluma *A. tumefaciens* GV3101 z OD_{600} A, ter optična gostota inokuluma *E. coli* DH5α (pKM 101)(pKP 80) kot OD_{600} E.

The fifth experiment	**Standard**	**Modification**
OD_{600} A+	0.8 to 1.2	
OD_{600} A	0.8 to 1.2	
OD_{600} E	0.5 to 0.8	
Mode	Dip	Spray
No of inoculations	2	
Interval between inoculations	5 or 6 days	5
Conjugation	No	
Concentration of surfractant	0.02 %	
Concentration of cultures	1x	

3.3.1.5 The sixst and the seventh experiement - 26 January 2009

The cell density and mode of inoculation are important factors which can affect the efficiency of the transformation. Drop-by-drop inoculation performed by Martinez-Trujillo et al. (2004) proved to be twice more efficient than inoculation by submersion. The percentage of transgenic plants they obtained by submerging the plants in the inoculum with an OD_{600} 0.8 was 0.57 ± 0.18 % compared to 1.12 ± 0.26 % obtained by drop-by-drop inoculation with the same inoculum. The same team of researchers reported that an OD_{600} higher than 2.0 produces transformation efficiency more than twice higher than an OD_{600} of 0.8. The transformation efficiency obtained by drop-by-drop inoculation at an OD_{600} of 0.8 was 1.03 ± 0.24 % and when the highly concentrated inoculum with an OD_{600} of more than 2.0 was used, the percentage of transgenic plants rose to 2.57 ± 0.32. Based on the results of Martinez-Trujillo et al. (2004) we decided to adapt their technique for co-inoculation and co-inoculation with previous conjugation. Before the inoculation all open flowers on all the plants used in this experiment had been removed to increase transformation efficiency.

Logemann et al. (2006) published the protocol for the floral dip where they used a bacterial culture grown to the lag phase on solid plates. They scratched bacteria from the plates and resuspended them in the medium to obtain the OD_{600} of about 2.0. Differently from Logemann et al. (2006), we used bacteria in the exponential phase at OD_{600} of about 1.6, when the most intensive growth occurs, and concentrated them 10- or 100-fold.

The positive control strain A+ was grown to the OD_{600} 1.564 (Table 4) and poured 65 ml into each of the three centrifuge tubes and centrifuged. The pellets were then resuspended in the infiltration medium (IM) as follows: for the 100-fold concentration in 0.65 ml of IM,

for the 10-fold concentration in 6.5 ml, and in 65 ml for the unconcentrated inoculum. Plants were inoculated with a pipette directly in the rosette and on the buds.

The strains for the co-inoculation were harvested at the OD_{600} 1.660 (A) and OD_{600} 0.888 (E) (Table 4). We poured GV3010 into three centrifuge tubes, 35 ml in each tube and the same was done with the DH5α (pKM 101)(pKP 80). The cultures were then centrifuged and the pellets were resuspended in a small amount of the infiltration medium. Then A and E pellets were mixed and IM was added to reach the final volume: for the 100-fold concentration in 0.70 ml of IM, for 10-fold in 7.0 ml IM, and in 70 ml of IM for the unconcentrated inoculum. The plants were inoculated with a pipette as previously described. The comparison between the standard procedure and the conditions of these experiments is summarised in Table 9.

Table 9: The comparison between the standard procedure and the modifications in the experiment with different concentrations and no conjugation. The optical density of *A. tumefaciens* GV3101 (pKP 80) inoculum is marked as OD_{600} A+, the optical density of *A. tumefaciens* GV3101 inoculum is marked as OD_{600} A, and optical density of *E. coli* DH5α (pKM 101)(pKP 80) inoculum as OD_{600} E.
Preglednica 9: Primerjava med standardnim postopkom in modifikacijami pri poskusu z različnimi koncentracijami in nekonjugiranima kulturama. Optična gostota inokuluma *A. tumefaciens* GV3101 (pKP 80) je označena z OD_{600} A+, optična gostota inokuluma *A. tumefaciens* GV3101 z OD_{600} A, ter optična gostota inokuluma *E. coli* DH5α (pKM 101)(pKP 80) kot OD_{600} E.

The sixsth experiement	**Standard**	**Modification**
OD_{600} A+	0.8 to 1.2	
OD_{600} A	0.8 to 1.2	
OD_{600} E	0.5 to 0.8	
Mode	Dip	Drops
No of inoculations	2	
Interval between inoculations	5 or 6 days	6
Conjugation	No	
Concentration of surfractant	0.02 %	
Concentration of cultures	1x	1x, 10x, 100x

The strains for the experiment with pre-conjugation were centrifuged down as described for the co-inoculation except the volumes were 50 ml. Both cultures were then resuspended in 5 ml of 0.9 % NaCl (w/v) and mixed together. We then added 1 ml of plain LB medium and centrifuged down again. Every pellet was thus composed of 50 ml of A and 50 ml of E culture. The pellets were resuspended in a small amount of plain LB medium and placed onto solid LB plates with a nitrocellulose membrane and left in the incubator at 28° C. After 4.5 hours the pellets were scratched and resuspended in 100 ml, 10 ml, or 0.1 ml of IM, depending on the desired final concentration. The inoculation with a pipette was performed as described previously. The control plants for the experiment with conjugation were inoculated as described for the A+ strain for the experiment without conjugation. The comparison between the standard procedure and the conditions of these experiments is summarised in Table 10.

Table 10: The comparison between the standard procedure and the modifications in the experiment with different concentrations and conjugation. The optical density of *A. tumefaciens* GV3101 (pKP 80) inoculum is marked as OD_{600} A+, the optical density of *A. tumefaciens* GV3101 inoculum is marked as OD_{600} A, and optical density of *E. coli* DH5α (pKM 101)(pKP 80) inoculum as OD_{600} E.
Preglednica 10: Primerjava med standardnim postopkom in modifikacijami pri poskusu z različnimi koncentracijami in konjugiranima kulturama. Optična gostota inokuluma *A. tumefaciens* GV3101 (pKP 80) je označena z OD_{600} A+, optična gostota inokuluma *A. tumefaciens* GV3101 z OD_{600} A, ter optična gostota inokuluma *E. coli* DH5α (pKM 101)(pKP 80) kot OD_{600} E.

The seventh experiement	**Standard**	**Modification**
OD_{600} A+	0.8 to 1.2	
OD_{600} A	0.8 to 1.2	
OD_{600} E	0.5 to 0.8	
Mode	Dip	Drops
No of inoculations	2	
Interval between inoculations	5 or 6 days	5
Conjugation	No	Yes
Concentration of surfractant	0.02 %	
Concentration of cultures	1x	1x, 10x, 100x

In both cases, with or without conjugation, the highly concentrated (100x) bacterial culture had negative effects on the plants. The plants inoculated with a dense bacterial culture were smaller and their leaves and flowers were deformed. The whole 26 January 2009 generation had an unusually low number of seeds (Figure 25, Table 20), regardless of the concentration of inoculum.

3.3.1.6 The eight experiement - 9 February 2009

In this experiment, we used both bacterial cultures grown to the same density at OD_{600} around 0.8 and a higher Concentration of the surfractant Silwet® L-77 (0.05 %). We used two negative controls (A- and E) and the preconjugated *E. coli* and *Agrobacterium* (AEC). The positive controls were not used due to technical reasons. The summary of experimental conditions for this experiment is presented in Table 11.

Table 11: The comparison between the standard inoculation method and inoculation with both cultures grown to the OD_{600}=0.8. The optical density of *A. tumefaciens* GV3101 (pKP 80) inoculum is marked as OD_{600} A+, the optical density of *A. tumefaciens* GV3101 inoculum is marked as OD_{600} A, and optical density of *E. coli* DH5α (pKM 101)(pKP 80) inoculum as OD_{600} E.
Preglednica 11: Primerjava med standardnim postopkom in modifikacijami pri poskusu, kjer sta bili obe kulturi uporabljeni pri optični gostoti OD_{600}=0.8. Optična gostota inokuluma *A. tumefaciens* GV3101 (pKP 80) je označena z OD_{600} A+, optična gostota inokuluma *A. tumefaciens* GV3101 z OD_{600} A, ter optična gostota inokuluma *E. coli* DH5α (pKM 101)(pKP 80) kot OD_{600} E.

The eight experiement	**Standard**	**Modification**
OD_{600} A+	0.8 to 1.2	NT*
OD_{600} A	0.8 to 1.2	0.8
OD_{600} E	0.5 to 0.8	0.8
Mode	Dip	
No of inoculations	2	3
Interval between inoculations	5 or 6 days	5 and 5
Conjugation	No	Yes
Concentration of surfractant	0.02 %	0.05 %
Concentration of cultures	1x	

* Not tested

The results in the Koumpena BSc thesis (2010) indicate that the most efficient donor-recipient ratio is OD_{600} 0.5 to 0.8 for *E. coli* and OD_{600} 0.8 to 1.2 for *A. tumefaciens*. The OD_{600} 0.5 to 0.8 means that the *E. coli* culture is in the exponential phase when intensive growth occurs. The number of *E. coli* cells in the culture at this phase is about 4 to $5x10^8$ cfu per millilitre at OD_{600} 0.5, and 6 to $7x10^8$ cfu per millilitre at OD_{600} 0.8. The growth curve for *A. tumefaciens* is a little different with a more gently sloping curve and the most intensive cell proliferation at OD_{600} 0.8 to 1.2, when cell density is around $1x10^9$ cfu per millilitre at OD_{600} 0.8 to 1.9 to $2x10^9$ cfu per millilitre at OD_{600} 1.2 (Koumpena BSc thesis, 2010).

The minimal donor-recipient ratio in the standard procedure is thus 1:5 at minimal OD_{600} 0.5 for *E. coli* cells and maximal OD_{600} 1.2 for *A. tumefaciens*. In this experiment we lowered the number of *A. tumefaciens* cells to OD_{600} 0.8, so the ratio was changed in favour of *E. coli* cells, and was about 1:1.4. This change was applied to check if a lowered donor-recipient ratio would give different results due to an increased chance for conjugation.

The concentration of the surfactant Silwet® L-77 is crucial for an efficient transformation, although it may cause damage to the plant tissue (Clough and Bent, 1998; Martinez-Trujillo et al., 2004; Chung at al., 2000; Logemann et al., 2006; Bartholmes et al., 2008; Curtis and Nam, 2001; Davis et al., 2009; Zhang et al., 2006). The study by Clough and Bent (1998) shows that levels of Silwet® L-77 between 0.02 % and 0.1 % give a 20-fold greater transformation rates compared to a 0.005 % Silwet® L-77 content. Different authors recommend v/v concentrations from 0.01 % (Davis et al., 2009), 0.02 % (Chung at al., 2000; Davis et al., 2009; Zhang et al., 2006), 0.03 % (Logemann et al., 2006), to 0.05 % (Clough and Bent, 1998; Martinez-Trujillo et al., 2004). Since none of the authors report

lethal effects on plants at a 0.05 % concentration of Silwet® L-77, we decided to increase its content in IM in order to increase transformation efficiency.

The inoculation procedure was the same as in the second experiment, but with two re-dips, 5 and 10 days after the first one. Immediately after the first dip, the plants were more bent than in other experiments and needed a longer period to reach their normal posture again. Moreover, drying shoots and leaves were observed a few days after the second dip. We attributed these observations to the higher concentration of surfactant and repeated exposure of the plants to it's toxic effects.

3.4 SELECTION OF TRANSGENIC SEEDS

Mature, dry siliques from inoculated plants were collected by individual clipping or by covering the plant with an inverted paper bag and tying it directly above the rosette (Figure 14), at around 2 months after the inoculation. The bags were placed on the plants three to four weeks after inoculation, when the plants were 7 to 8 weeks old. At this age the plants start to dry and siliques start to open, so the placed bag prevents the loss of the seeds. When the whole plant was completely dry, it was cut directly above the rosette, and the siliques were collected from the bag. Siliques were squished to release seeds and sieved to remove chaff and other remains of plant tissue. The seeds were collected and either stored for future use or further processed (imbibed in water and sterilized – see below).

In the first two experiments (7 October 2008 and 17 December 2008 plants) seeds were treated as follows: they were put in water at 4° C for 24 hours; the water was then removed and 70 % ethanol was added for 3 minutes. The ethanol was removed, and 25 % household bleach was added for 10 min. The bleach was then removed and the seeds were washed five times with sterile water. The seeds were then immersed in sterile water, vortexed so they were distributed evenly in the water and spread onto the plates by a pipette. We cut off the top of the pipette tip to enable the seeds to pass through it. The seeds were selected according to a short selective protocol (Harrison et al., 2006): they were spread on selective medium plates and left in the light for 4–6 hours at 21° C. They were then wrapped in aluminium foil for two days and stored in a dark place. The composition of the selective medium was as follows: MS medium 2.15 g/l (or MS salts 2.15 g/l + Gamborg B5 vitamin 1 ml (from stock solution 0,112 g/ml)), sucrose 10 g/l, MES 0.5 g/l, 0.8 g bacto agar, pH adjusted to 5.7 with 1N KOH. The selective medium was sterilized in an autoclave after the sterilization antibiotics were added: cefotaxime 200 µl/ml (from 200 mg/ml stock solution) and kanamycin 50 µl/ml (from 50 mg/ml stock solution). After two days in the dark, the plates were unwrapped and left for two days at a normal 16 hours light / 8 hours dark regime.

The seeds of the third experiment (6 January 2009 plants) were put in water at 4° C for 1.5 days (the first part of the selection) or 1 day (the second part of the selection). The water was removed and 70 % ethanol was added. After 10 min the ethanol was removed and 25 % household bleach with 0.05 % detergent Tween 80 was added for 10 min. The seeds were then rinsed five times with sterile water and spread on selective plates. The selection protocol was the same as before. Seeds of all the later generations were disinfected in the same manner as the first part of the third generation of seeds.

It should be noted here that the seeds were usually split in two batches in order to assay. One part of the seeds from the given experiment was left in the storage, and the other part was sterilised and prepared for the selection procedure as described above. In this regard, the selection procedure was always performed at two consecutive time points, the second of which followed a few days after the first. This split selection procedure was employed in order to reduce losses due to possible fungal infections (Zhang, 2006).

The number of seeds was estimated before spreading on the selective plates by weighing on a precise balance, according to the equation: 1,250 seeds = 25 mg. Despite the careful sieving some chaff and plant tissue remained mixed in with the seeds and might contributed to the weight, so we counted the seeds spread on the plates manually. A piece of paper with a 1x1 cm grid was placed under the selective plate to simplify the counting. *Arabidopsis* seeds are ellipsoid, light to reddish brown, and 0.3–0.5 mm long and can be easily distinguished from other plant tissue which is pale green or yellowish-brown and pointed. The number of seeds determined by counting was significantly lower than the number determined by weighting, probably because of the presence of impurities which contributed to the overall weight. Manually acquired numbers of seeds were used in Tables and calculations.

During the selection of plants of the last generation (9 February 2009 plants) a slimy, bacterial-like background growth was observed on the selective plates. To identify the bacteria samples were taken and streaked on bacteria-optimised media (LB and CM medium) with selective markers. The composition of CM medium was as follows: a-D-glucose 20 g/l, $(NH_4)_2SO_4$ 1 g/l, KH_2PO_4 1 g/l, $MgSO_4(7H_2O)$ 0.5 g/l, yeast extract 5 g/l, dH_2O. The selective markers and description of the procedure are in the results section.

Figure 14: The plants were wrapped in paper bags to collect seeds.
Slika 14: Rastline smo ovili s papirnatimi vrečkami, v katere so padala semena.

3.5 DETECTION OF THE TRANSGENIC PLANTLETS

Transgenic plants, detected four days after the spread on the selective plates, were greener than non-transgenic plants and were transferred to new selective plates to check for phenotype persistency. We transferred all the plants which were greener than average. Plants were transferred one by one with pincers onto the selective plates with the same composition as for the first selection and left for several days at a normal 16 hours light / 8 hours dark regime.

4 RESULTS

The transformation of *Arabidopsis thaliana* is usually carried out by the flower dip method and is known to be influenced by several factors including the developmental stage of inoculated plants, the number of inoculations, the composition of inoculation medium (Clough and Bent, 1998), the mode of inoculation (Clough and Bent 1998, Chung et al., 2000; Martinez-Trujillo et al., 2004), and – as with all plant transformations – the *Agrobacterium* strain and plasmid vector used (Hellens et al., 2000). In this work we pursued to employ past knowledge regarding optimal *A. thaliana* transformation while employing a novel protocol. We pursued to co-infect *A. thaliana* with a combination of an *Agrobacterium* disarmed strain and a conjugative *Escherichia coli* strain that would carry the binary vector. The disarmed strain used for this purpose was GV3101, the *E. coli* strain was DH5α, while the vector used was pKP80 (Pappas and Winans, 2003). The *E. coli* transgene-carrying strain and the *Agrobacterium* mediator were used directly in coinfection as a mixture of bacterial suspensions or after an interbacterial conjugation period which was carried out in order to allow *E. coli* to transfer the transgene vector to the *A. tumefaciens* mediator at optimal conditions. This would conceivably increase the number of *A. tumefaciens* transconjugants carrying the binary vector, and in turn increase maximal yields of plant transformants, since from previous experiments carried out with *N. tabacum*, it was proven that the route of transgene transfer at such biparental set-ups was from *E. coli* to *Agrobacterium* and then to the plant (Pappas and Winans, 2003). As a positive control, testifying to good plant receptivity, in all experiments GV3101 (pKP80) was used. Negative controls, testifying against unwarranted contamination in the transformation set-up, involved the use of GV3101 or DH5α (pKM101)(pKP80).

4.1 RESULTS OF THE FIRST EXPERIMENT (7 OCTOBER 2008 PLANTS)

The results for this experiment (the 7 October 2008 generation) are shown in Table 12. Out of 24 plants which were co-inoculated with GV3101 and DH5α (pKM 101)(KP 80) (AE), we got 30,467 seeds, 24,567 of which were destroyed by a fungal infection during the selection. From the remaining 5,900 seeds we got no transformants. The average seed number per plant was 1,269.

The eight plants inoculated with GV3101 (pKP 80) (A+) as positive controls had almost 3,000 seeds, 64 of which were transgenic (2.18 %). The discrepancies in the number of transformants between the selective plates were large: in the first part of the selection the percentage of the transgenic plants was 1.83 in the first plate and 4.80 in the second, whereas in the second part of the selection, where a single plate was used, it was 0.79 %. The average number of seeds per positive control (A+) plant was 367, and germination ratios were 100 % in all A+ plates. From the eight plants dipped in the negative control GV3101 (A-), and the twelve in DH5α (pKM 101)(KP 80) (E), we did not get any transgenic plants, as was expected. The percentage of vital (germinating) seeds on all the selective plates was very variable, ranging from 0 to 100 %. The plates with low or nonexistent percentage of germinating seeds were all infected with fungi, which overgrew the seeds and prevented germination or destroyed seedlings (selective plates 3 to 9 in the second part of the selection of co-inoculated plants).

Table 12: The transgenic seed assay for the first experiment. The assay was performed in two parts to reduce losses due to contamination. The seeds were sterilised and placed onto selective plates. After several days we counted the seedlings and transformants, and calculated the percentage of transformants per T_1 plants. *A. tumefaciens* GV3101 (pKP 80) positive control is marked as A+, *A. tumefaciens* GV3101 negative control is marked as A-, *E. coli* DH5α (pKM 101)(pKP 80) as E, and coinoculation with *A. tumefaciens* GV3101 and *E. coli* DH5α (pKM 101)(pKP 80) as AE.
Preglednica 12: Selekcija transgenih rastlin pri prvem poskusu. Selekcijo smo izvedli v dveh delih, da bi se izognili morebitnim izgubam zaradi kontaminacije. Semena smo sterilizirali ter jih nanesli na selekcijske plošče. Po nekaj dneh smo prešteli sejančke in transgene rastline in izračunali odstotek transgenih rastlin. *A. tumefaciens* GV3101 (pKP 80) kot pozitivna kontrola je označena z A+, *A. tumefaciens* GV3101 kot negativna kontrola je z A-, *E. coli* DH5α (pKM 101)(pKP 80) je označena z E, koinokulacija z *A. tumefaciens* GV3101 in *E. coli* DH5α (pKM 101)(pKP 80) pa z AE.

Experiment 1	Transgenic seed assay	Plate no.	No. of seeds on the selec-tive plate	No of T_1 plants	% of vital seeds	No of non-germina-ting seeds	No of trans-formants	% of transformants per plate/total
A+	1st part of the selection	1	876	876	100	0	16	1.83
		2	792	792	100	0	38	4.80
	Total for the 1st part		**1,668**	**1,668**	**100**	**0**	**54**	**3.24**
	2nd part of the selection	1	1,270	1,270	100	0	10	0.79
	Total for the 2nd part		**1,270**	**1,270**	**100**	**0**	**10**	**0.79**
	Total for A+		**2,938**	**2,938**	**100**	**0**	**64**	**2.18**
AE	1st part of the selection	1	865	813	93.99	52	0	0
		2	872	630	72.25	242	0	0
	Total for the 1st part		**1,737**	**1,443**	**83.07**	**294**	**0**	**0**
	2nd part of the selection	1	1,690	1,469	86.92	221	0	0
		2	2,508	2,248	89,63	260	0	0
		*3	2,928	134	4.58	2,794	0	0
		*4	6,383	0	0	6,383	0	0
		*5	3,484	0	0	3,484	0	0
		*6	4,890	0	0	4,890	0	0
		*7	1,258	0	0	1,258	0	0
		*8	3,942	493	12.51	3,449	0	0
		*9	1,647	113	6.86	1,534	0	0
	Total for the 2nd part		**28,730**	**4,457**	**15.51**	**24,273**	**0**	**0**
	Total for AE		**30,467**	**5,900**	**19.37**	**24,567**	**0**	**0**
A-	1st part of the selection	1	1,570	1,570	100	0	0	0
		2	562	562	100	0	0	0
	Total for the 1st part		**2,132**	**2,132**	**100**	**0**	**0**	**0**
	2nd part of the selection	1	1,890	1,890	100	0	0	0
		2	1,308	1,308	100	0	0	0
	Total for the 2nd part		**3,198**	**3,198**	**100**	**0**	**0**	**0**
	Total for A-		**5,330**	**5,330**	**100**	**0**	**0**	**0**
E	1st part of the selection	1	2,057	2,057	100	0	0	0
		2	1,493	1,493	100	0	0	0
		3	1,847	1,847	100	0	0	0
	Total for the 1st part		**5,397**	**5,397**	**100**	**0**	**0**	**0**
	2nd part of the selection	1	1,582	1,582	100	0	0	0
		2	2,086	2,086	100	0	0	0
	Total for the 2nd part		**3,668**	**3,668**	**100**	**0**	**0**	**0**
	Total for E		**9,065**	**9,065**	**100**	**0**	**0**	**0**

* Fungal contaminations

4.2 RESULTS OF THE SECOND EXPERIMENT (17 DECEMBER 2008 PLANTS)

From 5 plants dipped in the positive control strain (A+), we got 4,130 seeds and out of those 3,980 plants, 26 or 0.65 % were transgenic. The average number of seeds per plant was 826. Again, we found a large discrepancy in the percentage of transgenic plants between the first and the second part of the selection: in the first part of the selection we got 1.63 % of transgenic plants, whereas in the second part, we only got 0.54 %. There were large discrepancies even among the plates within the same part of the selection: in the first selection three plates were used and the percentages of transgenic plants were 2.67, 0.95, and 1.43. The plate with the lowest percentage (0.95) was infected with fungi. The results from the second part of the selection were much more even, ranging from 0.50 to 0.57 %.

From the 8 plants dipped in the preconjugated GV310 and DH5α (pKM 101)(pKP 80), we got 13,990 seeds, none of which were transgenic, and the average number of seeds was 1,110 per plant. Although seven out of nine selective plates were contaminated with fungi, the overall germination ratio was 99.37 % for the plants inoculated with the preconjugated inoculum, and 96.37 % for the control plants (Table 13).

Table 13: The transgenic seed assay for the second experiment. The assay was performed in two parts for a control (A+) as well as for the preconjugated cultures (AEC).
Preglednica 13: Selekcija transgenih rastlin pri drugem poskusu. Selekcija je potekala v dveh delih za kontrolne rastline (A+) kot tudi za rastline, inokulirane z predhodno konjugiranima kulturama (AEC).

Experiment 2	Transgenic seed assay	Plate no.	No. of seed on the selective plate	No of T1 plants	% of vital seeds	No of non-germinating seeds	No of transformants	% of transformants per plate/total
A+	1st part of the selection	1	210	150	71.43	60	4	2.67
		2	260	210	80.77	50	2	0.95
		3	110	70	63,64	40	1	1.43
	Total for the 1st part		**580**	**430**	**74.14**	**150**	**7**	**1.63**
	2nd part of the selection	1	1,300	1,300	100	0	7	0.54
		2	1,200	1,200	100	0	6	0.50
		3	1,050	1,050	100	0	6	0.57
	Total for the 2nd part		**3,550**	**3,550**	**100**	**0**	**19**	**0.54**
	Total		**4,130**	**3,980**	**96.37**	**150**	**26**	**0.65**
AEC	1st part of the selection	1	92	67	72.83	25	0	0
		2	74	60	81.08	14	0	0
		3	62	45	72.58	17	0	0
	Total for the 1st part		**228**	**172**	**75.44**	**56**	**0**	**0**
	2nd part of the selection	1	1,500	1,500	100	0	0	0
		2	1,500	1,500	100	0	0	0
		3	1,500	1,500	100	0	0	0
		4	1,500	1,500	100	0	0	0
		5	1,100	1,100	100	0	0	0
		6	1,550	1,550	100	0	0	0
	Total for the 2nd part		**8,650**	**8,650**	**100**	**0**	**0**	**0**
	Total		**8,878**	**8,822**	**99.37**	**56**	**0**	**0**

4.3 RESULTS OF THE THIRD AND FOURTH EXPERIMENTS (6 JANUARY 2009 PLANTS)

4.3.1 Results of the third experiment (preconjugated cultures)

The results for the plants inoculated with the preconjugated culture are shown in Table 14. From the 32 plants dipped in the preconjugated GV310 and DH5α (pKM 101)(pKP 80), we got 11,476 seeds, 4,023 of which germinated, and 2 were transgenic (0.05 %), which is comparable to the results of Koumpena et al. (2008). For the first part of the selection six selective plates were used and a little more than 3000 seeds were planted, but none of those were transgenic. The remaining seeds were used in the second part of the selection on eight plates, where two seedlings were transgenic. The germination ratio in the second part of the selection was much lower than in the first, only 10.81 %, ranging from less than 6 % to 20 %. The overall germination ratio was 35.06 % and the average number of seeds per plant was 359.

From the 8 plants dipped in the positive control strain, we got 5,800 seeds. 2,379 of these germinated (41.33 %), and 41 (1.72 %) were found to be transformants. As in previous experiments the variability between the first and the second part of the selection was very high: in the first part of the selection it was 100 %, whereas in the second part it was only 7 %. The average number of seeds was 725 per inoculated plant and the overall germination ratio was 41.02 %.

Table 14: The results from the third generation – 6 January 2009, dipped in preconjugated cultures of bacteria. The assay was performed in two parts for a control (A+) as well as for the preconjugated cultures (AEC).
Preglednica 14: Rezultati za tretjo generacijo rastlin (6. januar 2009), ki smo jih inokulirali s predhodno konjugiranima kulturama bakterij. Selekcija je potekala v dveh delih za kontrolne rastline (A+) kot tudi za rastline, inokulirane z predhodno konjugiranima kulturama (AEC).

Experiment 3	**Transgenic seed assay**	**Plate no.**	**No. of seed on the selective plate**	**No of T_1 plants**	**% of vital seeds**	**No of non-germinating seeds**	**No of transformants**	**% of transformants per plate/total**
A+	1st part of the selection	1	370	370	100	0	3	0.81
		2	540	540	100	0	9	1.67
		3	540	540	100	0	12	2.22
		4	680	680	100	0	15	2.21
	Total for the 1st part		**2,130**	**2,130**	**100**	**0**	**39**	**1.83**
	2nd part of the selection	1	1,300	91	7.00	1,209	1	1.10
		2	1,000	70	7.00	930	1	1.43
		3	770	81	10.52	689	0	0
		4	600	25	4.17	765	0	0
	Total for the 2nd part		**3,670**	**267**	**7.28**	**3,593**	**2**	**0.75**
	Total		**5,800**	**2,397**	**41.33**	**3,593**	**41**	**1.71**
AEC	1st part of the selection	1	540	540	100	0	0	0
		2	460	460	100	0	0	0
		3	540	540	100	0	0	0
		4	480	480	100	0	0	0
		5	640	640	100	0	0	0
		6	460	460	100	0	0	0
	Total for the 1st part		**3,120**	**3,120**	**100**	**0**	**0**	**0**
	2nd part of the selection	1	1,000	120	12.00	880	0	0
		2	1,200	70	5.83	1,130	1	1.43
		3	1,300	200	15.38	1,100	0	0
		4	900	115	12.78	785	1	0.87
		5	1,136	101	8.89	1,035	0	0
		6	1,300	132	10.15	1,168	0	0
		7	1,100	80	7.27	1,020	0	0
		8	420	85	20.24	335	0	0
	Total for the 2nd part		**8,356**	**903**	**10.81**	**7,453**	**2**	**0.22**
	Total		**11,476**	**4,023**	**35.06**	**7,453**	**2**	**0.05**

4.3.2 Results of the fourth experiment (non-preconjugated cultures)

The results for plants inoculated with the non-preconjugated culture are shown in Table 15. From the 32 plants dipped in the GV310 and DH5α (pKM 101)(pKP 80), out of 8,050 seeds, we got no transgenic plants. The overall germination ratio was 36.77 % or 2,960

germinating seeds, but again there was a very low percentage (3.05 %) of germinating seeds in the second part of the selection. The average number of seeds per plant was 252. From the 8 plants dipped in the control strain (A+), we got 3,565 seeds, 1,936 or 54.31 % of which germinated, and the average number of seeds per plant was 446. There were 11 transgenic plants (0.57 %). The germination ratio on the selective plate in the first part of the selection was 100 % and 6 (0.34 %) transgenic plants were identified, whereas in the second part the germination ratio was below 10 % and 5 transgenics (2.92 %) were grown.

Table 15: The results from the third generation – 6 January 2009, dipped in non-preconjugated cultures of bacteria. The assay was performed in two parts for a control (A+) as well as for the non-preconjugated cultures (AE).
Preglednica 15: Rezultati za tretjo generacijo rastlin (6. januar 2009), ki smo jih inokulirali z nekonjugiranima kulturama bakterij. Selekcija je potekala v dveh delih za kontrolne rastline (A+) kot tudi za rastline, inokulirane z dvema kulturama (AE).

Experiment 4	**Transgenic seed assay**	**Plate no.**	**No. of seed on the selective plate**	**No of T_1 plants**	**% of vital seeds**	**No of non-germinating seeds**	**No of transformants**	**% of transformants per plate/total**
A+	1st part of the selection	1	1,765	1,765	100	0	6	0.34
	Total for 1st part		**1,765**	**1,765**	**100**	**0**	**6**	**0.34**
	2nd part of the selection	1	1,100	78	7.09	1,022	2	2.56
		2	700	93	13.29	607	3	3.23
	Total for 2nd part		**1800**	**171**	**9.5**	**1,629**	**5**	**2.92**
	Total		**3,565**	**1,936**	**54.31**	**1,629**	**11**	**0.57**
AE	1st part of the selection	1	550	550	100	0	0	0
		2	610	610	100	0	0	0
		3	540	540	100	0	0	0
		4	480	480	100	0	0	0
		5	620	620	100	0	0	0
	Total for 1st part		**2,800**	**2,800**	**100**	**0**	**0**	**0**
	2nd part of the selection	1	1,000	78	7.8	922	0	0
		2	900	26	2.89	874	0	0
		3	400	10	2.5	390	0	0
		4	1,000	0	0	1,000	0	0
		5	800	31	3.88	769	0	0
		6	450	15	3.33	435	0	0
		7	700	0	0	700	0	0
	Total for 2nd part		**5,250**	**160**	**3.05**	**5,090**	**0**	**0**
	Total		**8,050**	**2,960**	**36.77**	**5,090**	**0**	**0**

4.4 THE PERCENTAGE OF TRANSGENIC PLANTS AND VITAL SEEDS

4.4.1 The percentage of transgenic plants in positive control plants (A+)

As explained in Materials and methods section, the selection procedure in the first four experiments was performed at two consecutive time points, the second of which followed a few days after the first. Generally, the percentage of transgenic plants in positive controls (A+) tends to be higher in the first part of the selection and lower in the second part (Figure 15). An average percentage of transgenic plants in the first part of the selection of A+ inoculated plants was 1.89, whereas in the second part was 1.07. The differences between the selective plates were very high regardless of the part of the selection (Annex A), ranging from 0.00 % to 4.8 % of transgenics per plate. The variability was higher in the first part of the selection: the standard deviation in this part was 1.25, and in the second 1.07. Transgenic plants were also obtained in plants co-inoculated with preconjugated *Agrobacterium* and *E. coli* in Experiment 3 (Table 10, selective plates 2 and 4 in the second part of the selection). Because this was the only case within the first four experiments, the comparison of differences between percentages of transgenic plants could not be made.

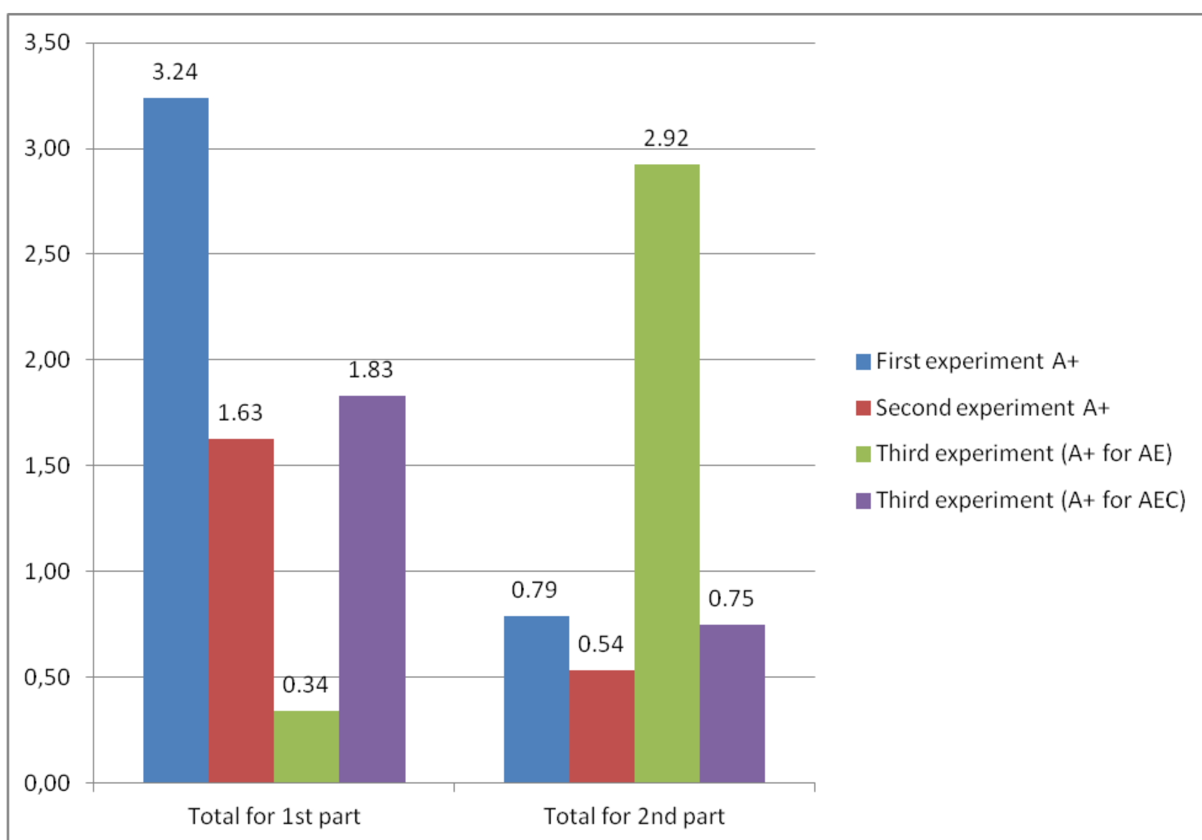

Figure 15: Total percentage of transgenic T_1 plants inoculated with GV310 (pKP80) as a positive control in the first four experiments depending on the part of the selection. Generally, the percentage of transgenic plants tends to be higher in the first part of the selection and lower in the second part.
Slika 15: Skupni odstotek transgenih T_1 rastllin, inokuliranih s pozitivno kontrolo GV310 (pKP80) v prvih štirih poskusih. Odstotek transgenih rastlin je bil v povprečju višji v prvem delu selekcije in nižji v drugem delu.

4.4.2 The percentage of vital seeds

The differences between the parts of the selection were also in the viability of seeds. The germination ratio for positive control plants in the first part of the selection was higher than in the second (Tables 7, 9, 11, and 12). The average germination ratio for A+ in the first part of the selection was more than 91.58 % and in the second part less than 44.91 % (Annex B). The variability of the results was much greater in the second part, the standard deviation was 47.48 and 14.14 in the first part.

The selection of plants co-inoculated with *Agrobacterium* and *E. coli* (AE) or with preconjugated *Agrobacterium* and *E. coli* (AEC) showed similar results. The percentage of vital seeds was on average higher in the first part of the selection and usually dropped significantly in the second part (Figure 16).

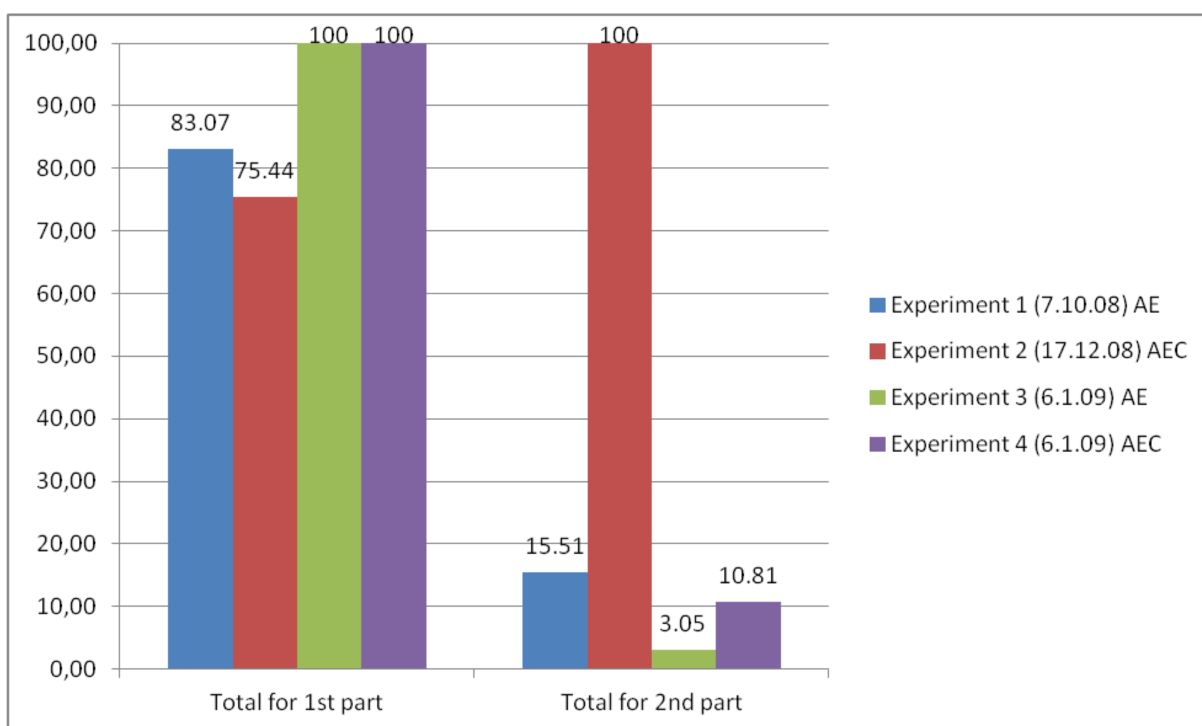

Figure 16: The total percentage of vital seeds in the first four experiments in regard to the part of the selection for plants co-inoculated with *Agrobacterium* and *E. coli* (AE) or co-inoculated with preconjugated *Agrobacterium* and *E. coli* (AEC).
Slika 16: Skupni odstotek kaljivih semen sočasno inokuliranih rastlin (AE), ter rastlin, inokuliranih s konjugiranima kulturama (AEC) v odvisnosti od dela selekcije za prve štiri poskuse.

4.5 RESULTS OF THE FIFTH EXPERIMENT (12 JANUARY 2009 PLANTS)

In this experiment 32 plants were used as a positive control and 56 plants for co-inoculation. The 32 plants used as a control yielded 3,718 seeds (116 per plant), the percentage of germinating seeds was 87 %, and 487 seeds did not germinate. Among the 3,231 germinating seeds, there were 111 transgenic plants or 3.44 % (Figure 17 and Table 16). Additionally, some mosaic plants were observed.

The co-inoculated plants yielded 10,202 seeds (182 per plant) and the percentage of germinating seeds was 87 % or 8,871 T_1 plants. Five plants from selective plates number 3, 5, and 6 were clearly transgenic, i.e. 0.06 % (Figure 18). We also observed one green plant on the plate 1, but the transgenicy of this plant is under the question, because it grew on the edge of the plate and might had not have been affected by the antibiotic selective marker. Upon the re-transfer onto a new selective plate this plant was contaminated and decayed before it could be undoubtedly recognised as truly transgenic.

The germination ratio was calculated as a ratio derived from plate 2 (positive control), where 400 plants were grown from every 460 seeds, which gives a germination ratio of 0.869, rounded to 0.87. The selection in this and in the following experiments was performed in one single part.

Table 16: The results from the spray experiment.
Preglednica 16: Rezultati poskusa inokulacije s pršenjem.

Experiment 5	**Transgenic seed assay**	**Plate No**	**No of seed on the selective plate**	**No of T_1 plants**	**No of non-germinating seeds**	**% of vital seeds**	**No of transformants**	**% of transformants per plate/total**
A+	1st part of the selection	1	880	765	115	87	24	3.14
		2	460	400	60	87	12	3.00
		3	1,128	980	148	87	31	3.16
		4	1,250	1,086	164	87	44	4.05
		Total	**3,718**	**3,231**	**487**	**87**	**111**	**3.44**
AE	1st part of the selection	1	2,463	2,143	320	87	0	0.00
		2	1,847	1,607	240	87	0	0.00
		3	1,284	1,117	167	87	1	0.09
		4	1,808	1,573	235	87	0	0.00
		5	1,000	870	130	87	3	0.34
		6	1,800	1,566	234	87	1	0.06
		Total	**10,202**	**8,871**	**1,331**	**87**	**5**	**0.06**

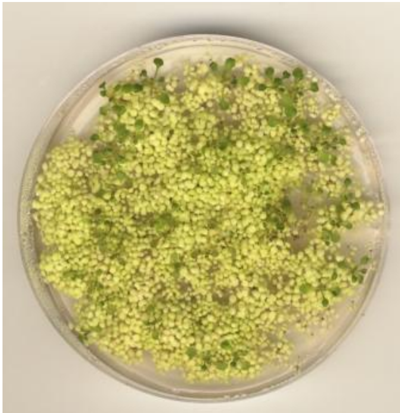

Figure 17: The selection of transgenic plants (green) which are clearly different from the non-transgenic plants (pale). These are the plantlets from the seeds of the fifth experiment (12 January 2009 generation of plants) sprayed with a positive control strain GV3101 (pKP80).
Slika 17: Selekcije transgenih rastlin (zelene barve), ki se očitno razlikujejo od netransgenih (svetle). Slika prikazuje rezultate petega poskusa - rastlinice generacije 12. januarj 2009, ki so bile inokulirane s pršenjem s pozitivno kontrolo GV3101 (pKP80).

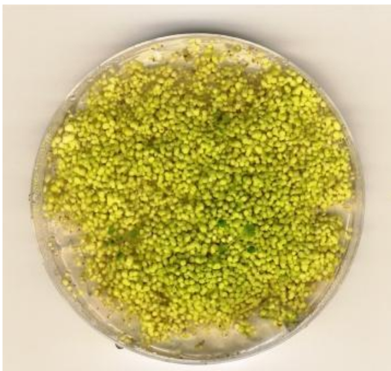

Figure 18: Three of five transgenic plants obtained by spray co-inoculation with *A. tumefaciens* and *E. coli*. Green transgenic plants are clearly distinguished from the pale non-transgenics.
Slika 18: Tri od petih transgenih rastlin, pridobljenih s sočasno inokulacijo s pršenjem z *A. tumefaciens* in *E. coli.* Zelene transgene rastline so dobro vidne med svetlimi netransgenimi rastlinami.

4.6 RESULTS OF THE SIXST AND SEVENTH EXPERIMENTS (26 JANUARY 2009 PLANTS)

4.6.1 Results of the sixsth experiment (experiment without conjugation)

The results of the experiment without prior conjugation are presented in Table 17. In this experiment we used 8 plants for every concentration and control. From the 8 plants inoculated with a 1x concentration we got only 204 seeds (25 per plant), 50.10 % or 104 of which germinated and none of which were transgenic. The corresponding control plants yielded 580 seeds (73 per plant), of which only 84 or 14.48 % germinated. None of the control plants inoculated with the 1x concentration yielded any clearly transgenic seeds – we observed only 4 poorly growing four-leaved mosaics.

The plants inoculated with a 10x concentration yielded 152 seeds, 68.42 % or 104 seeds germinated and out of those 10.58 % or 11 plants were transgenic, which is the highest percentage of transgenic plants of all the experiments performed. The percentage in control plants was 7.69 % or 2 plants out of 26. The percentage of germinating seeds in the control was very low, only 5.53 % or 26 plants out of 470 seeds.

The plants inoculated with a 100x concentrated culture yielded 390 seeds (49 per plant), out of 177 germinating seeds (germination ratio 57.09 %) 1 or 0.56 % was transgenic. Beside this transgenic plant we observed five mosaics. The control plants yielded 309 seeds (39 per plant); 69 seeds germinated (22.33 %) and none of them were transgenic.

Table 17: The results of the experiment 6. Plants, planted on 26.1.2009 were dipped in non-preconjugated cultures of bacteria (AE), which were concentrated 10 or 100-times or were used unconcentrated (1 x).
Preglednica 17: Rezultati poskusa št.6 z rastlinami generacije 26. januar 2009, inokuliranimi z nekonjugiranima kulturama (AE). Bakterijske kulture so bile koncentrirane 10 ali 100 krat, uporabili pa smo tudi nekoncentrirane kulture (1x).

Experiment 6 (26.1.2009 AE)	**Plate no.**	**No of seeds per selective plate**	**No of T_1 plants**	**% of vital seeds**	**Non-germinating seeds**	**No. of transgenic plants**	**% of transformants per plate/total**
A+ 1x	1	340	38	11.18	302	0	0
	2	240	46	19.17	194	0	0
	Total	**580**	**84**	**14.48**	**496**	**0**	**0**
A+ 10x	1	220	16	7.27	204	1	6.25
	2	250	10	4.00	240	1	10.00
	Total	**470**	**26**	**5.53**	**444**	**2**	**7.69**
A+ 100x	1	224	50	22.32	174	0	0
	2	85	19	22.35	66	0	0
	Total	**309**	**69**	**22.33**	**240**	**0**	**0**
AE 1x	1	104	52	50	52	0	0
	2	100	52	52	48	0	0
	Total	**204**	**104**	**50.10**	**100**	**0**	**0**
AE 10x	1	100	52	52	48	* N/D	* N/D
	2	100	52	52	48	* N/D	* N/D
	Total	**200**	**104**	**52**	**96**	**11**	**10.5**
AE 100x	1	180	25	13.89	155	* N/D	* N/D
	2	210	152	72.38	58	* N/D	* N/D
	Total	**390**	**177**	**57.09**	**213**	**1**	**0.56**

* N/D – no data

4.6.2 Results of the seventh experiment (experiment with conjugation)

The results are presented in Table 18. None of the control plants used in this experiment yielded any vital seeds. The number of seeds per parental plant was 55 (220 seeds from 4 plants) for the uncondensed culture, 107 (430 from 4 plants) for the 10x concentration, and no seeds from the four plants for 100x concentration.

The plants inoculated with the preconjugated *Agrobacterium* and *E. coli* yielded 280 seeds (35 per plant) for the 1x concentration, 301 seeds or 38 per plant for the 10x concentration and 170 seeds or 21 per plant for the 100x concentration. The germination ratio was 100 % in all cases.

Plants inoculated with 1x concentrated preconjugated culture yielded no transgenic seeds, although we observed 6 mosaic plants. A more condensed culture at a 10x concentration yielded one transformant (0.33 %) and 21 mosaics. From the plants inoculated with a 100x concentrated culture we got 1 transformant or 0.59 %.

Table 18: The results of the experiment 6. Plants, planted on 26.1.2009 were dipped in preconjugated cultures of bacteria (AE), which were concentrated 10 or 100-times or were used unconcentrated (1 x).
Preglednica 18: Rezultati poskusa št.6 z rastlinami generacije 26. januar 2009, inokuliranimi s konjugiranima kulturama (AE). Bakterijske kulture so bile koncentrirane 10 ali 100 krat, uporabili pa smo tudi nekoncentrirane kulture (1x).

Experiment 7 (26.1.2009 AEC)	**Plate no.**	**No of seeds per selective plate**	**No of T_1 plants**	**% of vital seeds**	**Non-germinating seeds**	**No. of transgenic plants**	**% of transgenic plants**
A+ 1x	1	100	0	0	100	0	0
	2	120	0	0	120	0	0
	Total	**220**	**0**	**0**	**220**	**0**	**0**
A+ 10x	1	210	0	0	210	0	0
	2	220	0	0	220	0	0
	Total	**430**	**0**	**0**	**430**	**0**	**0**
A+ 100x	1	0	0	0	0	0	0
	2	0	0	0	0	0	0
	Total	**0**	**0**	**0**	**0**	**0**	**0**
AEC 1x	1	140	140	100	0	0	0
	2	140	140	100	0	0	0
	Total	**280**	**280**	**100**	**0**	**0**	**0**
AEC 10x	1	121	121	100	0	N/D*	N/D
	2	180	180	100	0	N/D	N/D
	Total	**301**	**301**	**100**	**0**	**1**	**0.33**
AEC 100x	1	98	98	100	0	N/D	N/D
	2	72	72	100	0	N/D	N/D
	Total	**170**	**170**	**100**	**0**	**1**	**0.59**

* N/D – no data

4.7 RESULTS OF THE EIGHT EXPERIMENT (9 FEBRUARY 2009 PLANTS)

The plants inoculated with the negative control DH5α (pKP80)(pKM101) (E) yielded 680 seeds, all of which germinated. Surprisingly, there were 7 mosaic plants (1.03 %), which grew to the phase of four true leaves (Figures 20 and 21). Compared to the true transformants, these plants were growing slowly and they died later, after they were transferred into soil. The plants from another negative control, GV3101 (A-), yielded 3,240 seeds (the percentage of germinating seeds was 90 %) and 2,916 plants. As in the *E. coli*

negative control, we observed 11 mosaic plants among them, but no clearly transgenic plant.

From the plants inoculated with the preconjugated *E. coli* and *Agrobacterium* we got 636 seeds, the percentage of germinating seeds was 100 %, and we got one transgenic plant (0.16 %) (Table 19 and Figure 19), and one slow-growing mosaic plant. Neither this mosaic plant, nor the mosaics from the negative control mentioned before, grew in soil.

During the selection, we observed a slimy bacterial-like background growth on the selective plates of the negative control DH5α (pKP80)(pKM101) plants, as well as on the plates of the plants inoculated with the preconjugated GV3101 and DH5α (pKP80)(pKM101). A sample was taken and streaked on the selective plates on different media with a selective marker. On the CM medium at 30° C, the result was positive, on the LB medium with Rif and Gent at 30° C, it was negative, and on the LB medium with Ap and Spec at 30° C, three colonies grew. These colonies were transferred onto a fresh selective plate with the same composition and were incubated at 37° C, which is the most appropriate temperature for *E. coli*. The result in this case was negative, so we can exclude the contamination with *E. coli*.

Table 19: The results from the experiment 8 (9 February 2009 generation of plants).
Preglednica 19: Rezultati poskusa št.8 z rastlinami generacije 9. februar 2009.

Experiment 8 (OD=0.8)	**Plate no.**	**No of seeds per selective plate**	**No of T_1 plants**	**% of vital seeds**	**Non-germinating seeds**	**No. of transgenic plants**	**% of transgenic plants**
A-	1	1,800	1620	90	180	0	0
	2	1,440	1296	90	144	0	0
	Total	**3,240**	**2,916**	**90**	**324**	**0**	**0**
E	1	280	280	100	0	0	0
	2	400	400	100	0	0	0
	Total	**680**	**680**	**100**	**0**	**0**	**0**
AEC	1	86	86	100	0	0	0
	2	180	180	100	0	1	0.56
	3	210	210	100	0	0	0
	4	160	160	100	0	0	0
	Total	**636**	**636**	**100**	**0**	**1**	**0.16**

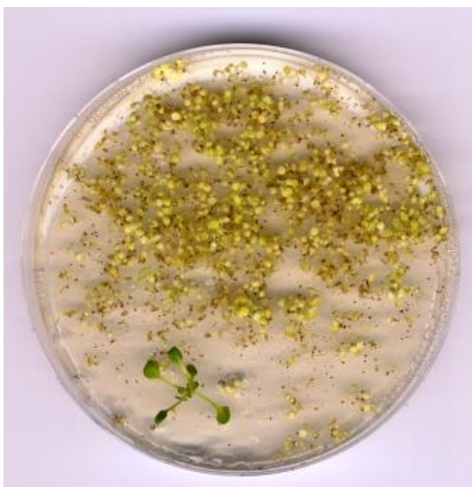

Figure 19: A transgenic plant from an experiment with OD_{600}=0.8.
Slika 19: Transgena rastlina iz poskusa, v katerem sta bili obe kulturi uporabljeni pri OD_{600}=0.8.

Figure 20: Mosaic plants from experiment eight which were transferred onto fresh selective plates.
Slika 20: Mozaične rastline iz osmega poskusa, ki smo jih prenesli na sveže selekcijsko gojišče.

Figure 21: Mosaic plants from experiment eight.
Slika 21: Mozaične rastline iz osmega poskusa.

4.8 SUMMARY OF THE RESULTS

The results of all the experiments conducted in this work (October 2008 – July 2009) are summarized in Table 20. The standard variations in the percentage of transgenic plants, the number of seeds per plant, and the percentage of germinating seeds were high in different experiments and also among the controls (Figures 22, 23, 24, 25, and 26). The lowest percentage of transformants was 0 % in all cases, the highest among the positive control plants (A+) inoculated with GV3101 (pKP 80) was 7.69 %, in the case of plants inoculated with a 10x bacterial concentration. The highest percentage of transgenic seeds in plants inoculated with the preconjugated culture was a 0.59 % (100-fold concentrated culture) and in non-preconjugated it was even higher, 10.58 % (10-fold concentrated culture).

Similarly, the seed number per plant and the number of vital seeds per plant was also highly variable, from no vital seeds per plant (positive control, 100x concentration), up to more than 1,200 seeds per plant. The percentage of germinating seeds was from 0 % to 100 % (Table 20).

Table 20: The summary of the results of the transformations. The columns represent the experiments with corresponding controls. The experiments are marked either as AE (Agrobacterium tumefaciens and Escherichia coli co-inoculation) or AEC (Agrobacterium tumefaciens and Escherichia coli co-inoculation with previous conjugation). 'A+' is a positive control, the Agrobacterium tumefaciens strain GV3101 (pKP 80). The negative controls are E. coli (E) or the Agrobacterium tumefaciens strain GV3101 (A-). The rows represent the number of seeds, the number of seeds per parental plant, the number of germinating seeds, the number of transgenic plants, the percentage of transgenic plants (calculated from the number of germinating seeds), the percentage of germinating seeds, and the number of parental plants.

Experiment	1st				2nd		3rd		4th		5th	
Generation of plants (date of planting)	7 October 2008				17 December 2008		6 January 2009		6 January 2009		12 January 2009	
Cultures used	AE	A+	E	A-	AEC	A+	AE	A+	AEC	A+	AE	A+
Number of parental plants	24	8	12	8	8	5	32	8	32	8	56	24
Total number of seeds	30,467	2,938	9,065	5,330	8,878	4,130	8,050	3,565	11,476	5,800	10,202	3,718
Total number of germinating seeds	5,900	2,938	9,065	5,330	8,822	3,980	2,960	1,936	4,023	2,397	8,871	3,231
Number of seeds per parental plant	1,269	367	755	666	1,110	826	252	446	359	725	182	155
Percentage of germinating seeds	19.37	100.00	100.00	100.00	99.37	96.37	36.77	54.31	35.06	41.33	87.00	87.00
Total number of transgenic plants	0	64	0	0	0	26	0	11	2	41	5	111
Percentage of transgenic plants	0.00	2.18	0.00	0.00	0.00	0.65	0.00	0.57	0.05	1.71	0.06	3.44

Continues

Continuation of Table 20

Experiment	6th		6th		6th		7th		7th		7th		8th		
Generation of plants (date of planting)	26 January 2009		26 January 2009		26 January 2009		26 January 2009		26 January 2009		26 January 2009		9 February 2009		
Cultures used	AE 1x	A+ 1x	AE 10x	A+ 10x	AE 100x	A+ 100x	AEC 1x	A+ 1x	AEC 10x	A+ 10x	AEC 100x	A+ 100x	AEC	E	A-
Number of parental plants	8	8	8	8	8	8	8	4	8	4	8	4	24	12	12
Total number of seeds	204	580	152	470	390	309	280	220	301	430	170	0	636	680	3,240
Total number of germinating seeds	104	84	104	26	177	69	280	0	301	0	170	0	636	680	2,916
Number of seeds per parental plant	26	73	19	59	49	38	35	55	38	107	21	0	27	57	270
Percentage of germinating seeds	50.10	14.48	68.42	5.53	57.09	22.33	100.00	0.00	100.00	0.00	100.00	0.00	100.00	100.00	90.00
Total number of transgenic plants	0	0	11	2	1	0	0	0	1	0	1	0	1	0	0
Percentage of transgenic plants	0.00	0.00	10.58	7.69	0.56	0.00	0.00	0.00	0.33	0.00	0.59	0.00	0.16	0.00	0.00

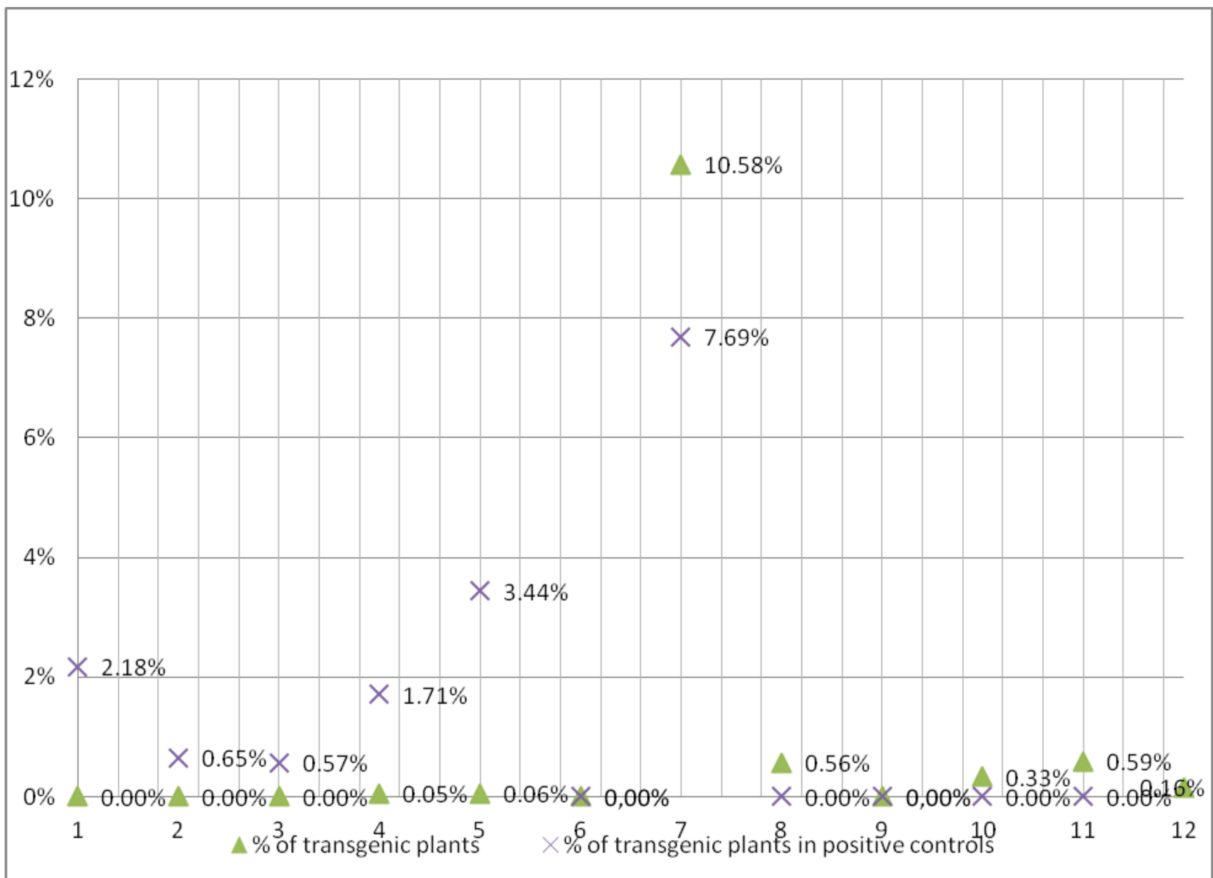

Figure 22: The average percentage of transgenic plants in the experiments and positive controls. The numbers indicate the generations of plants and the corresponding positive controls: 7 October 2008 (1), 17 December 2008 (2), 6 January 2009 AE (3), 6 January 2009 AEC (4), 12 January 2009 (5), 26 January 2009 AE 1x (6), 26 January 2009 AE 10 x (7), 26 January 2009 AE 100x (8), 26 January 2009 AEC 1x (9), 26 January 2009 AEC 10x (10), 26 January 2009 AEC 100x (11), and 9 February 2009 (12). In the last generation we used only two negative controls, as positive controls for selection plants from prevoius generations were used.

Slika 22: Povprečen odstotek transgenih rastlin v poskusih in pozitivnih kontrolah. Številke pomenijo generacijo inokuliranih rastlin in pripadajočih pozitivnih kontrol: 7. oktober 2008 (1), 17. december 2008 (2), 6. januar 2009 AE (3), 6. januar 2009 AEC (4), 12. januar 2009 (5), 26. januar 2009 AE 1x (6), 26. januar 2009 AE 10 x (7), 26. januar 2009 AE 100x (8), 26. januar 2009 AEC 1x (9), 26. januar 2009 AEC 10x (10), 26. januar 2009 AEC 100x (11) in 9. februar 2009 (12). V zadnji generaciji sta bili uporabljeni samo dve negativni kontroli.

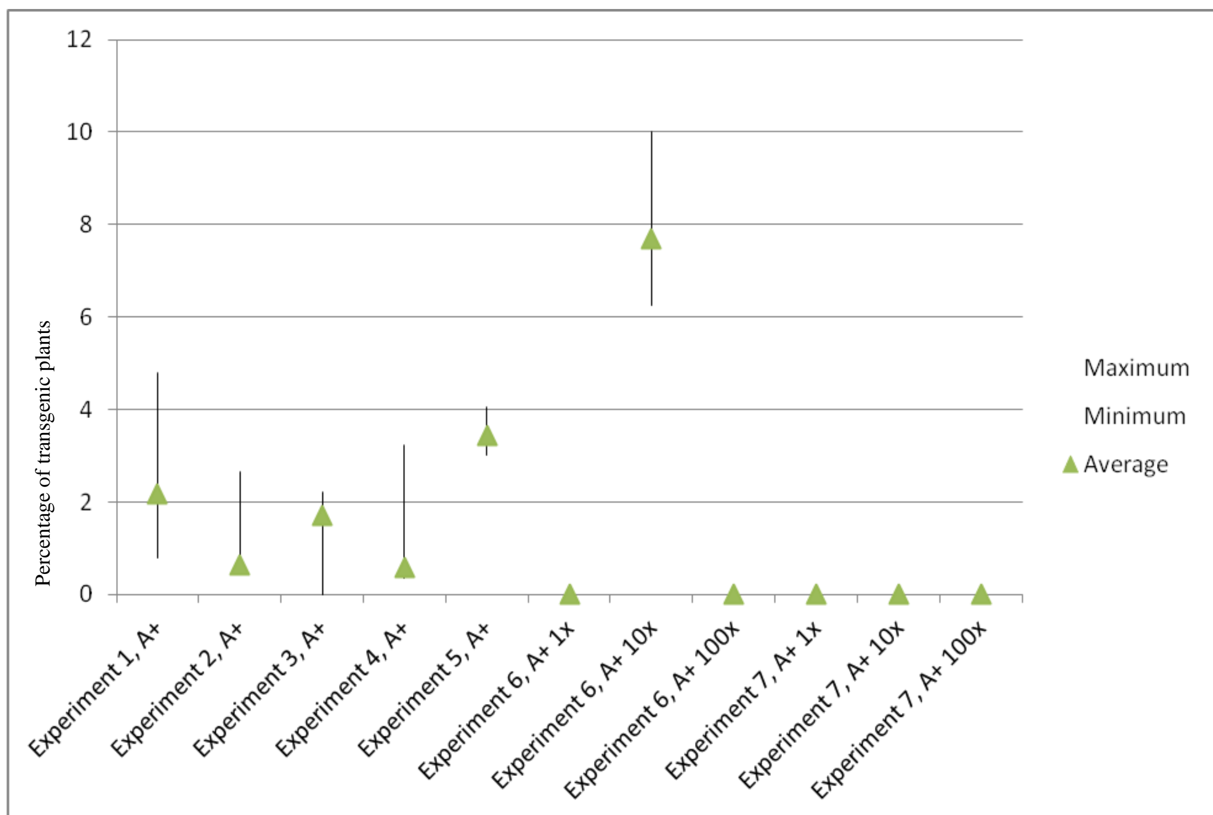

Figure 23: The maximum, minimum, and average percent of transgenic plants on selective plates in positive controls (A+).
Slika 23: Najvišji, najnižji in povprečen odstotek transgenih rastlin na selekcijskih ploščah pri pozitivnih kontrolah (A+).

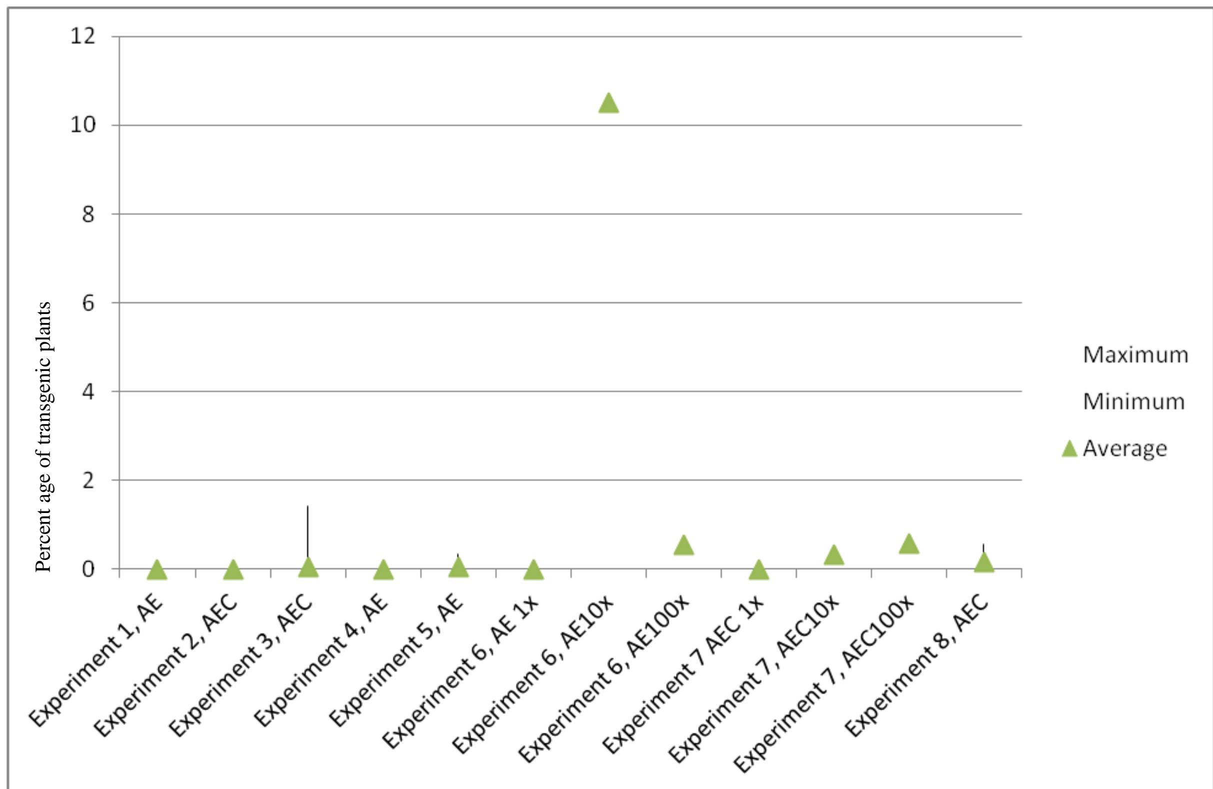

Figure 24: The maximum, minimum, and average percent of transgenic plants on selective plates in the experiments (AE and AEC).
Slika 24: Najvišji, najnižji in povprečen odstotek transgenih rastlin na selekcijskih ploščah pri poskusih (AE in AEC).

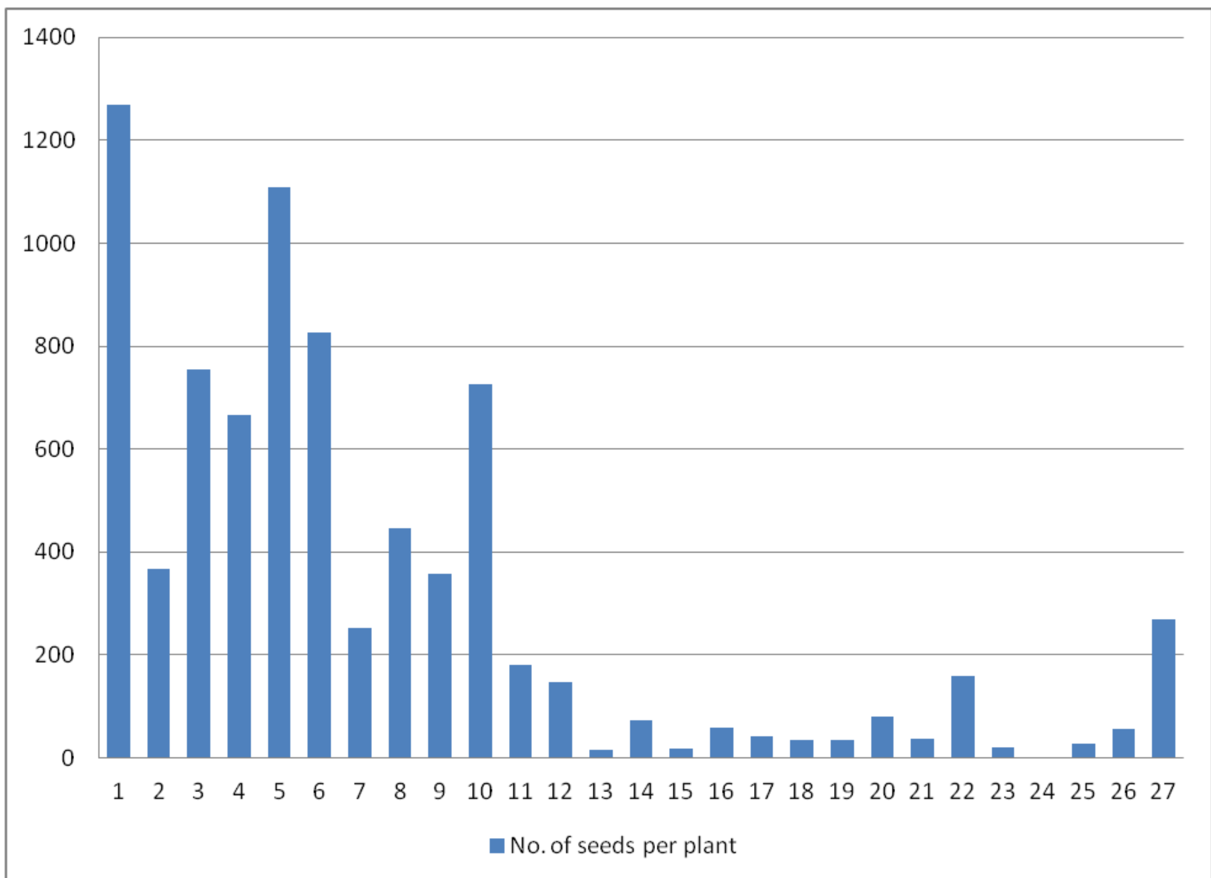

Figure 25: The number of seeds per plant in different generations. The columns represent generations as follows: 7 October 2008 AE (1), A+ (2), E (3), A- (4); 17 December 2008 AEC (5) A+ (6); 6 January 2009 AE (7), A+ (8), AEC (9), A+ (10); 12 January 2009 AE (11), A+ (12); 26 January 2009 AE 1x (13), A+ 1x (14), AE 10 x (15), A+ 10x (16), AE 100x (17), A+ 100 x (18), AEC 1x (19), A+ (20), AEC 10x (21), A+ 10x (22), AEC 100x (23), A+ 100x (24); 9 February 2009 AEC (25), E (26), and A- (27). The number of seeds in the first three generations (7 October 2008, 17 December 2008, and 6 January 2009) was within expectations, but later it dropped for unknown reasons.
Slika 25: Število semen na rastlino v različnih generacijah. Stolpci predstavljajo število semen v generacijah po zaporedju: 7. oktober 2008 AE (1), A+ (2), E (3), A- (4); 17. december 2008 AEC (5) A+ (6); 6. januar 2009 AE (7), A+ (8), AEC (9), A+ (10); 12. januar 2009 AE (11), A+ (12); 26. januar 2009 AE 1x (13), A+ 1x (14), AE 10 x (15), A+ 10x (16), AE 100x (17), A+ 100 x (18), AEC 1x (19), A+ (20), AEC 10x (21), A+ 10x (22), AEC 100x (23), A+ 100x (24); 9. februar 2009 AEC (25), E (26) in A- (27). Število semen v prvih treh generacijah 7. oktober 2008, 17. december 2008 in 6. januar 2009) je bilo v okviru pričakovanj, kasneje pa je število zaradi neznanega vzroka padlo.

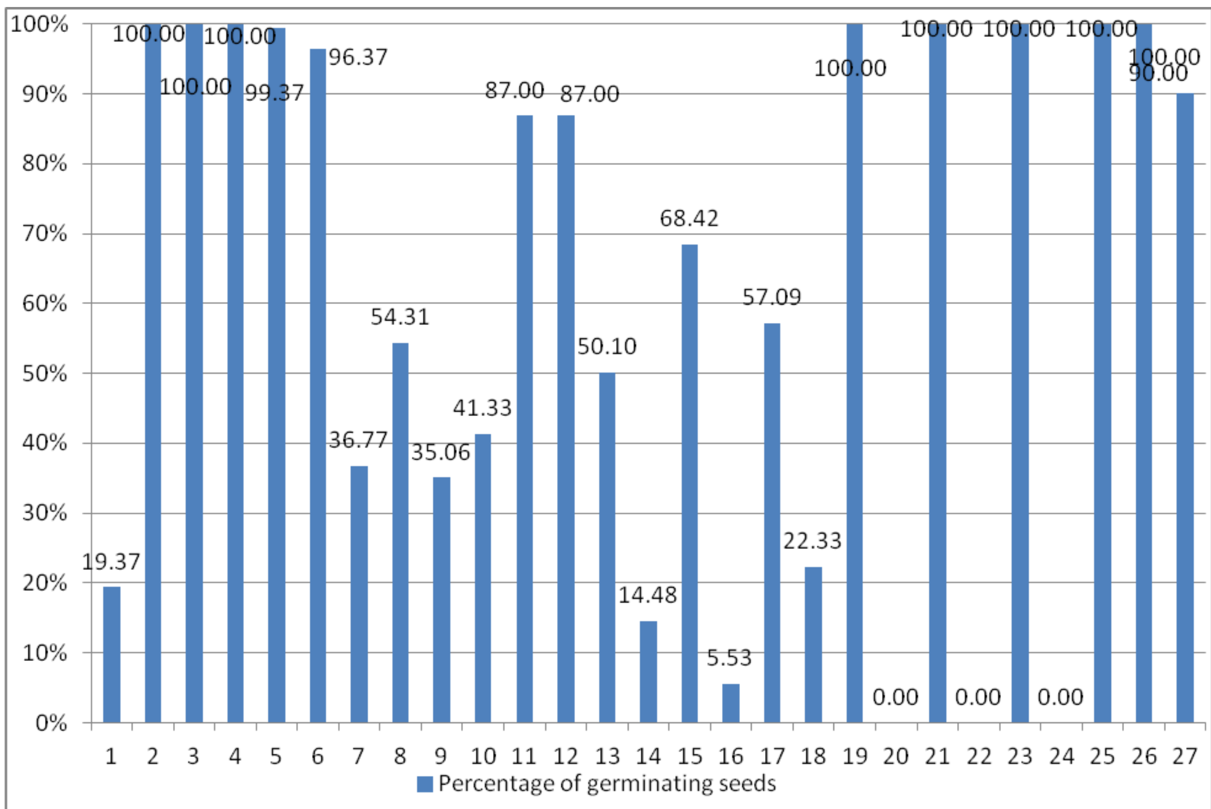

Figure 26: The percentage of germinating seeds. The percentage of germinating seeds ranges from 0 to 100 %. The columns represent generations as follows: 7 October 2008 AE (1), A+ (2), E (3), A- (4); 17 December 2008 AEC (5) A+ (6); 6 January 2009 AE (7), A+ (8), AEC (9), A+ (10); 12 January 2009 AE (11), A+ (12); 26 January 2009 AE 1x (13), A+ 1x (14), AE 10 x (15), A+ 10x (16), AE 100x (17), A+ 100 x (18), AEC 1x (19), A+ (20), AEC 10x (21), A+ 10x (22), AEC 100x (23), A+ 100x (24); 9 February 2009 AEC (25), E (26), and A- (27).

Slika 26: Odstotek kaljivih semen. Odstotek kaljivih semen varirira od 0 do 100 %. Stolpci predstavljajo število semen v generacijah po zaporedju: 7. oktober 2008 AE (1), A+ (2), E (3), A- (4); 17. december 2008 AEC (5) A+ (6); 6. januar 2009 AE (7), A+ (8), AEC (9), A+ (10); 12. januar 2009 AE (11), A+ (12); 26. januar 2009 AE 1x (13), A+ 1x (14), AE 10 x (15), A+ 10x (16), AE 100x (17), A+ 100 x (18), AEC 1x (19), A+ (20), AEC 10x (21), A+ 10x (22), AEC 100x (23), A+ 100x (24); 9. februar 2009 AEC (25), E (26) in A- (27).

5 DISCUSSION AND CONCLUSIONS

In this work, we attempted to obtain transgenic *A. thaliana* plants by co-inoculation with *E. coli* as a transgene donor and disarmed *A. tumefaciens* as intermediate strain, using alternations of the floral dip method. The floral dip is a simple, routinely used technique for obtaining transgenic plants, developed by Clough and Bent (1998), which gives an average of 1 % transformation efficiency (Clough and Bent, 1998; Zhang et al., 2006). Different techniques to increase the ratio of transformation have been attempted, but with no significant improvement (Logemann et al., 2006; Martinez-Trujillo et al., 2004; Zhang et al., 2006; Davis et al., 2009; Chung et al., 2000). Despite the low frequency of transformation, the average seed number per plant is usually high enough to get several transgenic seeds per inoculated plant.

Plant transformation by *E. coli – Agrobacterium* co-inoculation was first reported by Pappas and Winans (2003). In their work *N. tabacum* leaf explants were used as the transformation substrate; their simultaneous coinfection by the bacterial parents proved to be 30–40-fold less than the direct transformation from an *Agrobacterium* transgene carrying strain. When the bacterial parents where pre-mated prior to plant infection, the ratio rose to approximately threefold. The same principle was later adapted for *Arabidopsis* whole-plant flower-dip inoculation by Koumpena et al. (2008), aiming (i) to illustrate the success of this experimental set-up in an *in planta* application and (ii) to achieve better transformant yields, given that *Arabidopsis* generates substantial amounts of seeds (on average several hundred per plant) that permit detection of low transformant frequencies. Indeed, Koumpena and collaborators managed to reach a transformation ratio of about 0.002 %-0.004 % for direct co-infections which would rise 60-fold upon bacterial premating. Average transformability in those experiments with an infecting *Agrobacterium* was 2.56 %. Koumpena and collaborators also experimented with evaluating two different *Agrobacterium* transforming strains, with finding optimal donor-recipient ratios in *E. coli – Agrobacterium* filter matings that would precede the transformation step, and by assessing medium composition, temperatures at inter-bacterial conjugation and durations of matings (Koumpena et al., 2008; Koumpena et al., 2010).

Our intention in the present work was to further improve transformation efficiencies by employing all optimal conditions deriving from the previous work – i.e., use the best yielding *Agrobacterium* strain and mate it with *E. coli* donors at the most suitable temperature – and also to experiment with bacterial culture concentrations, and different modes of inoculation. The improvement of the transformation ratios would lead to better applicability of the method and its further development towards routine use for plant transformation.

5.1 THE PERCENTAGE OF TRANSGENIC SEEDS

The previous study by Koumpena et al. (2008) has shown that a 4–5-hour conjugation of *Agrobacterium* and *E. coli* strains at 28° C on a solid LB medium with a nitrocellulose filter can increase transformation efficiency up to several ten-folds. Plants co-inoculated with DH5α (pKP80)(pKM101) and *Agrobacterium* EHA101 yielded 0.001 % of transgenic

seeds, whereas plants inoculated with preconjugated cultures yielded 0.059 %. The results from our experiments suggest that the concentration of the bacterial culture is an equally important factor. We observed the increased transformation ratios at a 10x concentration in preconjugated and non-preconjugated cultures inoculated with drops (Figure 22). The beneficial effects of the concentrated cultures and drop-by-drop inoculation have been observed before by Martinez-Trujillo et al. (2004). They observed a transformation efficiency that was two times higher among plants inoculated with a 6x condensed culture, which had previously grown to the stationary stage of about OD_{600}=2.0. They also reported that the transformation ratio increased up to two times in plants inoculated by drops instead of submersion, although they performed four inoculations, first when the primary bolts were approximately 5 cm tall and then once every four days.

The higher transformation efficiency of 10x concentrated cultures may be due to the higher viscosity compared to the uncondensed culture. A more viscous medium persisted on the plant for a longer period and accelerated bacterial conjugation on the surface of the plant, which may explain the higher percentage of transformed plants in non-preconjugated cultures. Furthermore, in a more condensed culture the probability of bacteria entering floral buds is higher because more bacteria are available.

Drop-by-drop inoculation may also improve transformation efficiency. By applying the drops of the medium directly onto emerging buds as opposed to the whole plant submersion, we avoid the exposure of large parts of plant tissue to the toxic effects of surfactant and the pathogenic effects of *Agrobacterium*.

Compared to the 10x concentration, the 100x concentration did not prove to be very useful despite a relatively high percentage of transgenic plants (0.56 % for plants inoculated with a non-preconjugated and 0.59 % for preconjugated). In both cases, the condensed inoculum caused the deformation of the plants and consequently stunted growth as described later in the discussion.

5.2 SEED NUMBER PER PLANT

There was a huge variability in seed number per plant among the controls, the experiments and the experiments with preconjugated bacteria. The lowest number of seeds per plant was in the case of plants inoculated with a 100x concentration of the control strain *A. tumefaciens* GV3101 (pKP80), which produced not a single seed from four inoculated plants, and the highest was more than 1,200 (Table 20, Figure 25). A single *Arabidopsis* plant normally produces several hundred to several thousand seeds (Zhang et al., 2006). As we can see in Figure 25, the seed number per plant is not correlated with the mode of inoculation, nor with the concentration of bacteria, but with the generation of the plants. The average number of seeds per plant in the first three generations (7 October 2008, 17 December 2008 and 6 January 2009) is more than 600, but drops to an average of less than 100 in later generations. The seed reduction was observed in the control plants as well as in the experimental plants.

In their protocol, Zhang et al. (2006) suggest low light intensity and too much water as likely causes for a case when plants produce flowers but no seeds. The environmental

factors in the growing chamber (temperature 21° C, relative humidity 60-70 %, 16 hours of light and 8 hours of dark), the light intensity and the watering protocol were constant throughout all the generations so we believe they are not the reason for the reduction of the seed number in later generations. We also did not observe any reduction in the number of flowers in the 12 January 2009 and later generations.

The first generation (7 October 2009) was planted in sterilized Florrela green soil with 10 % (v/v) of perlite. The 17 December 2008 and all later generations were planted in a new soil, Potground P Klasmann. The 12 January 2009 and later generations were planted in Potground P Klasmann sterilized soil because we observed some insect larvae in pots of other plants in the growing chamber. Sterilization destroys the natural flora of the soil as well as the pest larvae, and may allow pathogenic species to overproliferate (Cooley et al., 2003), which could influence the plant performance and reduce the number of seeds. On the other hand, in the first generation (7 October 2009), which was also planted in sterilized soil, the number of seeds per plant was the highest in all generations.

Clough and Bent (1998) and Zhang et al. (2006) all emphasize the importance of healthy plants used for floral dip. One of the possible causes for the low number of seeds could be a latent infection with phytopathogenic fungi or viruses. The environment in the growing chamber – high humidity and warmth – is ideal for the development of fungi which could affect the plants' overall fitness and seed production.

The percentage of germinating seeds was also highly variable, from 0 % to 100 % (Table 20, Figure 26). The selection of transgenic seeds was performed in two parts for the first four experiments and we observed a drop in the percentage of vital seeds in the second part (Figures 15, 16, 17, and 18 and Tables 7, 9, 11, and 12). The reason for this is probably in the sampling procedure. The bag with the dry seeds was inverted so the seeds slipped out in to the beaker, where imbibition took place. The seeds in the bag were mixed with chaff and dry plant tissue. Heavy, vital seeds were at the bottom of the bag and slipped out first, whereas lighter and less vital seeds or empty shells were trapped in the chaff and were used for the second part of the selection.

There is also a big difference between the selective plates in the number of seeds and the germination percentage. The seeds in the water were spread on the plates with a pipette as described in Materials and methods. Excessive water with floating seeds was then poured or pipetted onto the next selective plate. Again, heavy vital seeds thus remained on the first plate, and lighter seeds and empty shells were transmitted onto the new plate.

The low percentage of germinating seeds was in some cases the consequence of fungal infections on the selective plates (Table 7) and cannot be attributed to a specific inoculation technique, concentration of bacteria, or other experimental variable. Even the inoculation with highly concentrated bacterial cultures (100x and 10x) did not affect the percentage of germinating seeds, in spite of a great effect on the number of seeds per plant.

5.3 SEED DISINFECTION

Two techniques are commonly used for *Arabidopsis* seeds sterilization: rinsing and vapour-phase sterilization. Rinsing includes alcohol treatment, bleach (sodium hypochlorite) with surfractants, and final rinsing with sterile water, whereas in vapour-phase sterilization chlorine fumes act as the disinfecting agent. Which technique is more efficient is a matter of dispute, because no study has been made considering the efficacy of *Arabidopsis* seed disinfection.

In their original paper, Clough and Bent (1998) first treated the seeds with a 95 % ethanol for 30-60 seconds, then immersed them in 50 % bleach (2.625 % sodium hypochlorite) containing 0.05 % Tween 20 for 5 min, and rinsed them three times with sterile water. Zhang et al. (2006) and Harrison et al. (2006) used a lower-percentage ethanol (70 %), Desfeux et al. (2000) used isopropanol instead, and Chung et al. (2000) completely omitted the use of alcohol. In their protocols, Zhang et al. (2006) as well as Harrison et al. (2006) used a prolonged exposure of seeds to sodium hypochlorite solution: 10 minutes instead of Clough and Bent's (1998) original 5 minutes.

Another seed sterilization technique is the vapour-phase sterilization. Clough and Bent (1998) placed seeds in a desiccator jar and just prior to sealing the jar, they put in a beaker with bleach to which a small amount of HCl was added to produce chlorine fumes. The seeds were left in the jar for 4–15 hours. The whole process was carried out under a fume hood because of the toxic effects of chlorine gas. The same method was used by Ye et al. (1999) and Desfeux et al. (2000).

The specific mode of action of alcohols as disinfectants is not known in details, but researchers think that they cause membrane damage by dissolving lipids, protein denaturation, and subsequent obstruction of cell metabolism (McDonnell and Russell, 1999). The 70 % ethanol is known to be more effective than 95 % or higher, probably because it passes the bacterial membrane more efficiently and denaturises not only the membrane proteins, but also the cytoplasmatic proteins (McDonnell and Russell, 1999). The isopropyl alcohol (isopropanol) is considered slightly more efficient against bacteria than ethyl alcohol (ethanol), probably because of its greater lipophilic properties (McDonnell and Russell, 1999). The higher alcohols are more germicidal than ethyl alcohol: their effectiveness is in correlation with their molecular weight. Since alcohols of molecular weight higher than propyl alcohol are not soluble in water in all proportions (Pelczar et al., 1986), they are not very widely used.

The surfractants like Tween 20 or Tween 80 are nonionic detergents which reduce surface tension and ease the way for the disinfectants to penetrate the membranes and increase their efficiency (Helenius et al., 1979).

Sodium hypochlorite and chlorine fumes belong to the class of halogen-releasing disinfectants. They are highly active oxidising agents and they destroy the protein structure, form chlorinated derivatives of nucleotide bases, and disrupt oxidative phosphorylation (McDonnell and Russell, 1999). Depending on the concentration of chlorine and the pH these substances are sporocidal as well (McDonnell and Russell,

1999). The antimicrobial effect of chlorine is based on the formation of hypochlorous acid in water. The hypochlorous acid is then decomposed and releases oxygen, which is a strong oxidising agent and it destroys the cellular constituents of bacteria. The disinfecting effects are also the consequence of the binding of chlorine to the proteins in the cell membrane and to the enzymes (Pelczar et al., 1986).

As mentioned, no data exists on the efficiency of *Arabidopsis* seed sterilization or on possible detrimental effects of disinfecting agents on seed vigour. Extensive research on seed sterilisation and disinfecting has been made on cereals, pulses, and other agriculturally important plants (Cantore et al., 2009; Van Der Wolf et al., 2008; Piernas and Guiraud, 1997), which may be used as a guide for research on *Arabidopsis* seeds.

We used the rinsing method of disinfection for our experiments because of its safety and simplicity in comparison to the vapour-phase sterilisation. Vapour-phase sterilisation is potentially hazardous because of the formation of the highly toxic chlorine gas and there is no indication that it is more effective than rinsing.
Despite the sterilisation of seeds, some bacteria may survive on or in the seed, so 200 μl/ml of the antibiotic cefotaxime was added to the selective medium to prevent bacterial growth on the selective plates. Cefotaxime has a relatively low toxicity for plants and is widely used to prevent bacterial growth in plant tissue cultures (Okkels and Pedersen, 1987; Danilova and Dolgikh, 2004). Instead of cefotaxime some authors use carbenicilin (Zhang et al., 2006).

5.4 SEED SPREADING ON THE SELECTIVE PLATES

The seeds were released from siliques and spread with a pipette on selective plates as was described in the Materials and methods section. Despite the intensive sieving some chaff remained mixed in with the seeds and caused some problems with the spreading of seeds as it got stuck in the tip of the pipette. The plant chaff which was spread on the plates with the seeds could be another possible source of fungal contamination as the phytopathogenic fungi embedded in the plant's tissue could survive the sterilisation process.

The spreading with a pipette has another disadvantage: the number of seeds sucked up into a 1 ml pipette tip was very variable and it was difficult to prepare selective plates with approximately equal number of seeds. The number of seeds per selective plate varied from some 60 to more than 6,000 and at higher densities the estimation of the seed number was difficult.

As explained in section 5.3 (Percentage of the germinating seeds), the pipetting technique also had an effect on the percentage of the germinating seeds on the plate.

5.5 CONTAMINATIONS ON THE SELECTIVE PLATES

Fungal growth on the selective plates is most probably the consequence of poor seed disinfection. Despite thorough disinfection of seeds some bacteria and fungi survive in the seed and cause contamination of the seedlings. The selection of transformants was carried

out in the growth chamber, which ensures appropriate environmental conditions (temperature 21° C, relative humidity 60-70 %, 16 hours of light and 8 hours of darkness) not only for *Arabidopsis* seedlings, but also for different species of microorganisms. The selective medium is rich in sugars and the selective plates are sealed with Parafilm, which ensures high humidity, prevents the plate from drying, and retains a sterile environment.

The seed disinfection is crucial for a successful selection, but the standard disinfection procedure with ethanol and bleach may be harmful to the seeds if they are exposed for too long. As explained later in section 6.1, Method improvements, some alternative procedures exist, although they are not optimised for *Arabidopsis* seed disinfection.

5.6 EFFECT OF HIGHLY CONCENTRATED BACTERIAL CULTURES

Highly concentrated bacterial cultures had negative effects on plant performance and reproductivity. The plants inoculated with a 100x concentrated bacterial culture, whether it was preconjugated or not, were smaller and had shrunken and deformed leaves, flowers, and siliques. The same effect was observed in the plants inoculated with a 100x concentration of the positive control GV3101 (pKP80). The 100x concentrated bacterial culture was like a dense paste and was applied with a pipette directly into the inflorescences, although it later slipped down the stem to the leaves. No similar effect was observed at lower concentrations, e.g. 10x or lower.

Stunted growth could be attributed to the high concentration of bacteria which lead to the obstruction of the plants' stomata. The condensed culture later dried up and formed a thin film on the surface of the plants. The dense bacterial paste obstructed gas exchange through the stomata, covered parts of leaf surfaces, and prevented a normal photosynthesis. Furthermore, *Agrobacterium* itself is a plant pathogen and is able to enter plant cells and cause damage. The *E. coli* bacteria has also been proven to be able to enter plant tissues and form colonies (Solomon et al., 2002; Seo and Frank, 1999; Takeuchi and Frank, 2000; Cooley et al., 2003), which could further enhance the pathological effects of *Agrobacterium* infection and decreased photosynthesis.

These plants also had an unusually low average number of seeds per plant (Table 20, Figure 25), although we cannot directly link the low seed number with the high concentration of bacteria. The whole of the 26 January 2009 generation, as well as the 9 February 2009 generation, had a very small amount of seeds per plant. As discussed in the previous section, the exact reason for the low seed number is unknown.

5.7 MOSAICISM

The mosaic plants had some green and some pale leaves or green spots on the pale leaves; their growth was slower and had a different appearance than true transformants (Figures 17 and 18). Mosaicism of T_1 plants was observed in several cases; especially surprising were the mosaics from the plants inoculated with *E. coli* and *A. tumefaciens* GV3101 as negative controls. The significance of these plants is not known and further analysis of the plant

genome should be performed with molecular methods to detect if the marker gene has integrated.

In our last experiment, where we used both bacterial cultures grown to OD_{600}=0.8, we got some positive results in the plants inoculated with the negative control, the *E. coli* strain DH5α (pKP80)(pKM101). Out of 680 plants, 7 plants (1.03 %) were mosaics. These plants were not as green as the true transformants obtained in positive controls, but they were not pale enough to be considered as clearly non-transgenic.

In a previous study by Koumpena et al. (2008), similar results were obtained: 4 out of 2,160 plants or 0.19 % were mosaics. They were contributed to the cross-contamination of plants in the growing chamber, which would be possible by insects as vectors, or by touching between individual plants. Pappas and Winans (2003) also detected some slow-growing calli from tobacco leaf explants inoculated with *E. coli* DH5α carrying the IncP-type system, but all of these calli failed to grow when subcultured to new selective plates. The significance of mosaic plants from the study of Koumpena et al. (2008) and the slow-growing calli from the experiments by Pappas and Winans (2003) is not known as no further research has been done yet. Mosaic plants were also recorded in another negative control, plants inoculated with *A. tumefaciens* strain GV3101 in experiment 8. Out of 2916 plantlets, 11 were mosaics (0.38 %).

The significance and exact cause of mosaicism is not known yet, although there are some suggestions as to what might be the reason. One of the possible causes of mosaicism of *E. coli* inoculated plants might be the infection of plant embryos with *E. coli* expressing the *ntp*II gene. The bacteria could survive on the T_0 plant, infected by the developing seed and embryo of the T_1 plant. Bacterial colonies embedded in the plant tissue could result in mosaicism of the plantlets. There are reports that *E. coli* is able to survive on a plant and infect the developing seeds. Cooley et al. (2003) performed experiments with *E. coli* on *Arabidopsis* and their results show that the inoculation of *Arabidopsis* roots with the *E. coli* strain O157:H7 leads to the contamination of the entire plant. Even if they infected plant seedlings they were then able to recover bacteria from seeds and siliques of dried mature plants some 60 days after germination.

Mosaicism was also observed among the plants inoculated with the positive control GV3101 (pKP80). The reason for mosaicism may be an infection of the plant embryo in the stage of a small group of cells after fertilization. *Agrobacteria* could infect and transform some of the cells instead of transforming an ovule, which would lead to chimeric mosaic plants. As was demonstrated by several researches, the primary target of the *Agrobacterium* in the floral dip technique are the plant's female reproductive tissues (Desfeux et al., 2000; Ye et al., 1999), and the progeny is mostly hemizygous with T-DNA inserted at one allele on a given locus (Feldmann et al., 1991; Bechtold, 1993). Desfeux et al. (2000) carried out experiments with a mutant *Arabidopsis* variety in which carpel fusion is incomplete at the apex and the gynoecium remains open rather than becoming enclosed as in wild-type plants. With this mutant line, the transformation ratios were 6 times higher than in wild-type plants. This means that for a successful transformation, the *Agrobacterium* must have an open access to ovules. It is well known that flower initiation in *Arabidopsis* occurs continuously in an indeterminate spiral at each floral apex. With the

floral dip technique, we thus inoculate flowers in different developmental stages. Since the gynoecium is open for a relatively short interval of approximately 3.5 days and it closes before the stigma becomes receptive for the pollen and before the anthers develop (Smyth et al., 1990), the infection with *Agrobacterium* must occur before pollination. The *Agrobacteria* may remain in the ovary until pollination and until an embryo develops, and then they infect the cells of the developing embryo. Ye et al. (1999), who also reported several cases of mosaicism in transgenic seeds obtained by vacuum infiltration, suggests a similar explanation.

We also observed several cases of mosaicism in the plants inoculated with a preconjugated and non-preconjugated mixture of *Agrobacterium* GV3101 and *E. coli* DH5α (pKP80)(pKM101): in a spray experiment and a positive control, in plants inoculated with 10x concentrated preconjugated bacteria, and in plants inoculated with both cultures grown to OD=0.8. In the case of the 10x concentrated and preconjugated inoculum, the number of mosaic plants was much higher than the number of clearly transgenic plants – 21 mosaic plants and only one that was completely green. In all these cases, the cause was probably the combination of an infection with *E. coli* and a post-fertilization infection with *Agrobacterium*.

5.8 THE EFFECT OF THE SURFRACTANT CONCENTRATION

In the experiment on the sixth generation of plants (9 February 2009), we used a higher concentration of the surfractant Silwet® L-77 (0.05 %) than in other experiments (0.02 %), and we observed some negative effects on the plants. After the first inoculation, the plants were more bent than in the other experiments and after the second and third inoculations we observed drying shoots, which we attributed to the higher concentration of the surfractant and repeated exposure. We did not observe any damage on plant tissues or any other negative effects on plants or their growth which could be attributed to Silwet® L-77 in experiments where 0.02 % concentration was used.

Surfractants reduce the surface tension of liquids and may damage the plant's cuticle and epidermis. Clough and Bent (1998), who used 0.05 % Silwet® L-77 in their experiments, also observed drying shoots and recommended a very careful use of the surfractant. Moreover, Desfeux et al. (2000) reported the deformation of pistils and flower mortality at an even lower concentration, 0.03 %. Despite the toxicity of Silwet® L-77, we cannot completely omit its use or significantly lower its dose, since it has been proven that the levels between 0.02 % and 1.0 % give about 20 times greater rates of transformation than at 0 % or 0.005 % (Clough and Bent, 1998).

5.9 METHOD IMPROVEMENTS AND FURTHER RESEARCH

5.9.1 Method improvements

5.9.1.1 Seed disinfection and contamination prevention

One of the major problems of the floral dip technique is still the prevention of contamination of the T_1 seeds on the selective plates. The selective medium is rich in sugars and offers ideal conditions for fungal growth.

Until now, no research has been done on *Arabidopsis* seeds disinfection, but there are many reports on disinfection of commercial plant seeds (Miché and Balandreau, 2001; Caetano-Anollés et al., 1990; Cantore et al., 2009; Van Der Wolf et al., 2008; Piernas and Guiraud, 1997), which can be used as guidance for research on *Arabidopsis* seeds. The potential disinfecting agents for seed disinfection are phenol and phenolic compounds, higher alcohols, chlorine and iodine compounds, quaternary ammonium compounds, aldehydes, ethylene oxide (Pelczar et al., 1986), essential oils (Cantore et al., 2009; Van Der Wolf et al., 2008), organic acids and plant extracts (Van Der Wolf et al., 2008), commercial fungicides, and physical agents such as heat (Narichika and Yoshihiko, 2002) and gamma irradiation (Cuero et al., 1986; Maity et al., 2009). The latter is inappropriate for genetic studies since gamma irradiation causes DNA damage (Sellins and Cohen, 1987). There are some unpublished reports on microwave sterilisation of *Arabidopsis* seeds, which is claimed to be extremely effective (over 99 %) (Franco, 1993, personal information). Care should be taken when applying this technique since the microwaves can cause damage to DNA (Sagripanti et al., 1987; Sagripanti and Swicord, 1986; Lai, 1996; Banik et al., 2003) and microwave treatment may produce false results.

Another interesting approach to contamination prevention is selection under non-aseptic conditions. Davis et al. (2009) used chromatography quartz and chromatography silicon dioxide sands saturated with MS basal salts medium without sucrose placed on petri dishes. Davis et al. (2009) also tried to select transformed seedlings on soil but were unsuccessful. Conversely, in 2011, Das and Joshi successfully performed *Arabidopsis* seedling selection in agropeat-filled soil which was pretreated with an antibiotic solution, without any previous seed sterilization. Soil is a complex mixture of organic and inorganic components and its composition varies greatly even within the same batch. The development and standardisation of the selective procedure in the soil is thus almost impossible; for a successful selection a more defined medium should be used.

The selection can be further improved by disinfection of smaller batches. The seeds should be carefully selected, separated from chaff, counted, and then disinfected in a small test tube. This approach is more labour-intensive, but it would reduce the possibility of contamination originating from chaff and the remains of the inoculated plant tissue. This approach would also significantly improve seed distribution on the selective plates as the number of seeds would be more even. That would also ease the determination of transgenic plants. Zhang et al. (2006) recommend resuspension of the sterilised seeds in 0.05 % agarose (40 µL of seeds per ml of agarose) to reach a more uniform spread on the plate.

5.9.1.2 Plant growth

The plants of the maternal generation were grown as was described in the Materials and methods section. In their protocol, Zhang et al. (2006) recommend a different approach which allows uniform and maximal seed germination. Seeds are either suspended in 0.05 % (w/v) sterile agarose or sterilised and placed on a solid MS medium and left at 4° C for 3 days. Agarose-stratified seeds are then spread onto wet soil, 20–30 seeds in a 10 x 10 cm pot, while seeds on the MS medium are placed in the growing chamber (16 hours light/8 hours dark, 20° C) and left for 2 weeks. After 2 weeks the plantlets are transferred to the soil, with 12 seedlings per pot. The soil in the pots should be covered with a nylon mesh, which prevents the soil from dropping into the inoculation medium during the inoculation, which in turn prevents the contamination of the medium and consequently the inflorescences with the soil bacteria.

Considering the inoculation procedure, Zhang et al. (2006) recommend re-dipping after a 7-day interval, compared to Clough and Bent (1998), who recommend an interval of 5–6 days. The plants are completely wrapped with stretch foil after the dip and left for 16–24 hours. In our protocol, we merely covered the plants with plastic bags and left them covered for 24 hours.

5.9.1.3 The inoculation procedure

An interesting simplification of the classic floral dip was introduced by Davis et al. (2009). Instead of centrifuging down the bacterial culture and resuspending it in the infiltration medium, plants are dipped directly into the initial culture with a surfractant which is added just before the dip. This procedure is not suitable for co-inoculation because of the different sensitivity of bacteria to the selective markers presented in the media, but it is very versatile for the standard inoculation with *A. tumefaciens*. This protocol shortens and simplifies the dipping procedure and reduces the cost because there is no need to prepare the infiltration medium and centrifuge the growth medium.

5.9.2 Further research

5.9.2.1 Determination of the integration of the transgene

The growth of dozens of transgenic plants obtained in this work, by either direct agrobacterial-mediated transfer or biparental transfer, was monitored at the hosting laboratory in subsequent subculturings on selective plates. Well-grown transgenic plants were finally transferred to soil pots. The plants were let to mature and grow siliques, which were collected for future use. Additionally, plant tissues from the same plants were cut off and put to deepfreeze, in order to be analyzed by molecular techniques (Southern hybridisation and PCR) in the near future. This will prove (i) the presence and stable integration of the transgene and (ii) its copy-number in each of the plants' genomes.

5.9.2.2 Inoculation methods

Several different inoculation techniques and concentrations were tested in this work; further employment of the most yielding conditions that were found, alone or in combinations, could predictably increase transgenic frequencies even more. For instance, the spray method in combination with the preconjugated *E. coli* and *A. tumefaciens* and the combination of *E. coli* and *A. tumefaciens* at regular, 10-fold or even 100-fold concentrated cultures, should absolutely be tested, provided that culture viscosity does not obstruct the nozzle of the spray can. Additionally, the mere use of 10- and 100-fold concentrated cultures ought to be tested again by either dipping or drop-by-drop inoculation in order to verify their high capacity to yield increased transgenic numbers. Particularly for 100x cultures, the repetition of the experiment is needed in order to ascertain if such an increased bacterial concentration indeed imposes a negative effect in plant growth or impaired seed development.

6 CONCLUSION

Plant transformation by co-inoculation is a novel method for obtaining genetically modified plants. It simplifies cloning and strain construction as it circumvents the need to construct *Agrobacterium* strains carrying binary vectors. Instead of *Agrobacterium*, *E. coli* cells are the source of the transgene-carrying plasmid.

Several improvements were introduced in the standard floral dip procedure, which was used as a positive control in our experiments, and in the co-inoculation procedure. Compared to the standard inoculation with *A. tumefaciens*, concentrated cultures, repetitive inoculation, drop-by-drop inoculation, and the improved floral spray protocol were introduced. Concentrated cultures were used in concentrations of 10- and 100-fold, and an unconcentrated culture was used as a standard. The cultures in this experiment were applied with a pipette directly onto the buds and into the rosette instead of being used for plant submersion. Split inoculation was used in the first experiment – instead of the second dip after 5 or 6 days, we performed two re-dips, one 3 days after the first, and the another one 4 days after the second dip. Clough and Bent (1998) reported detrimental effects on plants dipped at intervals of every four or less days over a period of two weeks and lower transformation efficiency (0.5 % or lower). We observed no such effects and a transformation efficiency of 2.18 %, which is comparable to the standard floral dip at an interval of 5 or 6 days (Clough and Bent, 1998). The floral spray technique, first reported by Chung et al. in 2000, was also improved. Chung and co-workers sprayed the plants from the distance of 20–30 cm three times at interval of 8 hours and reached a transformation efficiency of an average 2.41 %. We sprayed the plants from a distance of 4-5 cm twice at an interval of 5 days. This approach proved to be effective and yielded 3.44 % of transgenics.

Improvements were also introduced in the co-inoculation technique: the best-yielding *A. tumefaciens* strain (GV3101) was employed and left to mate with the *E. coli* donor strain *in planta* or prior to plant infection, at conditions optimal for this particular set of parents. Additionally, concentrated cultures, split inoculation, spraying, a changed donor-recipient ratio, and a higher Silwet® L-77 content were applied, as before. Finally, a donor-recipient ratio shift was attempted in one experiment, where both cultures contributed to the transformation at almost equal cell ratios. In all experiments conducted in this work, a novel rapid method for seed identification was followed (Harrison et al., 2006). This method shortens the time needed for identification of transgenic plantlets from 7 to 10 days to only 4 days.

Overall, inoculation by spraying and raising the bacterial cell concentrations 10x to 100x proved to be the most promising introduction in our technique development for both *At*mt infections and co-infections. The high culture concentration, in combination with drop-by-drop inoculation that protects large areas of the plant tissue from the surfractant, seems to be the most favourable for co-inoculation with *Agrobacterium* and *E. coli*. Particularly in the last case, the previously recorded rarely occurring transgenesis instances were now raised to that of lower levels of regular *At*mt infections, thereby testifying to the applicability of this novel method.

7 SUMMARY (POVZETEK)

7.1 SUMMARY

Arabidopsis thaliana is a widely used model plant in genetic research and is routinely transformed by using *Agrobacterium*-mediated transformation methods. It belongs to the mustard or crucifer family (Brassicaceae) which comprises many economically important species. *Agrobacterium tumefaciens* is a soil phytopatogenic bacterium which causes crown gall disease in dicot plants by transfer and integration of a segment (T-DNA) of its tumour inducing (Ti) plasmid into the host's genome, resulting in the formation of tumours. The removal of T-DNA genes from the plasmid prevents tumour formation but it does not affect the ability of DNA transfer into the host – these plasmids are termed 'disarmed'. Another crucial component for a successful *Agrobacterium*-mediated gene transfer are the *vir* genes which can reside on separate plasmids called binary Ti vector systems. The desired genes are located on T-DNA, provided on a small shuttle vector that can replicate in both *E. coli* and *A. tumefaciens*. The shuttle vector must carry the selectable markers appropriate for selection in *E. coli* and *A. tumefaciens*.

Agrobacterium-mediated transformation methods involve vector and transgene construction steps in *Escherichia coli* and later the introduction of the desired constructs into *A. tumefaciens* for further transfer to the plant. To avoid this step, a novel method was introduced by Pappas and Winans (2003): *Nicotiana tabacum* leaf discs were transformed using a conjugative *E. coli* strain as the T-DNA and transgene carrying host and a disarmed *A. tumefaciens* strain as an *in situ* conjugal mediator for the transfer of the transgene to the plant. Later, this technique was adapted for the transformation of *Arabidopsis thaliana* using the floral dip method: an optimal media composition, strain combination, mating duration, and temperature; and *E. coli* : *A. tumefaciens* ratios were tested (Koumpena et al., 2008). In the present study, different inoculation techniques, concentrations of bacterial cultures, and preconjugated and non-preconjugated cultures were tested in order to increase transformation efficiency and with that the possible applications of the *E. coli* – *A. tumefaciens* co-inoculation principle.

The *Arabidopsis thaliana* plants, sown in the soil, were inoculated approximately 30 days after planting and reinoculated one or two times after several days with *A. tumefaciens* GV3101 and *E. coli* DH5α (pKM101)(pKP80) or with *A. tumefaciens* GV3101 (pKP80) as a positive control. *A. tumefaciens* and *E. coli* cultures were harvested at appropriate OD_{600} and then either left in the incubator for conjugation or resuspended in the inoculation medium and used for the inoculation of the plants. Different inoculation techniques (floral dip, spray, or drop-by-drop inoculation), inoculum concentrations (1x, 10x, 100x), repetitions (one or two reinoculations), surfactant concentration (0.02 or 0.05 %), and OD_{600}s of the bacterial cultures at the harvest were tested. Control plants were inoculated with *A. tumefaciens* GV3101 (pKP80) in the same manner. Seeds from the transformed plants were collected some 7-8 weeks after inoculation and screened for transgenics on the selective plates with kanamycin as a selective marker.

The concentration of bacterial cultures in association with drop-by-drop inoculation proved to be most effective way to reach high percentages of transgenic plants. The highest percentage of transgenic seeds was 10.58, yielded by plants inoculated with non-preconjugated 10-fold concentrated inoculum. The same concentration of preconjugated cultures yielded 0.33 % transgenic seeds. The concentrated inoculum proved to be efficient also in plants inoculated with the preconjugated culture as plants inoculated with 100-fold concentrated inoculum yielded 0.59 % of transgenics, which was the highest percentage among the preconjugated cultures. The 100-fold concentrated non-preconjugated inoculum yielded 0.56 % of transgenics. The highest percentage in positive control plants was 7.69 % in plants inoculated with a 10-fold concentrated culture. The use of a very dense 100-fold concentrated inoculum had detrimental effects on plant health: plants were smaller and had shrunken and deformed leaves, which was not observed in the 10-fold or unconcentrated inoculum. Unconcentrated non-preconjugated co-inoculated plants yielded transgenic seeds only in the spray experiment, where transformation efficiency was 0.06 %. The corresponding positive control in this experiment yielded 3.44 % of transgenics. Out of four experiments (2nd, 4th, 7th – with a 1x concentration, and 8th) where plants were inoculated with preconjugated unconcentrated cultures two yielded transgenic seeds. The percentage of transgenics was 0.05 in the 4th experiment and 0.16 in the 8th experiment. In the 8th experiment both cultures were grown to the OD_{600} 0.8. The plants inoculated with *A. tumefaciens* GV3101 (pKP80) as a positive control in many cases yielded no transformants (26 January 2009 generation both 1x and 100x concentrated controls, and 10x concentrated control for AEC experiment). Otherwise the percentage of transgenic positive control plants inoculated by dipping was from 0.57 to 2.18.

The percentage of germinating seeds and the number of seeds per plant were highly variable. The average number of seeds per plant dropped after the 3rd generation for unknown reasons. The percentage of germinating seeds was ranging from 0 to 100, with large differences also between the selective plates. In some cases the low germination ratio can be attributed to fungal infections of the selective plates.

Among the clearly transgenic plants some mosaic plants were observed in several cases. Mosaic plants were greener than clearly non-transgenic plants, and persisted longer on the selective plates. In comparison with the clearly transgenic plants they grew slower, were not well developed, and not uniformly green. Mosaics appeared among co-inoculated plants and in positive and negative controls, and their exact cause is not yet known.

The *Agrobacterium* - *E. coli* co-inoculation technique proved to be efficient in generating genetically modified plants. *E. coli* cells are the source of the transgene-carrying plasmid, which is especially convenient when large plasmids which are unstable in *Agrobacterium* strains are used. The technique simplifies cloning and strain construction as it circumvents the need to construct *Agrobacterium* strains carrying binary vectors.

7.2 POVZETEK

Navadni repnjakovec (*Arabidopsis thaliana*) je zelnata trajnica iz družine križnic (Brassicaceae), v katero je uvrščenih mnogo pomembnih kmetijskih rastlin. *A. thaliana* je zaradi majhnega genoma in kratkega juvenilnega obdobja pomembna modelna rastlina na področju genetike in biotehnologije, prav tako pa jo je mogoče preprosto transformirati s pomočjo bakterije *Agrobacterium tumefaciens*.

Agrobacterium tumefaciens je po Gramu-negativna talna bakterija, ki povzroča karakteristična tumorska tkiva pri dvokaličnicah. Bakterija vstavi del svojega genoma t.i T-DNA, ki je del Ti plazmida (tumour inducing), v genom gostiteljske rastline. Transformirane celice postanejo maligne in tvorijo tumorje, iz katerih se sproščajo opini, ki služijo kot hrana bakterijam (Tzfira in Citovsky, 2008).

Escherichia coli je eden najbolje proučenih organizmov, saj jo je preprosto gojiti in manipulirati ter je na voljo v mnogih različnih sevih. *E. coli* je ena izmed mnogih vrst bakterij, ki so sposobne konjugacije in s tem prenosa genetskega materiala v druge bakterijske vrste (Sussman, 1997).

Gensko spremenjene rastline lahko pridobimo na dva načina: s transformacijo z *Agrobacterium tumefaciens* in z metodami neposrednega vnosa genov (biolistika in elektroporacija). Prednost metode vnosa genov z *A. tumefaciens* je, da lahko transformiramo tudi cele rastline in ne zgolj izoliranih rastlinskih tkiv kot pri metodah neposrednega vnosa genov. Transformacija rastlin z *A. tumefaciens* zahteva vstavitev želenega gena na plazmidu v *E. coli* in v naslednji fazi prenos plazmida v *A. tumefaciens*, ki je sposobna gen prenesti v rastlinski genom. Ena izmed težav te tehnike je tudi nestabilnost velikih genskih fragmentov v *A. tumefaciens* (Shibata in Liu, 2000).

Gojenje rastlinskih tkiv in celic v *in vitro* pogojih povzroča somaklonsko variabilnost, poleg tega pa se *in vitro* kulture pogosto okužijo z glivami in plesnimi, ki jih uničijo. Mnogo raziskovalcev se je zato trudilo najti način, kako se izogniti gojenju rastlin in rastlinskih tkiv v tkivnih kulturah. Feldmann in Marks (1987) sta uspešno transformirala kaleča semena navadnega repnjakovca, kasneje pa so Bechtold in sod. (1993) uvedli transformacijo navadnega repnjakovca z vakuumsko infiltracijo. Vakuumsko metodo sta še poenostavila Clough in Bent (1998), ki sta nadzemne dele cvetoče rastline navadnega repnjakovca preprosto potopila v suspenzijo celic agrobakterije. Ta preprosta metoda je znana pod imenom "floral dip" in se sedaj rutinsko uporablja za transformacijo navadnega repnjakovca. Poleg izjemne preprostosti je prednost metode tudi, da ne povzroča zaznavnih sprememb, ki bi bile posledica somaklonske variabilnosti (Labra in sod., 2004).

Pappas in Winans (2003) sta razvila novo metodo za transformacijo navadnega tobaka (*Nicotiana tabacum*). Listne izsečke tobaka (*N. tabacum*), ki je poleg repnjakovca še ena pomembna modelna rastlina, sta transformirala s pomočjo *E. coli*, ki je predstavljala gostitelja T-DNA in transgena, ter razoroženege seva *A. tumefaciens*, ki je bil *in situ* konjugacijski partner in mediator transfera genov v rastlinsko tkivo. Prednost in novost te metode je, da se izognemo kloniranju in konstrukciji sevov *A. tumefaciens*, kar je posebej

priročno, ko želimo uporabiti velike fragmente DNA, ki so v *A. tumefaciens* pogosto nestabilni. Pappas in Winans (2003) sta dokazala, da je s to metodo možno v *in vitro* pogojih, ki so prilagojeni rastlinskemu tkivu in so precej drugačni od naravnega okolja *E. coli*, učinkovito transformirati listne izsečke tobaka.

Koumpena in sod. (2008) so metodo kasneje prilagodili za transformacijo navadnega repnjakovca z metodo floral dip in dokazali, da lahko *E. coli* in *A. tumefaciens* uporabimo tudi za *in planta* transformacijo celih rastlin. Učinkovitost takšne transformacije je bila sicer precej nizka, zato smo jo v pričujočem delu poskušali izboljšati z različnimi modifikacijami metode.

Uporabili smo seve bakterij, ki so se v poskusu Koumpene in sodelavcev (2008) izkazali za najbolj učinkovite, ter testirali vpliv predhodne konjugacije bakterij na trdnih gojiščih, koncentracijo bakterij, način inokulacije, ter koncentracije detergenta na učinkovitost transformacije. Predvidevali smo, da bi ti faktorji lahko pozitivno vplivali na učinkovitost transformacije.

Materiali in metode

Semena navadnega repnjakovca (*Arabidopsis thaliana*) ekotip Columbia 1 smo čez noč vernalizirali v vodi v hladilniku na temperaturi 4° C. Naslednji dan smo jih posejali v lončke ter pokrili s prozornim plastičnim pokrovom, ki je vzdrževal primerno zračno vlago, ter jih postavili v rastno komoro (21° C, relativna vlažnost 60-70 %, 16 ur svetlobe in 8 ur teme). Po 5-7 dneh, ko so rastlinice dosegle stadij dveh listov, smo po štiri rastline presadili v nove lončke, v katerih so rasle do konca poskusa.

Prvo inokulacijo smo izvedli ob starosti približno 30 dni, ko so imele rastline primarne poganjke višine 2-10 cm in nekaj odprtih cvetov. Druga inokulacija je bila v večini primerov izvedena po petih ali šestih dneh, le v prvem in osmem poskusu smo izvedli tri inokulacije. Pri prvem poskusu je bila druga inokulacija po treh dneh in tretja štiri dni za prvo, pri osmem poskusu pa sta si inokulaciji sledili v zamiku petih dni.

Bakterije smo gojili v tekočem LB gojišču z ustreznimi selekcijskimi antibiotiki pod naslednjimi pogoji: *A. tumefaciens* na temperaturi 27° C v stresalnem inkubatorju (180 obratov na minuto) in *E. coli* na 37° C v navadnem inkubatorju. Nekaj ur preden je kultura *A. tumefaciens* dosegla željeno gostoto, smo *E. coli* kulturo prestavili v stresalni inkubator, da smo dosegli sinhronizacijo in sta bili obe kulturi ob inokulaciji primerne gostote.

Uporabili smo *E. coli* sev DH5α z *Inc*N-type konjugacijskim sistemom (plazmid pKM101) in plazmidom pKP80, ki nosi *npt*II transgene za resistenco na kanamicin in *Inc*N *ori*T regijo, ki z *Inc*N *tra* sistemom omogoča mobilnost (Pappas in Winans, 2003). *A. tumefaciens* seva, uporabljena v poskusih, sta bila GV3101 in GV3101 (pKP80). *E. coli* kulturo smo uporabili, ko je dosegla OD_{600} 0.5–0.8, *A. tumefaciens* pa pri 0.8–1.2. for *A. tumefaciens*, saj je to po ugotovitvi Koumpene in sod. (2008) najprimernejše razmerje za konjugacijo. Kulture smo centrifugirali in raztopili v infiltracijskem mediju (IM), ki smo mu dodali detergent. Pri poskusih s predhodno konjugacijo bakterij smo po centrifugiranju kulturi nanesli na trdno gojišče s filtrom in jih za 4 do 5 ur pustili v inkubatorju na

temperaturi 28° C, ter jih nato raztopili v infiltracijskem mediju. V nekaterih poskusih smo medij koncentrirali 10 ali 100 krat.

Rastline smo potopili v IM za dve minuti ali pa nekajkrat popršili z razdalje nekaj cm, oziroma smo IM nanesli s kapalko neposredno na popke in v rozeto. Po inokulaciji smo rastline za 24 ur pokrili s prozorno plastično vrečko, ki je zagotavljala primerno vlažnost. Druga ali tretja inokulacija rastlin je potekala po enakem postopku.

Kot pozitivno kontrolo (oznaka A+) smo uporabili *A. tumefaciens* sev GV3101 (pKP80), kot negativni pa *A. tumefaciens* GV3101 (oznaka A-) in *E. coli* DH5α (pKM 101)(pKP 80) (oznaka E). Sočasna inokulacija z *A. tumefaciens* GV3101 in *E. coli* DH5α (pKM 101)(pKP 80) je označena z AE, sočasna inokulacija s predhodno konjugacijo pa z AEC.

Semena inokuliranih rastlin smo pobrali približno dva meseca po inokulaciji, ko so bili luski popolnoma suhi. Rastline smo v starosti 7 do 8 tednov ovili v papirnate vrečke, v katere so padala zrela semena in odpadli luski. Semena smo presejali in jih sterilizirali ter jih s pipeto nanesli na selekcijsko gojišče s kanamicinom kot selekcijskim markerjem. Selekcija je potekala po skrajšanem selekcijskem protokolu (Harrison in sod., 2006).

Število semen lahko ocenimo s tehtanjem na podlagi enačbe 1250 semen = 25 mg. Navkljub skrbnemu čiščenju je med semeni ostalo precej plev in ostankov rastlinskega tkiva, kar bi lahko vplivalo na rezultat zatehte, zato smo se odločili, da število semen določimo s štetjem. Pod selekcijske plošče z nanesenimi semeni smo vstavili papir z mrežo kvadratov s stranicama 1x1 cm, ki je olajšala štetje.

Selekcijo smo izvedli po skrajšanem selekcijskem protokolu (Harrison in sod., 2006), ki traja le štiri dni. Po preteku štirih dni smo vse rastlinice, ki so bile bolj zelene od povprečja, prenesli na nove selekcijske plošče in jih pustili rasti še nekaj dni v rastni komori, da smo dobili potrditev njihove transgenosti. Selekcijo smo običajno opravili v dveh delih, da smo se izognili izgubi celotne količine semen v primeru okužbe selekcijskih plošč s plesnijo.

Rezultati

Prvi poskus 7. oktober 2008

Rastline, uporabljene v prvem poskusu, smo inokulirali po enaki metodi kot Koumpena in sod. (2008), ki temelji na standardni metodi, ki sta jo razvila Clough in Bent (1998), in je prilagojena za sočasno inokulacijo z *A. tumefaciens* in *E. coli*. Standardni postopek je bil uporabljen kot kontrola (A+, A- in E) za sočasno inokulacijo (AE). Razvojni stadij rastline je izjemno pomemben za uspešno transformacijo rastline in ker se ne pojavijo vsi cvetovi hkrati, je treba postopek ponoviti. Clough in Bent (1998) priporočata ponovitev po 5 ali 6 dneh, v našem poskusu pa smo prvo reinokulacijo izvedli po treh dneh in drugo štiri dni kasneje. Ker je pretirano izpostavljanje kulturi *A. tumefaciens* za rastline lahko škodljivo (Clough in Bent, 1998), smo se odločili, da drugo inokulacijo izvedemo samo s kapljicami medija na popke in v rozeto rastline, prva in tretja inokulacija pa sta bili izvedeni klasično, s potapljanjem rastlin v medij.

Postopek se je izkazal za učinkovitega pri pozitivni kontroli (A+), kjer smo dobili 2,18 odstotka transgenih rastlin, oziroma 64 transgenih rastlin od skoraj 3000 semen. Pri sočasno inokuliranih rastlinah nismo dobili pozitivnih rezultatov, vendar moramo pri tem upoštevati, da je velik delež semen (80 %) iz teh rastlin propadel zaradi plesni na gojišču. Pričakovano pri rastlinah, inokuliranih z negativnima kontrolama (A- in E), nismo dobili nobenega transgenega semena.

Drugi poskus 17. december 2008

V drugem poskusu smo kulturi *A. tumefaciens* GV310 in *E. coli* DH5α (pKM 101) (pKP 80) predhodno konjugirali, kot je opisano v Materialih in metodah in rastline inokulirali s potopitvijo v IM za dve minuti. Rastline, inokulirane s pozitivno kontrolo (A+), so imele 0,65 % transgenih semen, pri sočasno inokuliranih rastlinah pa nismo zaznali nobenega transgenega semena.

Tretji in četrti poskus 6. januar 2009

Tretji in četrti poskus smo izvedli na rastlinah, posejanih 6 januarja. Zaradi precejšnjih izgub semen prvega poskusa, negativnega rezultata drugega poskusa in nizkega odstotka transformiranih semen pri pozitivni kontroli smo se odločili, da enaka postopka izvedemo še enkrat. Priprava kultur, konjugacija in potapljanje rastlin je bilo izvedeno kot v prvih dveh poskusih, le da je bila reinokulacija v obeh primerih samo ena, in sicer pet dni po prvi inokulaciji.

Med semeni rastlin, inokuliranih s konjugiranima kulturama, sta bili dve transgeni, kar predstavlja 0,05 %, pri kontrolnih rastlinah pa je bil odstotek transgenih 1,72. Rastline, ki so bile inokulirane z nekonjugiranima kulturama, niso imele transgenih semen, pri kontrolnih rastlinah pa je bil odstotek transgenih 0,57.

Peti poskus 12. januar 2009

Chung in sod. (2000) in Ye (2008, neobjavljeno) so poročali o precej visokih odstotkih transgenih rastlin navadnega repnjakovca, pridobljenih s škropljenjem, zato smo se odločili, da preizkusimo, ali je metoda primerna tudi za sočasno inokulacijo. Bakterijske kulture smo pripravili kot v prejšnjih poskusih, za inokulacijo pa smo uporabili pršilko za rože, ter rastline popršili z razdalje nekaj centimetrov. Med sočasno inokuliranimi rastlinami smo dobili 0,06 % transgenih semen, med kontrolnimi rastlinami pa 3,44 % ter nekaj mozaičnih rastlin.

Šesti in sedmi poskus 26. januar 2009

Gostota bakterijske kulture in način inokulacije sta pomembna faktorja, ki lahko vplivata na učinkovitost transformacije (Martinez-Trujillo in sod., 2004; Logemann in sod., 2006). V teh poskusih smo uporabili konjugirane in nekonjugirane kulture koncentracij 1x, 10x in 100x, ter kapljično inokulacijo s pipeto. Kulture smo gojili in konjugirali kot v prejšnjih

poskusih, po centrifugaciji pa smo jih raztopili v manjši količini IM, da smo dobili koncentrirane raztopine, ter rastline inokulirali s pipeto.

Med rastlinami, ki smo jih inokulirali z nekonjugirano kulturo so bili rezultati sledeči: pri inokulaciji z nekoncentrirano kulturo nismo dobili nobenega transgenega semena, pri 10x koncentrirani kulturi pa je bil odstotek kar 10,58, kar je tudi najvišji odstotek v vseh poskusih. Pri 100x koncentraciji je bil odstotek transgenih rastlin 0,56. Pri kontrolnih rastlinah smo transgene rastline opazili le v primeru inokulacije z 10x koncentrirano kulturo (7,69 %), v ostalih primerih smo opazili le mozaične rastline.

Med rastlinami, ki so bile inokulirane s konjugiranima kulturama, smo pri 10x koncentraciji dobili 0,33 % transgenih semen ter 21 mozaičnih, pri 100x koncentraciji pa 0,59 %. V primeru uporabe nekoncetriranih konjugiranih kultur ter pri vseh kontrolnih rastlinah nismo dobili nobenega transgenega semena, le nekaj mozaičnih.

Osmi poskus 9. februar 2009

V tem poskusu smo uporabili obe kulturi ob dosegu optične gostote OD_{600} 0.8 ter povišano koncentracijo detergenta. Uporabili smo prekonjugirani kulturi (AEC) ter dve negativni kontroli (A- ter E), pozitivne kontrole (A+) pa zaradi tehničnih razlogov nismo uporabili.

Rezultati Koumpene (Koumpena, 2010) kažejo, da je najboljše razmerje med *E. coli* in *A. tumefaciens* približno 1:5, kar je pri OD_{600} 0.5 do 0.8 za *E. coli* ter OD_{600} 0.8 do 1.2 za *A. tumefaciens.* V tem poskusu smo razmerje spremenili v korist *E. coli* na približno 1:1,4 in sicer da bi ugotovili, ali je lahko tako razmerje ugodnejše za konjugacijo.

Koncentracija detergenta je prav tako pomemben faktor, saj koncentracija med 0,05 in 0,1 % tudi do dvajsetkrat poveča uspešnost transformacije (Clough in Bent, 1998). Previsoka koncentracija lahko poškoduje rastline (Clough in Bent, 1998; Martinez-Trujillo in sod., 2004; Chung in sod., 2000; Logemann in sod., 2006; Bartholmes in sod., 2008; Curtis in Nam, 2001; Davis in sod., 2009; Zhang in sod., 2006). Nobeden od avtorjev ni poročal, da bi koncentracija 0,05 % povzročala poškodbe na rastlinah in zato smo se odločili, da preizkusimo, ali pozitivno vpliva na uspešnost transformacije.

Inokulacija je potekala s potapljanjem rastlin kot v drugih poskusih, vendar v dveh ponovitvah po petih in desetih dneh. Takoj po inokulaciji smo opazili, da so rastline povešene, po nekaj dneh pa smo opazili sušeče poganjke in liste. Oboje smo pripisali povišani koncentraciji in večkratni izpostavljenosti detergentu.

Rastline, ki smo jih inokulirali s konjugiranima kulturama, so imele eno transgeno seme (0,16 %), ter eno mozaično rastlino. Presenetljivo, 7 mozaičnih rastlin smo zaznali tudi med rastlinami, inokuliranimi z negativno kontrolo DH5α (pKP80)(pKM101) (E), ter med rastlinami, inokuliranimi z GV3101 (A-) negativno kontrolo, kjer smo jih zaznali 11.

Razprava in sklepi

Namen te raziskave je bil povečati učinkovitost transformacije navadnega repnjakovca z metodo potapljanja cvetov s sočasno inokulacijo z razoroženim sevom *A. tumefaciens* in *E. coli* kot donorjem transgena.

O možnosti transformacije rastlin s sočasno inokulacijo z *A. tumefaciens* in *E. coli* sta prva poročala Pappas in Winans (2003), ki sta uporabila listne izsečke tobaka. Učinkovitost takšne transformacije je bila 30 do 40 krat nižja od učinkovitosti klasične transformacije z *A. tumefaciens*, vendar sta jo s predhodno konjugacijo bakterij izboljšala za približno trikrat. Isti princip so kasneje uporabili Koumpena in sod. (2008) in dokazali, da je to metodo možno prilagoditi za tehniko potapljanja cvetov in z njo transformirati cele rastline. Preizkusili so različna seva *A. tumefaciens* in našli optimalno razmerje med *E. coli* in *A. tumefaciens* ter najprimernejše pogoje za konjugacijo bakterij in sestavo medija ter pridobili 0.002 %-0.004 % transgenih rastlin (Koumpena in sod., 2008; Koumpena 2010). V naši raziskavi smo poskušali najti primerno koncentracijo inokulacijskega medija ter najprimernejši način inokulacije, ki bi odstotek transgenih rastlin povišali.

Odstotek transgenih rastlin

Koumpena in sod. (2008) so dokazali, da 4 do 5 urna konjugacija *E. coli* in *A. tumefaciens* na trdnem LB gojišču z nitroceluloznim filtrom poviša uspešnost transformacije za nekaj desetkrat. Rezultati naše raziskave kažejo, da sta koncentracija inokuluma ter inokulacija s kapljicami prav tako ključna za uspešno transformacijo. Pri rastlinah, inokuliranih z 10x koncentriranim inokulumom, smo namreč opazili višje odstotke transformiranih semen ne glede na to ali sta bili kulturi prej konjugirani ali ne.

Povišano učinkovitost transformacije z 10x koncentrirano kulturo morda lahko pripišemo večji viskoznosti. Bolj viskozna kultura se na rastlini zadrži dlje časa in pospeši konjugacijo bakterij na površini rastline. To morda lahko pojasni višji odstotek transgenih rastlin ob uporabi nekonjugiranih kultur v primerjavi s konjugiranimi. Pri uporabi bolj koncentriranih kultur je tudi možnost okužbe popkov z bakterijami večja, saj je bakterij preprosto več kot pri manjši koncentraciji.

Ugodne učinke kapljične inokulacije na transformacijo so opazili že Martinez-Trujillo in sod. (2004). S kapljično inokulacijo se izognemo izpostavljanju cele rastline toksičnim učinkom detergenta ter patogenim učinkom agrobakterij.

V primerjavi z 10x koncentracijo se 100x koncentracija navkljub relativno visokemu odstotku transgenih rastlin (0.56 % pri rastlinah, inokuliranih z nekonjugiranima in 0.59 % pri rastlinah, inokuliranih s konjugiranima kulturama) ni izkazala za zelo učinkovito. V obeh primerih je namreč močno koncentriran inokulum povzročil deformacijo listov, poganjkov in luskov ter zakrnelo rast. Enake simptome smo opazili tudi pri rastlinah, inokuliranih s 100x koncentrirano pozitivno kontrolo GV3101 (pKP80).

Močno koncentrirana kultura bakterij je bila zelo viskozna in gosta in je prekrila rastlinsko tkivo ter ovirala izmenjavo plinov ter fotosintezo. Inokulum se je kasneje na rastlini posušil

in tvoril tanek film. *A. tumefaciens* rastlinski patogen, ki škoduje rastlinam, pa tudi za *E. coli* bakterije je dokazano, da lahko vstopijo v rastlinsko tkivo in tvorijo kolonije (Solomon in sod., 2002; Seo in Frank, 1999; Takeuchi in Frank, 2000; Cooley in sod., 2003), kar bi lahko še pospešilo škodljive vplive agrobakterije.

Te rastline so imele tudi nenavadno nizko število semen na rastlino, čeprav števila semen ne moremo neposredno povezati s koncentracijo inokuluma. Celotna generacija rastlin, posejanih 26. januarja 2009, je imela nenavadno nizko število semen. Število semen na rastlino je bilo sicer zelo variabilno, vendar kaže, da ni povezano z načinom inokulacije, koncentracijo ali katero od drugih spremenljivk, temveč od generacije rastlin. Povprečno število semen je namreč iz generacije v generacijo vztrajno padalo pri vseh rastlinah, ne glede na to ali so bile inokulirane z konjugiranima ali nekonjugiranima kulturama ali s kontrolnim sevom. V prvih treh generacijah je povprečna količina semen nad 600 na rastlino, v kasnejših generacijah pa pade na manj kot 100. Zhang in sod. (2006) omenja, da je slaba osvetljenost in presežek vode lahko vzrok, da rastline sicer cvetijo, vendar nimajo semen. Razmere v rastni komori in režim zalivanja so bili sicer med vsemi poskusi enaki in jih nismo spreminjali, zato verjetno niso vzrok za padec števila semen. Clough in Bent (1998) ter Zhang in sod. (2006) poudarjajo pomen zdravstvenega stanja rastlin na uspešnost transformacije. Eden od možnih vzrokov za nizko število semen bi lahko bila latentna infekcija rastlin z glivami ali virusna okužba.

Odstotek kaljivih semen je bil prav tako izjemno variabilen. V primerih, ko smo selekcijo opravili v dveh delih, je bil odstotek kaljivih semen v drugem delu selekcije navadno precej nižji kot v prvem delu. Vzrok je verjetno v postopku: suha semena smo stresli v vodo za vernalizacijo. Pri tem so težja, vitalna semena iz vrečke spolzela prej, prazne lupine in lažja, slabše kaljiva semena pa so večinoma ostala ujeta v plevah in smo jih uporabili v drugem delu selekcije.

Precejšnje razlike v odstotku kaljivih semen so bile tudi med posameznimi selekcijskimi ploščami. Vernalizirana semena smo s pipeto nanesli na selekcijsko gojišče ter odpipetirali odvečno vodo z plavajočimi semeni. Težja, kaljiva semena so ostala na selekcijski plošči, lažja in manj vitalna pa so plavala v tekočini in smo jih prenesli na novo ploščo.

V nekaterih primerih je bil nizek odstotek kaljivosti posledica okužbe selekcijskih plošč s plesnimi. Odstotka kaljivih semen ne moremo povezati s specifično koncentracijo, načinom inokulacije ali katerokoli drugo spemenljivko. Razvoj plesni na gojišču je najverjetneje povezan z nepopolno dezinfekcijo semen. Za razkuževanje semen se uporabljajo etanol, belilo ter detergenti, ki pa lahko poškodujejo semena, če je izpostavljenost predolga. Navkljub pazljivi dezinfekciji spore gliv in plesni preživijo v semenu in med selekcijo okužijo rastlinice.

Zaenkrat ni nobenih poročil o učinkovitosti različnih metod dezinfekcije semen navadnega repnjakovca, obstaja pa precej raziskav dezinfekcije semen kmetijsko pomembnih rastlin (Miché in Balandreau, 2001; Caetano-Anollés in sod., 1990; Cantore in sod., 2009; Van Der Wolf in sod., 2008; Piernas in Guiraud, 1997), ki bi lahko služile kot ideje za raziskave na repnjakovcu. Poleg različnih kemičnih sredstev kot so fenol in fenolne spojine, višji alkoholi, klorove in jodove spojine (Pelczar in sod., 1986), so potencialno uporabne tudi

fizikalne metode, kot so gama žarki (Cuero in sod., 1986; Maity in sod., 2009) in mikrovalovi (Franco, 1993, osebna informacija). Tretma s fizikalnimi agensi lahko povzroči poškodbe DNA (Sagripanti in sod., 1987; Sagripanti in Swicord, 1986; Lai, 1996; Banik in sod., 2003) in zato je vprašljiva njegova primernost za genetske študije.

Drug pristop k preprečevanju okužb na gojišču je selekcija nerazkuženih semen v nesterilnih pogojih na kromatografskem pesku (Davis in sod., 2009) ali celo v zemlji s selekcijskim markerjem (Davis in sod., 2009; Das in Joshi, 2011), vendar taki pristopi še niso standardizirani in tudi ne vedno uspešni.

Mozaične rastline

Mozaične rastline so imele nekaj zelenih in nekaj svetlih listov ali pa zelene pege na drugače svetlih listih. Rasle so počasneje kot transgene rastline in so se razlikovale od njih kot tudi od netransgenih rastlin. Mozaične rastline so se pojavile v kar nekaj primerih, posebej presenetljiv je bil pojav mozaikov med rastlinami, inokuliranimi z negativnimi kontrolami *E. coli* in *A. tumefaciens* GV3101.

V študiji Koumpena in sod. (2008) so prav tako zaznali mozaike in jih pripisali kontaminaciji v rastni komori. Pappas in Winans (2003) sta pri svojih poskusih med listnimi izsečki, inokuliranimi z *E. coli* DH5α z IncP-type sistemom, zaznala nekaj počasi rastočih kalusov, ki pa so propadli po prenosu na sveže selekcijsko gojišče. Pomen teh rastlin in kalusov še ni znan, zato so potrebne nadaljnje raziskave.

Natančen vzrok za nastanek mozaičnih rastlin ni znan, možnih razlag je kar nekaj. Eden možnih vzrokov je okužba zarodka rastline z *E. coli*, ki izraža *ntp*II gen. Bakterije bi lahko preživele na T_0 rastlini in okužile semena ter T_1 rastlino. Obstajajo namreč poročila, da lahko *E. coli* preživi na repnjakovcu in okuži nastajoča semena (Cooley in sod., 2003).

Mozaične rastline smo opazili tudi med rastlinami, inokuliranimi z GV3101 (pKP80) pozitivno kontrolo. Vzrok bi bil lahko okužba rastlinskega embrija v stadiju nekaj celic. Primarna tarča okužbe z agrobakterijo pri tehniki potapljanja cvetov je sicer ovarij matične rastline (Desfeux in sod., 2000; Ye in sod., 1999) in potomci so večinoma hemizigotni s T-DNA na enem lokusu (Feldmann in sod., 1991; Bechtold, 1993). Agrobakterije bi lahko preživele v ovariju in kasneje okužile že nastajajoči embrio, kar bi povzročilo nastanek mozaičnih rastlin. Ye in sod. (1999), ki je prav tako poročal o mozaičnih rastlinah po vakuumski infiltraciji, je ponudil podobno razlago za njihov nastanek. Mozaiki so se pojavili tudi pri sočasno inokuliranih rastlinah, kjer bi vzrok lahko bil kombinacija zgoraj naštetih razlogov.

Sklepi

V standardno in v sočasno inokulacijsko metodo genskega spreminjanja navadnega repnjakovca s potapljanjem cvetov smo uvedli nekaj izboljšav. Preizkusili smo inokulacijo s koncentriranimi kulturami, večkratno ponovitev inokulacije, inokulacijo s kapljicami, ter inokulacijo s škropljenjem. Pri poskusu s koncentriranimi kulturami smo uporabili 10 in 100-krat koncentrirane kulture, ter nekoncentrirane kulture, ki so bile uporabljene kot

standard. Zaradi viskoznosti koncentriranih kultur smo rastline inokulirali po kapljicah s pipeto neposredno v rozeto in na popke. Večkratno ponovitev inokulacije smo uporabili v prvem poskusu. Namesto reinokulacije po 5 ali 6 dneh, smo rastline ponovno inokulirali po 3 dneh ter še enkrat čez 4 dni. Clough in Bent (1998) sta sicer poročala o škodljivih učinkih prepogoste izpostavljenosti rastlin kulturi agrobakterij in nižjim odstotkom transgenih rastlin, pridobljenih na ta način. V našem poskusu teh negativnih učinkov nismo zaznali, učinkovitost transformacije pri kontrolnih rastlinah pa je bila 2,18 %, kar je primerljivo z rezultati standardne metode. Izboljšali smo tudi metodo inokulacije s škropljenjem cvetov, o kateri so prvi poročali Chung in sod. (2000). Chung in sod. (2000) so cvetove repnjakovca poškropili trikrat z razdalje 20 do 30 cm v intervalu 8 ur ter dosegli 2,41 % transgenih rastlin. V našem poskusu smo rastline poškropili dvakrat v intervalu petih dni z razdalje 4-5 cm neposredno v rozeto rastline. Metoda se je izkazala za učinkovito, saj smo pri pozitivni kontroli dosegli 3,44 % transgenih rastlin. Izboljšave smo uvedli tudi v metodo sočasne inokulacije. Uporabili smo sev *A. tumefaciens* (GV3101), ki se je izkazal za najbolj primernega za konjugacijo z *E. coli*, ter ju konjugirali v optimalnih pogojih (Koumpena in sod., 2008; Koumpena, 2010). Kot pri kontrolnih rastlinah smo tudi pri sočasni inokulaciji preizkusili različne koncentracije, ponovitev inokulacije, škropljene, ter dodatno še spremenjeno razmerje med donorji ter recipienti. Za identifikacijo transgenih rastlin smo v vseh primerih uporabili kratki selekcijski protokol, ki omogoča identifikacijo v štirih dneh (Harrison in sod., 2006). Inokulacije rastlin s koncentriranimi kulturami ter inokulacija s škropljenjem so se izkazali za najbolj obetajoče pristope za sočasno inokulacijo in klasično inokulacijo. Visoka koncentracija kultur v kombinaciji s kapljično inokulacijo prepreči stik večine površine rastline z agrobakterijo in detergentom, s čimer se izognemo toksičnim učinkom le-teh. S to tehniko smo dosegli odstotke transgenih rastlin, primerljive z odstotki, pridobljenimi s klasično metodo, kar nakazuje, da je primerna za praktično uporabo.

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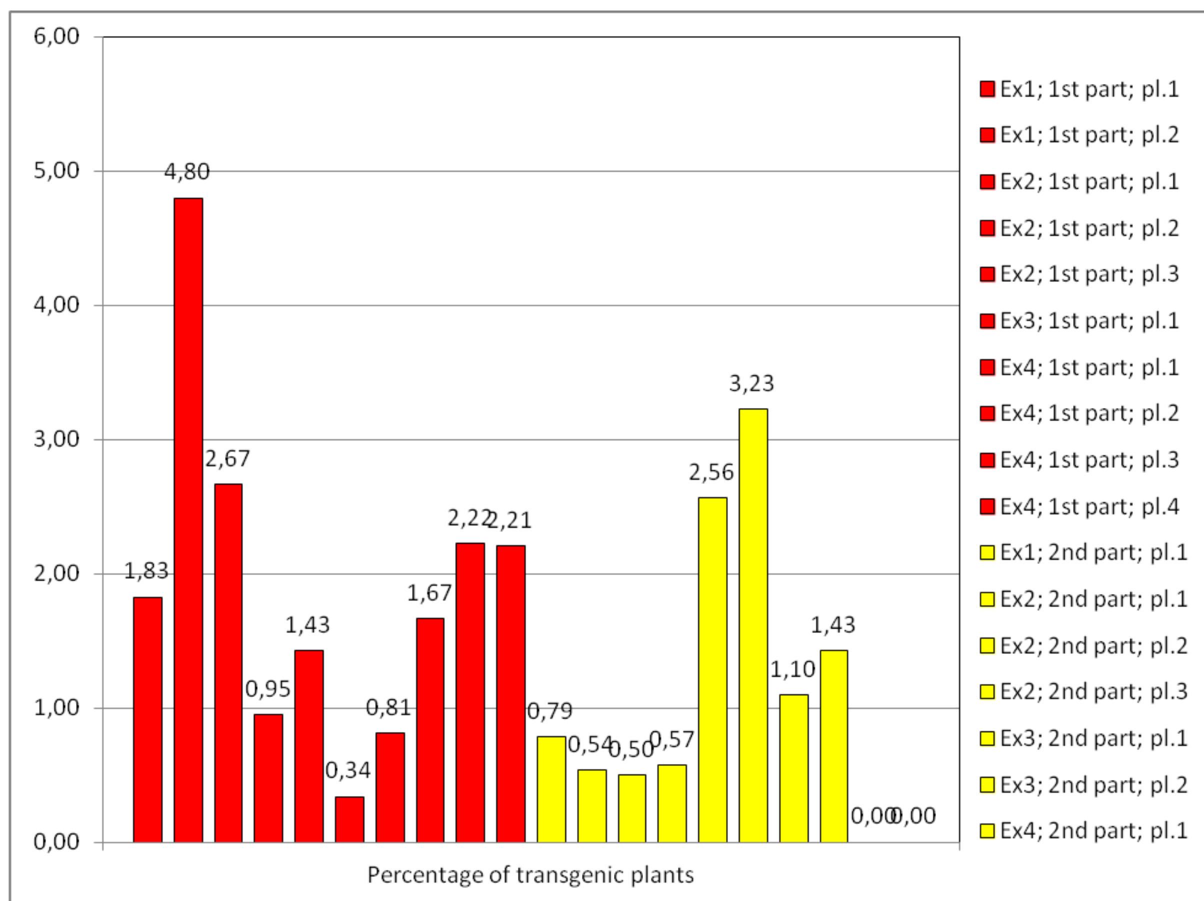
ANNEX A

The percentage of transgenic T_1 control plants (A+) on different selective plates and in different parts of the selection for the first four experiments. The red bars represent the percentage of transgenic plants on the selective plates from the first part of the selections and the yellow bars show results from the second part. The percentages of transgenic plants are generally higher in the first part of the selection and lower in the second part. The differences between the plates are bigger in the first part of the selection, whereas the second part is more uniform. The bars represent experiments in the order listed in the key on the right side of the graph. The experiments are labeled as follows: Ex1; 1st part; pl.1 – first experiment, first part of the selection, selective plate 1. The further experiments are labeled in the same manner.

Odstotek transgenih kontrolnih T_1 rastlin (A+) na selekcijskih ploščah v obeh delih selekcije. Obravnavani so prvi štirje poskusi. Rdeči stolpci predstavljajo odstotek transgenih rastlin na selekcijsih ploščah v prvem delu selekcije, rumeni pa v drugem delu selekcije. Odstotek transgenih rastlin je splošno v prvem delu višji, v drugem pa nižji. Razlike med posameznimi ploščami so bolj izražene v prvem delu selekcije, v drugem delu pa so manjše. Stolpci predstavljajo poskuse po vrsti kot so v legendi na desni strani grafa. Poskusi so označeni po ključu: Ex1; 1st part; pl.1 – prvi poskus, prvi del selekcije, selekcijska plošča 1. Ostali poskusi so označeni po istem principu.



ANNEX B

The percentage of vital seeds from control plants (A+) on the selective plates in regard to the part of the selection. Red bars represent the percentage of vital seeds in the first part of the selection, while yellow bars represent the second part of the selection. The percentage of vital seed is generaly higher in the first part of the selection and drops in the second part. The bars represent experiments in the order listed in the key on the right side of the graph. The experiments are labeled as follows: Ex1; 1st part; pl.1 – first experiment, first part of the selection, selective plate 1. The further experiments are labeled in the same manner.

Odstotek kaljivih semen pri kontrolnih rastlinah (A+) v odvisnosti od dela selekcije. Rdeči stolpci predstavljajo odstotek v prvem delu selekcije, rumeni pa v drugem delu. Odstotek kaljivih semen je v povprečju višji v prvem delu selekcije in nižji v drugem. Stolpci predstavljajo poskuse po vrsti kot so v legendi na desni strani grafa. Poskusi so označeni po ključu: Ex1; 1st part; pl.1 – prvi poskus, prvi del selekcije, selekcijska plošča 1. Ostali poskusi so označeni po istem principu.