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New Approaches to *Agrobacterium tumefaciens*-Mediated Gene Transfer to Plants

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<http://dx.doi.org/10.5772/66465>

Abstract

Agrobacterium tumefaciens, a plant pathogen, is commonly used as a vector for the introduction of foreign genes into plants and consequent regeneration of transgenic plants. *A. tumefaciens* naturally infects the wound sites in dicotyledonous plants and induces diseases known as crown gall. The bacterium has a large plasmid that induces tumor induction, and for this reason, it was named tumor-inducing (Ti) plasmid. The expression of T-DNA genes of Ti-plasmid in plant cells causes the formation of tumors at the infection site. The molecular basis of *Agrobacterium*-mediated transformation is the stable integration of a DNA sequence (T-DNA) from Ti (tumor-inducing) plasmid of *A. tumefaciens* into the plant genome. *A. tumefaciens*-mediated transformation has some advantages compared with direct gene transfer methods such as integration of low copy number of T-DNA into plant genome, stable gene expression, and transformation of large size DNA segments. That is why manipulations of the plant, bacteria and physical conditions have been applied to increase the virulence of bacteria and to increase the transformation efficiency. Preculturing explants before inoculation, modification of temperature and medium pH, addition chemicals to inoculation medium such as acetosyringone, changing bacterial density, and co-cultivation period, and vacuum infiltration have been reported to increase transformation. In this chapter, four new transformation protocols that can be used to increase the transformation efficiency via *A. tumefaciens* in most plant species are described.

Keywords: *Agrobacterium tumefaciens*, gene transfer, gamma radiation, magnetic field, squirting cucumber's fruit juice, osmotic pressure

1. Introduction

Genetic engineering could use the genetic resources that exist in nature without any limitation. With the use of these techniques, a gene cloned to an organism from any living being (human,

animal, plant, and microorganisms) could be easily transferred. For instance, a gene cloned from bacteria could be placed in the plant cell to give resistance to the pests, and this transfer gives an opportunity for the production of the organisms expressed as “genetically modified” or transgenic. These transferred genes replicate with the natural plant genes after they are placed in the organism and produce protein. The process of the studies on modern technology includes (i) recognizing, (ii) characterizing, (iii) isolating, and (iv) transferring desired genes to new hosts.

The basis of the techniques used for gene transfer to plants is the integration of a DNA segment including the gene of interest into chromosomes of the plant cells and thereof the recovery of transgenic plants from transformed cells by using tissue culture methods. In general, the rate of transformed cells in tissue is quite low. That is why the prerequisite of success in gene transfer is high-frequency shoot regeneration. Most commonly used technique in gene transfer to plants is the bacterium *Agrobacterium tumefaciens*. *A. tumefaciens* is known as a “natural genetic engineer of plants” due to this trait [1]. *Agrobacterium*-mediated transformation method has been a widely used gene transfer method. The advantages of the method are wide host range of plants: agronomically and horticulturally important crops including soybean, cotton, rice, wheat, flowers, and various trees [2] and transferring a small copy number of the transfer-DNA (T-DNA) into the cytoplasm and resulting in stable integration into plant chromosome. Although it has merits as compared to other transformation methods, such as particle bombardment, electroporation, and silicon carbide fibers, it is still hard to achieve high transformation efficiency and gene expression using this method.

2. Molecular mechanism of *A. tumefaciens*-mediated DNA transfer

Agrobacterium, of the family *Rhizobiaceae*, is a genus of Gram-negative bacterium that genetically transforms host plants and causes crown gall tumors at wound sites [3] (**Figure 1**). *Agrobacterium* can transfer DNA to a broad group of organisms: plants, fungi such as yeasts, ascomycetes, and basidiomycetes, and protist such as algae [4, 5]. *Agrobacterium* is usually classified by the disease symptomology (type of opine) and host range. The genetic mechanism of

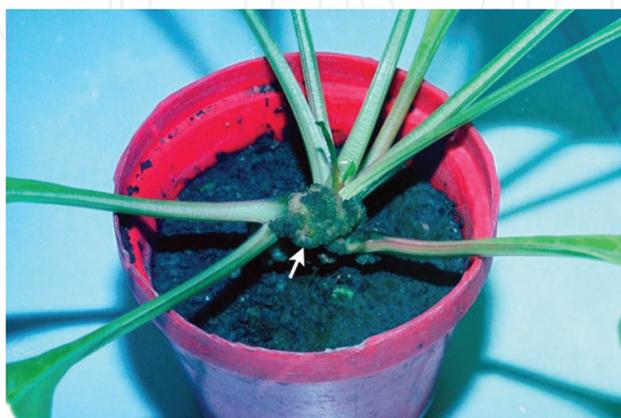


Figure 1. Crown gall in sugar beet caused by wild (oncogenic) *Agrobacterium* strain.

host range determination is still obscure, but it was reported that several virulence (*vir*) genes on the tumor-inducing (Ti) plasmid, *virC* [6], *virF* [7], and *virH* [8] were involved in determination for the range of plant species.

Bacterial recognition of monosaccharide and phenolic compounds secreted by the plant wound site initiates the tumor induction. "Activated" *Agrobacterium* transfers a particular gene segment called transfer DNA (T-DNA) from the Ti plasmid. After T-DNA is stably integrated into the chromosomal DNA in the nucleus of the host plant, genes for opine synthesis and tumor-inducing factors on the T-DNA are transcribed in the infected cells. This expression of the foreign gene in the host plant results in neoplastic growth of the tumors, providing increased synthesis and secretion of opine for bacterial consumption [9]. Opine is the condensation of an amino acid with a keto acid or sugar and is a major carbon and nitrogen source for *Agrobacterium* growth. Different *A. tumefaciens* strains produce different opine phenotypes of crown gall tumors because a particular opine expressed in the tumor is used for particular bacterial growth. Most common *Agrobacterium* strains produce an octopine or a nopaline form of opines [10]. Octopine and nopaline are derivatives of arginine. Agropine is discovered in octopine-type tumors, and it is derived from glutamate [11].

The studies on (virulent) *Agrobacterium* strains that create tumor have demonstrated that a small and round DNA molecule that exists in bacterium with a size of 150–250 kb creates tumor and opine synthesis [1]. This DNA molecule that exists in *A. tumefaciens* bacterium is called Ti (tumor inducing) plasmid [12]. Several components of *Agrobacterium* are necessary for transferring the piece of bacterial DNA into the plant cell [13]. These are the following:

1. The region on the Ti plasmid is called the transfer DNA (T-DNA) [14]. The T-DNA region is a small segment that integrates into the plant genome by being transferred from bacterium into the plant cell [15]. Previous studies have demonstrated that some genes in the T-DNA region (TMS1, TMS2 and TMS3) induce tumor genesis and opine synthesis in infected cells [16].

The T-DNA region of octopine and nopaline type plasmids is marked by right and left borders of 25 bp long random nucleotide sequences, and they have the genes that form tumor [17]. Previous studies have shown that any DNA segment inserted between these borders is easily transferred into the plant cell. Besides, it has been found out that removing the genes that forms tumor from the T-DNA region via restriction enzyme does not affect gene transfer into the plant cell [1]. The plasmids with no genes that form tumor are named as nononcogenic Ti-plasmids.

2. The second condition that plays an important role in gene transfer from *Agrobacterium* to the plant cell is the virulence (*vir*) region that is outside of the T-DNA and close to the left border with a nearly 25 kb length. Previous studies have shown that *vir* region contains six main genes (*virA*, *virB*, *virC*, *virD*, *virE*, and *virG*) [18]. *VirA* codes for a receptor that detects and correlates with phenolic compounds leaking out of damaged plant cells, and as a consequence, *virG* is stimulated. Stimulated *VirG* takes charge of the transcription operator task for itself and the other *vir* genes. *virC* enables to separate from the borders, while *virD* gene provides the regeneration of the T-DNA strand; *virB* and *virE* genes facilitate the move of the T-DNA from the bacterium to the plant cell [19].

3. The third condition that is influential in gene transfer into the plant cells is the compounds coded by three loci (*chvA*, *chvB* and *pscA*) in the chromosomes of *Agrobacterium*, being of great importance for the bacterium to attach to the plant cell and to respond to the specific chemical (chemotaxis) [20].

3. Structure and function of Ti plasmid

Ti plasmid contains 35-kb virulence (*vir*) region, which is composed of seven loci (*virA*, *virB*, *virC*, *virD*, *virE*, *virG*, and *virH*). Vir proteins are involved in signal recognition, transcriptional activation, conjugal DNA metabolism, intercellular transport, nuclear import, and probably T-DNA integration into the plant chromosome. Expression of *vir* genes is triggered by phenolic compounds, which are secreted from the wound site of the host plant. After bacterial and plant cell binding, *virA* gene within the bacterium is activated by signal molecules, such as sap with acidic pH (5.0–5.8), phenolic compounds, acetosyringone, and lignin or flavonoid precursors secreted from plant wound [21]. Monocyclic phenolics, such as acetosyringone, are the most effective *vir* gene inducers [22]. Uninjured plants do not produce these phenolic compounds or produce them at low levels. Phenolic compound is dramatically increased in the wounded plant and enhances transferring T-DNA during bacterial infection. Sugars also assist activation of the major phenolic-mediated wound-signaling pathway. The major role of the VirA coupled with VirG protein is activating other *vir* genes. VirA activates VirG, which is a cytoplasmic DNA-binding protein and works as a transcriptional factor to induce the expression of other *vir* genes. Heterologous system, VirD1 and VirD2, proteins act like endonucleases that cut between the third and fourth base pairs of 24 bp right and left border repeats of the T-DNA bottom strand [23]. A linear single-stranded copy of the T-DNA region is oriented from 5' to 3' direction, initiating at the right T-DNA border and terminating at the left border [24]. Howard and Citovsky [25] reported the structural model of the T-strand when it is transferred out of the bacterium into the plant cells. They suggested a protein-nucleic acid complex called a T-complex. This T-DNA transport intermediate has at least three components: a T-strand molecule, VirD2, and VirE2 single-strand DNA-binding protein. Citovsky et al. [26] showed that VirE coats the single-stranded DNA and forms strong, stable unfolded VirE2-ssDNA complex that is protected from external nucleolytic activity. VirC1 also helps generate a T-strand when VirD1 and VirD2 are limiting. VirD2 and VirE2 have specific nuclear localization signal (NLS) to drive T-complex into the nucleus. The direct relevance of the NLS for T-strand transfer to the host plant nucleus was confirmed by NLS deletion mutants of VirD2 and VirE that resulted in reduced T-DNA expression and tumor formation [27]. The T-DNA transfer system is similar with interbacterial conjugative transfer system of broad host range plasmids. Eleven *virB* genes in the Ti-plasmid make proteins that seem to be involved in T-DNA transfer [28]. VirB proteins are primarily linked with the cytoplasmic and periplasmic membranes to be a part of putative trans-membrane pore or channel for transferring bacterial DNA to another bacterium or host plant [29]. The main proteins for pilus formation were identified, and they are studied to find the mechanism for pili formation [30]. Three VirB proteins (VirB1, VirB2, and VirB5) are the structural subunits of the promiscuous conjugative pilus structure. VirB1, VirB2 and VirB5 needed each other for the stability and cellular localization [31].

Another main component of Ti plasmid is T-DNA, which is actually integrated into the plant cell chromosome. The T-DNA is on average 25 kb, ranging from 10 to 30 kb in size. T-DNA region is flanked and delineated by two 25-bp direct repeats, known as the right border and left border [21]. These border sequences are highly homologous and are targets of the border-specific endonuclease (VirD1/VirD2). Excised single strand of T-DNA from the Ti plasmid is exported from the bacterial cell to the plant cell by the activity of the other *Agrobacterium* Vir proteins through pili. The studies have shown that a deletion of segment in the left border does not affect genetic material transfer from the bacterium into the plant cell, while the right border nucleotide sequence of the T-DNA is of vital importance for *Agrobacterium* pathogenicity, and the transfer of the T-DNA is directed from the right to left border by creating polarity [32]. Wild-type T-DNA also has genes that are involved in plant hormone synthesis in the host plant. They are *tml*, *tms*, and *tmr* regions for leafy tumor, shooty tumor, and rooty tumor, respectively, in a plant wound site [33]. After T-DNA is integrated into the host plant, opine is synthesized, and then secreted out and imported into *Agrobacterium*. The absorbed opine molecule is catabolized by a specific enzyme in *Agrobacterium* and degraded into amino acid and the sugar moieties, which can be used as carbon and energy sources for bacterial growth. The Ti plasmid also has other components: opine catabolism region, conjugal transfer region, and vegetative origin of replication of the Ti plasmid (*oriV*).

The successful expression of the transgene depends on where the T-DNA integrates within the chromosome. T-DNA can be inserted near or far from transcriptional activating elements or enhancers, resulting in the activation of T-DNA-carried transgenes. The failure of transgene expression (gene silencing) can be caused by methylation or posttranscriptional gene silencing of multiple copies of transgenes. RNAs from these transgene copies may interfere with each other and then be degraded. This is one of the important merits of *Agrobacterium*-mediated transformation method because fewer gene copies are integrated compared to the direct gene transfer method (e.g., polyethylene glycol liposome-mediated transformation, electroporation, or particle bombardment) [34].

4. Adoption of plant molecular biology

A. tumefaciens has been used for plant genetic engineering extensively. Plants were genetically engineered for the purpose of developing resistance to herbicides, insect, or virus, tolerance to drought, salt, or cold, and increasing the yield [35]. The *Agrobacterium*-mediated transformation method has not only been used for commercial purpose but also for basic biology research to test study gene regulation or protein function in transgenic plants [36]. The *Agrobacterium*-mediated transformation method was improved by the strategy of developing modern binary Ti plasmid. Ti plasmids have been engineered to separate T-DNA and *vir* regions into two distinct plasmids, resulting in a binary vector and a *vir* helper plasmid, respectively [37]. Many *Agrobacterium* strains containing nononcogenic *vir* helper plasmids are called disarmed plasmid. LBA 4404, GV3101 MP90, AGL0, EHA101, and its derivative strain EHA 105 bacterial strains have been commercially developed to have disarmed plasmid [4]. The wild-type Ti plasmid was around 200 kb, and the sizes of the processed binary

vector from wild-type Ti plasmid were reduced to less than 10 kb, which resulted in increasing transformation efficiency. Binary vector has a replication origin for both *Escherichia coli* and *Agrobacterium*, an antibiotic selectable marker for bacteria and plants, a reporter gene, such as β -glucuronidase (GUS), luciferase, or green fluorescent protein (GFP), and a T-DNA region containing a multicloning site in which genes of interest can be inserted into. In the binary vector system, T-DNA region on the binary vector from the bacterium is transformed into the host plant with the help of another plasmid containing *vir* genes [38].

5. *Agrobacterium*-mediated plant transformation protocol development

Transformation efficiency can be increased through the manipulation of either the plant or the bacterium by enhancing competency of plant tissue and *vir* gene expression, respectively [2]. To increase the virulence of bacterium by inducing the *vir* gene expression, temperature, media pH, chemical inducers such as acetosyringone [2] has been tested. These factors likely enhance bacterial pili formation required for gene transfer between bacteria and host plants. Manipulation of other factors such as bacterial density, co-cultivation duration, surfactant, and vacuum infiltration has also increased transformation efficiency in many experiments [39].

Temperature is an important environmental factor that mainly affects transfer pilus (T-pilus; pili) biogenesis in *Agrobacterium*. *Agrobacterium* produced higher amounts of exocellular assembly of the major T-pilus components, VirB2 and VirB5, at 20°C and expression of VirB2 and VirB5 was inhibited at 26°C to 28°C [40]. Schmidit-Eisenlohr et al. [31] reported that optimized virulence gene induction was detected from the *Agrobacterium* grown on an agar plate at 20°C. Co-cultivation temperature effects on plant transformation have been studied. Dillen et al. [41] tested optimal co-cultivation temperature for *Nicotiana tabacum* and *Phaseolus acutifolius* transformation and highest GUS expression was detected at 19°C or at 22°C co-cultivation. A temperature of 22°C resulted in higher GUS expression than at 28°C in tobacco *Agrobacterium* mediated-transformation study [42]. It implies that low temperature during co-cultivation induces pili formation and results in high T-DNA transformation efficiency into host plants.

Other factors affecting transformation efficiency were studied. Whalen et al. [43] first reported that Silwet L-77 could be used to increase the susceptibility of *Arabidopsis* leaves to *Agrobacterium* infection by reducing surface tension with low phytotoxicity to allow aqueous droplets to spread evenly over leaf surface and to penetrate the stomatal opening. Due to these characteristics, Silwet L-77 enhances entry of bacteria into relatively inaccessible plant tissues. Silwet L-77 (0.001%) resulted in the development of disease phenotypes and toxicity at concentrations above 0.1% in *Arabidopsis* transformation study. Surfactant was reported as a most critical factor for increasing GUS activity. GUS activity was enhanced with 0.01% Silwet L-77 treatment, and highest activity was detected at 0.05%. When the concentration was greater than 0.05%, most of the immature wheat embryos could not survive [44]. Curtis and Nam [45] compared other surfactants, Pluronic F-68 and Tween-20, to Silwet L-77. It was confirmed that 0.05% (v/v) Silwet L-77 treatment was the most beneficial.

Vacuum infiltration has also been used mostly as an aid for efficient *Agrobacterium* inoculation for flowering stage of *Arabidopsis* [46], *Brassica napus* [47], or Chinese cabbage [48] transformation. Kapila et al. [49] reported that vacuum infiltration, ranging from 1 to 0.1 mbar for 20 min application to *Proteus vulgaris* leaf transformation via *A. tumefaciens*, resulted in high transient expression, and all infiltrated leaves showed high GUS expression sectors. Dillen et al. [41] used the vacuum infiltration method on tobacco leaves and *P. vulgaris* leaf transformation to increase transformation efficiency. Vacuum and Silwet L-77 combination effect has been studied in *Arabidopsis* flower dipping method and tobacco leaf disk transformation study, and vacuum with the low concentrations of the surfactant together resulted in the highest transformation efficiency in both studies [50].

Effect of bacterial growth phase and cell density on transformation efficiency had been considered as an important factor also. In standard protocol, cells are grown to the stationary phase ($OD_{600\text{ nm}} \approx 2\text{--}2.4$), pelleted and resuspended in inoculation medium to stationary or log or mid-log phase ($OD_{600\text{ nm}} \approx 0.1\text{--}1.15$). High concentrations of bacteria at the stationary phase have normally been used for rice, legume, and tobacco transformation [49], and low concentrations of bacteria at the log or mid-log phase have been used for broccoli [51], cabbage [52], wheat [44], cottonwood [53], and tobacco [54]. Clough et al. [46] reported that different bacterial concentration ranging from 0.15 to 1.75 of $OD_{600\text{ nm}}$ resulted in different transformation efficiency in *Arabidopsis* transformation.

Co-cultivation duration also affects transformation efficiency. Co-cultivation for 2–5 days has been normally used in *Agrobacterium*-mediated transformation under various co-cultivation temperature [55]. Coculture for 3 days resulted in high transformation efficiency and reached to a maximum at day 5 in citrange (*Citru sinensis* L. Osbeck \times *Poncirus trifoliata* L. Raf.) [56]. Co-cultivation period of more than 5 days caused bacterial overgrowth and decreased the transformation efficiency. Many transformation experiments in different plant species, such as tea (*C. sinensis* L.), cauliflower, white spruce (*Picea glauca*), and citrange, showed that 2–3 days of co-cultivation gave rise to higher transformation efficiency under room temperature [2]. Therefore, 2–3 days co-cultivation has been routinely used in most transformation protocols, since longer co-cultivation causes bacterial overgrowth, which covers the leaf tissue and brings toxicity under room temperature co-cultivation condition.

Bacteria preculture on minimal media for 3 days has been routinely practiced to induce pili formation before co-cultivation. Clough et al. [46] compared *vir* genes induction in liquid medium culture to the plate culture for 20 h at 19°C. However, they reported that two different preculture methods showed similar transformation in *Arabidopsis*.

Acetosyringone (AS) is a phenolic compound produced from wounded plant cells. AS concentration has been known as a very important factor affecting transformation efficiency. Fullner and Nester [57] reported that *Agrobacterium* did not produce pili without 200 μM AS at both 19°C and 25°C. Results from a wheat inflorescence transformation experiment showed that T-DNA cannot be transformed to the plant tissue without AS [39]. These results indicated that AS is the main factor in the low temperature co-cultivation condition that induces VirB protein, which is a subunit of pili. Various optimal concentrations of AS depended on the plant species. But in most experiments, optimal AS concentrations for different plant species are in the range of 50–400 μM AS.

6. New methods for high-transformation frequency via *A. tumefaciens*

6.1. Utilizing explant's negative atmospheric pressure for increased gene transformation

In the study conducted by Beyaz et al. [58], the aim was to increase transformation efficiency in flax (*Linum usitatissimum* L.) by increasing osmotic pressure of the tissue as plant material flax cultivars "Madaras," "Clarck," and "1886 Sel." were used in the study. Sterilized seeds got cultured on Murashige and Skoog (MS) [59] medium for germination and seedling establishment. *A. tumefaciens* strain, GV2260 harboring plasmid p35S GUS-INT that contains neomycin phosphotransferase II (*npt-II*) gene, was used for inoculation. GV2260 strain carrying p35S GUS-INT plasmid was grown overnight in a liquid Nutrient Broth (NB) medium containing 50 mg l^{-1} kanamycin and 50 mg l^{-1} rifampicin at 28°C in a rotary shaker (180 rpm) (0.6 of $\text{OD}_{600 \text{ nm}}$) and used for transformation. In the study, conventional transformation method in which hypocotyls were directly cultured on co-cultivation medium after inoculation with $500 \mu\text{l}$ bacterial solution for 20 min was compared to the method in which 7-old-day flax seedlings having cotyledon leaves without root system dried for 35 min in laminar flow were inoculated with $500 \mu\text{l}$ bacterial solution for 20 min (**Figure 2**). In both the transformation methods, after inoculation, hypocotyl segments— 0.5 cm in length—were cultured on co-cultivation medium for 2 days. Then, the explants were transferred to a regeneration medium supplemented with 1 mg l^{-1} BAP, 0.02 mg l^{-1} NAA, 100 mg l^{-1} kanamycin, and 500 mg l^{-1} augmentin and cultured for 4 weeks in a culture room at a temperature of $24 \pm 1^\circ\text{C}$. Shoots were transferred to a rooting medium containing 3 mg l^{-1} indole-butyric acid (IBA) and 100 mg l^{-1}

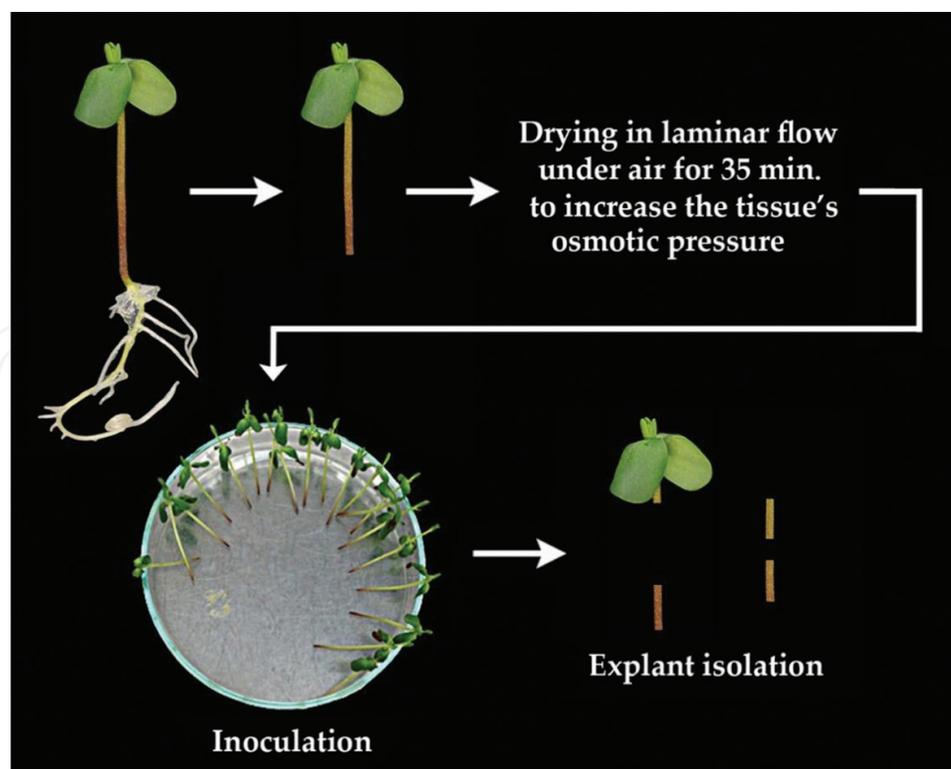


Figure 2. Explant isolation from inoculated 7-old-day flax seedlings having cotyledon leaves without root system.

kanamycin in Magenta vessels to culture for 3 weeks at $24 \pm 1^\circ\text{C}$. After root formation, plantlets were transferred to pots in a growth room for 3 weeks to recover putative transgenic plants. The presence of neomycin phosphotransferase II (*npt-II*) gene in transformants was confirmed by PCR.

The lowest results were noted in the first inoculation method in which hypocotyls were directly cultured on co-cultivation medium after inoculation (**Table 1** and **Figure 3a**). Keeping 7-day-old seedlings having cotyledon leaves without root system under air in laminar flow was aimed to enable seedlings to intake bacterial solution rapidly toward inner cells by increased osmotic pressure and consequently to increase the transformation efficiency. The highest results of all characters examined in all cultivars were obtained from a newly described inoculation method in which 7-day-old sterile flax seedlings having cotyledon leaves and no root system were inoculated with 500 μl bacterial solution for 20 min after drying in sterile cabin for 35 min (**Table 1** and **Figure 3b**). Shoot regeneration percentage, mean shoot number per explant, mean shoot number per petri dish, mean shoot number rooted per Magenta vessels, total plant number growing in soil, total PCR+ plant number, and transformation efficiency were recorded in a newly described transformation method as 70.83, 1.58, 29.50, 27.00, 97.66, 82.00, and 84.19%, while they were 34.50, 1.09, 9.25, 6.91, 18.00, 5.66, and 30.45% in routinely used transformation method (**Table 1**).

Transformation efficiency was recorded as 84.19% from hypocotyl explants excised from inoculated 7-day-old sterile seedlings having cotyledon leaves without a root system, while it was

Cultivars	Inocu. method	Regeneration (%)	Mean shoot number per explant	Mean shoot number per petri dish	Mean shoot number rooted per Magenta vessel	Total plant number growing in soil ¹	Total PCR+ plant number ²	Transfor. efficiency (%) (2/1 × 100)
"Madaras"	1	41.00 ^b	1.09 ^b	11.25 ^b	8.50 ^b	25.00 ^b	8.00 ^b	30.76 ^b
	2	71.25 ^a	1.65 ^a	30.00 ^a	28.75 ^a	104.00 ^a	88.00 ^a	84.61 ^a
"Clarck"	1	42.50 ^b	1.03 ^b	10.50 ^b	8.25 ^b	18.00 ^b	6.00 ^b	33.33 ^b
	2	73.75 ^a	1.65 ^a	33.25 ^a	28.75 ^a	103.00 ^a	82.00 ^a	79.61 ^a
"1886 Sel."	1	20.00 ^b	1.15 ^b	6.00 ^b	4.00 ^b	11.00 ^b	3.00 ^b	27.27 ^b
	2	67.50 ^a	1.46 ^a	25.25 ^a	23.50 ^a	86.00 ^a	76.00 ^a	88.37 ^a
Mean of cultivar^s	1	34.50	1.09	9.25	6.91	18.00	5.66	30.45
	2	70.83	1.58	29.50	27.00	97.66	82.00	84.19

Values followed by the different letters in a column are significantly different at the 0.01 level. Each value is the mean of 4 replications containing 25 explants per replication. All experiments were repeated 2 times.

1. Hypocotyls were directly transferred to co-cultivation medium after inoculation with 500 μl bacterial solution for 20 min.

2. Seven-day-old sterile seedlings having cotyledon leaves without root system were dried in sterile cabin for 35 min. Then, they were inoculated with 500 μl bacterial solution for 20 min. Finally, hypocotyls excised from inoculated seedlings were placed on co-cultivation medium.

Table 1. The development of shoots from hypocotyls inoculated with *A. tumefaciens* with two different methods on selection medium 4 weeks after culture initiation and transgenic plant development.

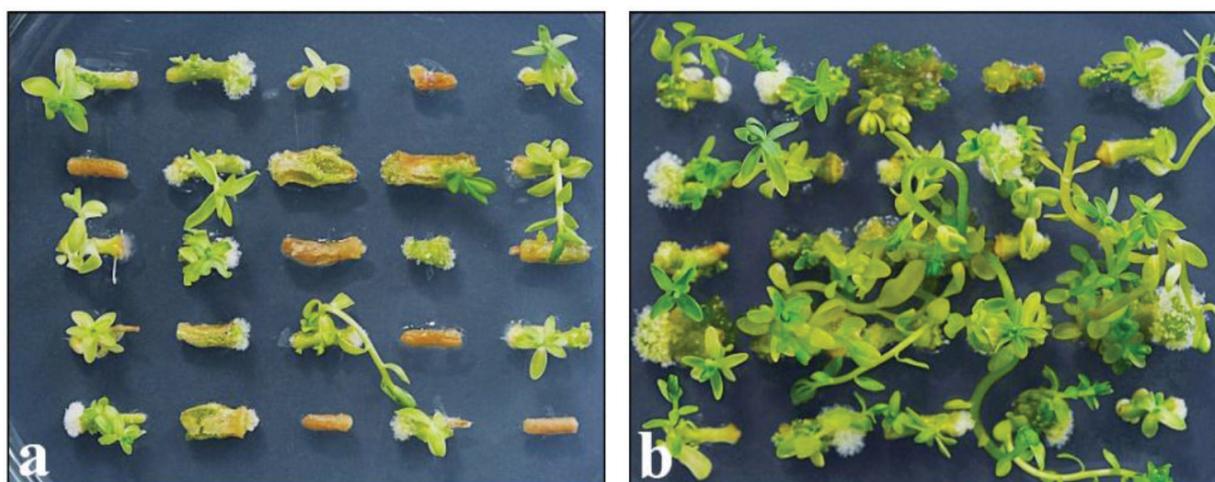


Figure 3. Shoot regeneration from hypocotyl explants of cv. "Madaras" inoculated by two different methods (a) first inoculation method, (b) second inoculation method on selection medium 4 weeks after culture initiation.

30.45% in routinely used transformation method where hypocotyls were directly cultured on selection medium after inoculation (**Table 1**). In the study, a new inoculation method was based on enabling seedlings to intake bacterial solution rapidly toward inner cells by increased osmotic pressure of explants to increase the number of cells inoculated and consequently to higher transgenic shoots was developed.

6.2. The effect of squirting cucumber (*Ecballium elaterium* (L.) A. Rich) fruit juice on *A. tumefaciens*-mediated transformation

Squirting cucumber (*E. elaterium* (L.) A. Rich.), from the cucumber family, contains cucurbitacins such as α -elaterin (cucurbitacin E), β -elaterin (cucurbitacin B), elatericine A (cucurbitacin D), and elatericine B (cucurbitacin I) [60] that are poisonous and showed antibacterial activities [61]. However, it was found out that mature fruit juice of the plant stimulated growth of *A. tumefaciens* and increased gene transfer frequency in tobacco [62]. That was why, this study supported by a project numbered 113O280 from the Scientific and Technological Research Council of Turkey (TUBITAK) was conducted to determine the effect of squirting cucumber (*E. elaterium* (L.) A. Rich.) fruit juice on *A. tumefaciens*-mediated gene transfer in flax (*L. usitatissimum* L.).

Flax cultivar "Madaras" obtained from "Northern Crop Science Laboratories," North Dakota, USA, was used in the study. Seed sterilization was achieved according to the protocol described by Yildiz and Er [63] and then seeds were sown on Murashige and Skoog (MS) [59] medium for germination and further seedling development. The mature fruits of squirting cucumber were squeezed manually and fruit juice collected in the glass jar was filter-sterilized by using 0.45 μm filters and stored at -20°C . Different squirting cucumber fruit juice concentrations (0-control, 200, 400, 800, and 1600 $\mu\text{l l}^{-1}$) were added to regeneration medium after autoclaving in order to determine the most effective concentration on shoot regeneration from hypocotyl explants. The highest shoot regeneration was recorded in 400 $\mu\text{l l}^{-1}$ squirt-

ing cucumber fruit juice concentration. *A. tumefaciens* strain GV2260 harboring plasmid p35S GUS-INT that contains neomycin phosphotransferase II (*npt-II*) gene was used for inoculation. GV2260 strain carrying p35S GUS-INT plasmid was grown overnight in a liquid NB (Nutrient Broth) medium containing 50 mg l⁻¹ kanamycin, 50 mg l⁻¹ rifampicin, and different squirting cucumber fruit juice concentrations (0-control, 200, 400, 800 and 1600 µl l⁻¹) at 28°C in a rotary shaker (180 rpm) (0.6 of OD_{600 nm}) and used for transformation. Hypocotyl explants of 7-day-old flax seedlings were inoculated with bacteria in a liquid medium having different squirting cucumber fruit juice concentrations (0-control, 200, 400, 800 and 1600 µl l⁻¹) for 20 min. After inoculation, hypocotyl explants were transferred to a solid medium containing 1 mg l⁻¹ BAP, 0.02 mg l⁻¹ NAA, and 400 µl l⁻¹ squirting cucumber fruit juice for co-cultivation for 2 days in a culture room at a temperature of 24 ± 1°C. Explants were then transferred to the medium that had the same content as co-cultivation, supplemented with 100 mg l⁻¹ kanamycin and 500 mg l⁻¹ duocid for selection for 4 weeks. Regenerated shoots were transferred to a rooting medium supplemented with 3 mg l⁻¹ indole-butyric acid (IBA) and 100 mg l⁻¹ kanamycin in Magenta vessels for 3 weeks at 24 ± 1°C. After root formation, plantlets were transferred to pots in a growth room for 3 weeks to recover putative transgenic plants. The presence of neomycin phosphotransferase II (*npt-II*) gene in transformants was confirmed by PCR.

At the end of the study, it was determined that 400 µl l⁻¹ squirting cucumber fruit juice added to bacterial growth and inoculation medium was found the most effective fruit juice concentration on gene transformation frequency. The highest shoot regeneration percentage on selection medium, having antibiotics was recorded 54.00% as the highest from the medium containing 400 µl l⁻¹ fruit juice 4 weeks after culture initiation. It was 41.25% in control treatment having no fruit juice. The highest shoot number per explant was recorded in the treatment where 1600 µl l⁻¹ fruit juice was used as 2.06. From the medium having 400 µl l⁻¹ fruit juice, 1.16 shoots per explant were recovered. The highest shoot length was noted as 1.73 cm from growth medium containing 1600 µl l⁻¹ fruit juice. Total shoot number per petri dish was noted as 12.53 as the highest from medium containing 400 µl l⁻¹ fruit juice (**Table 2**). In 1600 µl l⁻¹ fruit juice concentration in which the highest results were recorded in shoot number per explant and the highest shoot length per explant, total shoot number per petri dish was achieved as 7.75—the lowest.

After a 4-week-cultivation on selection medium, rooted explants were directly transferred to soil by skipping *in vitro* rooting stage (**Figure 4a**). From the medium having 400 µl l⁻¹ fruit juice, 11.00 rooted explants were transferred to soil, and finally, 3.00 putative plantlets were grown in soil, reached maturity, and all were morphologically normal (**Figure 4b**). On the other hand, 5.57 putative transgenic plantlets were grown from a medium containing 800 µl l⁻¹ fruit juice. Out of 8.70 rooted explants were transferred to soil, only 1.86 putative transgenic plants were grown in soil in control application where no fruit juice was used (**Table 2**). After PCR analysis, all the plants grown in soil from selection medium containing 400 µl l⁻¹ fruit juice were confirmed to be transgenic, while two plants were found transgenic from medium having 800 µl l⁻¹ fruit juice. The highest transformation efficiency was noted as 100.00% in 400 µl l⁻¹ fruit juice treatment. In control where squirting cucumber fruit juice was not used, transformation efficiency was 0.00% meaning that no transgenic plants were recovered (**Table 2**).

Fruit j. concent. ($\mu\text{l l}^{-1}$)	Regeneration (%)	Shoot number per explant	The highest shoot length per explant (cm)	Total shoot number per petri dish	The number of rooted explants transferred to soil	The number of putative transgenic plants growing in soil ¹	The number of PCR+ transgenic plants ²	Transfor. efficiency (%) ($2/1 \times 100$)
0	41.25 ^b	1.17 ^b	1.24 ^b	9.53 ^b	8.70 ^{ab}	1.86 ^c	0.00 ^b	0.00
200	49.00 ^a	1.04 ^b	1.05 ^b	10.19 ^{ab}	9.00 ^{ab}	2.65 ^b	1.00 ^b	37.73
400	54.00 ^a	1.16 ^b	1.50 ^a	12.53 ^a	11.00 ^a	3.00 ^b	3.00 ^a	100.00
800	42.00 ^b	1.07 ^b	0.97 ^b	8.98 ^b	7.65 ^b	5.57 ^a	2.00 ^{ab}	35.90
1600	38.75 ^c	2.06 ^a	1.73 ^a	7.75 ^c	6.25 ^c	2.00 ^c	0.00 ^b	0.00

Values followed by the different letters in a column are significantly different at the 0.01 level.

Each value is the mean of 5 replications containing 10 explants per replication. All experiments were repeated 2 times.

Table 2. The effect of squirting cucumber fruit juice on shoot regeneration from hypocotyl explants inoculated with *A. tumefaciens* on selection medium containing 100 mg l⁻¹ kanamycin and 500 mg l⁻¹ duocid 4 weeks after culture initiation and recovery of transgenic plants.

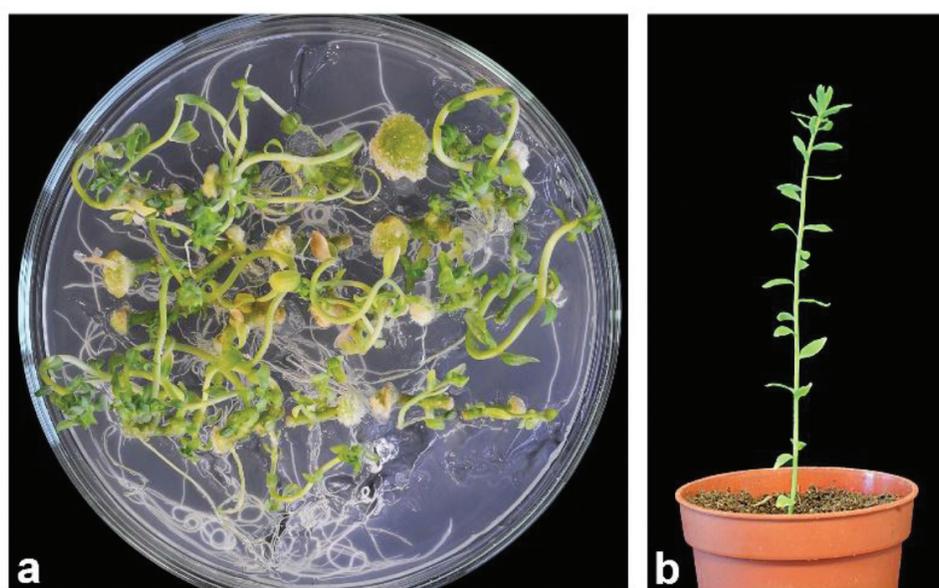


Figure 4. Shoot regeneration from hypocotyl explants of cv. “Madaras” inoculated with *A. tumefaciens* on selection medium containing 100 mg l⁻¹ kanamycin, 500 mg l⁻¹ duocid, and 400 µl l⁻¹ fruit juice 4 weeks after culture initiation (a) and PCR-confirmed transgenic plant growing in soil (b).

Results showed positive effects of squirting cucumber’s fruit juice on regeneration and transformation at 400 µl l⁻¹ concentration as compared to control. At fruit juice concentrations over and below 400 µl l⁻¹ in the culture medium, shoot regeneration and transformation were hindered significantly. Kanamycin-resistant shoots were formed in a medium containing high concentration (100 mg l⁻¹) of kanamycin that prevents the escaped shoots in the selection medium efficiently. PCR analysis confirmed that all raised plants were transgenic in the medium having squirting cucumber’s fruit juice at a concentration of 400 µl l⁻¹.

The data presented here clearly indicates that the addition of squirting cucumber’s fruit juice to bacterial growth, inoculation, and co-cultivation media improved the transformation frequency of flax significantly. We conclude that squirting cucumber’s fruit juice induces *vir* genes leading to increased transformation efficiency.

6.3. Use of magnetic field strength for high-transformation frequency via *A. tumefaciens*

Exposure of seeds to magnetic field for a short time was found to help in accelerated sprouting and growth of the seedlings. It was reported that magnetic conditions stimulated plant growth [64–66]. The current study was aimed to examine the effects of magnetic field strength on *A. tumefaciens*-mediated gene transfer in flax (*L. usitatissimum* L.). Seeds of flax cv. “Madaras” obtained from “Northern Crop Science Laboratories,” North Dakota, USA, were used. First, seeds were exposed to different magnetic field strengths (0-control, 75, 150, and 300 mT) for 24 h. Then, they were surface sterilized with 40% commercial bleach containing 5% sodium hypochlorite at 10°C for 12 min with continuous stirring, and they were washed three times with sterile distilled water at the same temperature. Sterilized seeds were germinated on MS medium in Magenta vessels. Hypocotyl explants excised from 7-day-old seedlings were used

for regeneration. GV2260 line of *A. tumefaciens* having p35S GUS-INT plasmid containing *npt-II* gene that determines kanamycin resistance was used in transformation studies. Hypocotyls were kept in petri dishes containing 50 ml sterile water with 500 μ l bacterial solution during 20 min for inoculation. Inoculated hypocotyls were then cultured on MS medium containing 1 mg l⁻¹ BAP, 0.02 mg l⁻¹ NAA, for co-cultivation for 2 days in culture room at a temperature of 24 \pm 1°C. Then, explants were transferred to selection medium containing 1 mg l⁻¹ BAP, 0.02 mg l⁻¹ NAA, 50 mg l⁻¹ kanamycin, and 500 mg l⁻¹ duocid and cultured for 4 weeks. Shoots were kept in a rooting medium containing 3 mg l⁻¹ indole-butyric acid (IBA) and 100 mg l⁻¹ kanamycin in Magenta vessels for 3 weeks at 24 \pm 1°C. Then, plantlets were transferred to pots in a growth room for 3 weeks to obtain putative transgenic plants. The presence of the *npt-II* gene was verified by PCR analysis in candidate plants.

The highest results with respect to regeneration percentage, shoot number per explant, the highest shoot length per explant, total shoot number per petri dish, the number of rooted explants transferred to soil, the number of putative transgenic plants growing in soil, and the number of PCR+ transgenic plants were recorded in the treatment, where seeds were exposed to 75 mT magnetic field strength as 82.00%, 2.40, 3.40, 27.60, 16.40, 12.60, and 8.00, respectively. In control application where no magnetic field strength was used, the lowest results were obtained in all characters examined. Out of 12.60 putative transgenic plants growing in soil, 8.00 was found PCR positive (**Figure 5**).

Results clearly showed that 75 mT magnetic field strength increased *A. tumefaciens*-mediated transformation frequency in flax. At 75 mT magnetic field strength, out of 12.60 putative transgenic plants, 8 were confirmed transgenic after PCR analysis that meant 63.49% transforma-

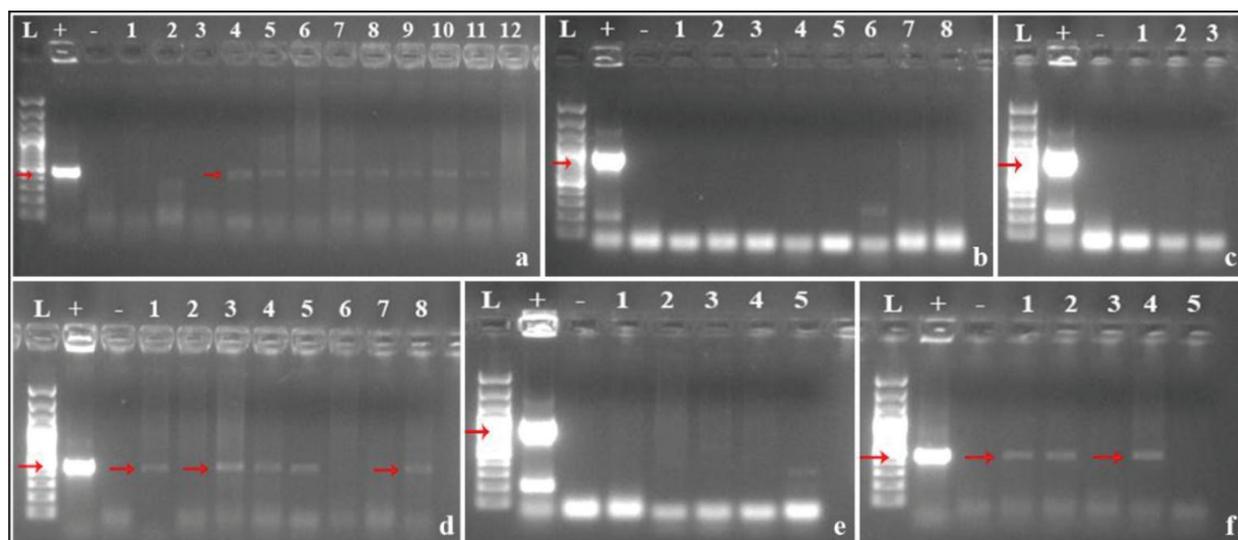


Figure 5. PCR analysis of genomic DNA from putative transgenic plants cv. "Madaras" for amplification of 458 bp *npt-II*. L DNA ladder 100 bp, + Plasmid as a positive control. Water as a negative control. (a) Detection of the *npt-II* gene in 75 mT magnetic field strength, (b) detection of *chv* gene in 75 mT magnetic field strength, (c) detection of *chv* gene in 300 mT magnetic field strength, (d) detection of the *npt-II* gene in 150 mT magnetic field strength, (e) detection of the *chv* gene in 150 mT magnetic field strength, (f) detection of the *npt-II* gene in 300 mT magnetic field strength.

Mag. field stren. (mT)	Regeneration (%)	Shoot number per explant	The highest shoot length per explant (cm)	Total shoot number per petri dish	The number of rooted explants transferred to soil	The number of putative transgenic plants growing in soil ¹	The number of PCR+ transgenic plants ²	Transfor. efficiency (%) (2/1 × 100)
0	41.25 ^b	1.17 ^c	1.24 ^c	9.53 ^d	8.70 ^d	1.86 ^d	0.00 ^d	0.00
75	82.00 ^a	2.40 ^a	3.40 ^a	27.60 ^a	16.40 ^a	12.60 ^a	8.00 ^a	63.49
150	78.00 ^a	2.36 ^a	2.76 ^b	24.80 ^b	15.60 ^b	8.40 ^b	5.00 ^d	59.52
300	46.00 ^b	1.70 ^b	2.54 ^b	12.80 ^c	9.20 ^c	5.50 ^c	3.00 ^c	54.54

Values followed by the different letters in a column are significantly different at the 0.01 level.

Each value is the mean of 5 replications containing 10 explants per replication. All experiments were repeated 2 times.

Table 3. The effect of magnetic field strengths on transgenic shoot regeneration and recovery of transgenic plants from hypocotyls inoculated with *A. tumefaciens* on selection medium containing 100 mg l⁻¹ kanamycin and 500 mg l⁻¹ duocid 4 weeks after culture initiation and recovery of transgenic plants.

Gamma dose (Gy)	Regeneration (%)	Shoot number per explant	The highest shoot length per explant (cm)	Total shoot number per petri dish	The number of rooted explants transferred to soil	The number of putative transgenic plants growing in soil ¹	The number of PCR+ transgenic plants ²	Transfor. efficiency (%) (2/1 × 100)
0	41.25 ^c	1.17 ^c	1.24 ^c	9.53 ^d	8.70 ^c	1.86 ^a	0.00 ^d	0.00
40	70.00 ^a	2.20 ^b	1.79 ^a	30.80 ^a	13.20 ^a	25.00 ^a	25.00 ^a	100.00
80	66.00 ^b	2.11 ^b	1.58 ^b	27.85 ^a	14.00 ^a	25.00 ^a	22.00 ^b	88.00
120	61.00 ^b	2.06 ^a	1.57 ^b	25.13 ^a	12.20 ^b	25.00 ^a	20.00 ^c	80.00

Values followed by the different letters in a column are significantly different at the 0.01 level.

Each value is the mean of 5 replications containing 10 explants per replication. All experiments were repeated 2 times.

Table 4. The effect of gamma radiation on transgenic shoot regeneration from hypocotyls inoculated with *A. tumefaciens* on selection medium containing 100 mg l⁻¹ kanamycin and 500 mg l⁻¹ duocid 4 weeks after culture initiation.

tion efficiency (**Table 3**). To our knowledge, this was the first study indicating that magnetic field strength could increase *A. tumefaciens*-mediated transformation.

This study was supported by a project number 113O280 from the Scientific and Technological Research Council of Turkey (TUBİTAK).

6.4. The effect of gamma radiation on *A. tumefaciens*-mediated transformation

Gamma rays give rise to cytological, biochemical, physiological, and morphological changes in cells and tissues via producing free radicals in cells [67]. Although higher doses of gamma radiation were inhibitory [68], lower doses are stimulatory. Low doses of gamma rays have been reported to increase cell proliferation, germination, cell growth, enzyme activity, stress resistance, and crop yields [69–72].

In the study supported by the project number 113O280 from the Scientific and Technological Research Council of Turkey (TUBİTAK), the effects of gamma radiation of radioactive cobalt (^{60}Co) γ rays on *A. tumefaciens*-mediated gene transfer to flax were examined. Flax seeds of cv. “Madaras” were irradiated with different gamma doses (0-control, 40, 80, and 120 Gy), and then surface sterilized by using the protocol described in Section 6.3. Sterilized seeds were then sown in Magenta vessels having MS medium for germination. Hypocotyls of 7-day-old seedlings were used for regeneration. GV2260 line of *A. tumefaciens* having p35S GUS-INT plasmid containing *npt-II* gene that determines kanamycin resistance was used in transformation studies. Hypocotyls were kept in petri dishes containing 50 ml sterile water with 500 μl bacterial solution during 20 min for inoculation. Inoculated hypocotyls were then cultured on MS medium containing 1 mg l^{-1} BAP, 0.02 mg l^{-1} NAA, for co-cultivation for 2 days in a culture room at a temperature of $24 \pm 1^\circ\text{C}$. Then, explants were transferred to selection medium containing 1 mg l^{-1} BAP, 0.02 mg l^{-1} NAA, 50 mg l^{-1} kanamycin, and 500 mg l^{-1} duocid, and cultured for 4 weeks. Shoots were then transferred to a rooting medium having 3 mg l^{-1} indole-butyric acid (IBA) and 100 mg l^{-1} kanamycin in Magenta vessels for 3 weeks at $24 \pm 1^\circ\text{C}$. Plantlets were transferred to pots to develop for 3 weeks. The presence of the *npt-II* gene in putative transgenic plants was verified by PCR analysis.

The highest results were recorded in 40 Gy gamma dose with respect to the number of putative transgenic plants growing in soil, the number of PCR+ transgenic plants, and transformation efficiency as 25.00, 25.00, and 100.00% (**Table 4**). It could be concluded that low gamma radiation increased transformation efficiency significantly compared with control application where no gamma radiation was used.

7. Conclusion

A. tumefaciens as a plant pathogen naturally infects the wound sites in dicotyledonous plants and induces disease known as crown gall, and this bacterium has been widely used for the introduction of foreign genes into plants and consequent regeneration of transgenic plants. However, *A. tumefaciens*-mediated gene transfer is quite difficult in most of the plant species.

The success of genetic transformation via *A. tumefaciens* is limited due to the fact that plant's defence mechanism will be active when pathogen attacks. That is why manipulations of the plant and bacterium and physical conditions have been applied to increase the virulence of bacterium and to increase the transformation efficiency. To our knowledge, the four new transformation protocols described in this chapter are new and not reported elsewhere before. And newly reported protocols can be easily used to increase the transformation efficiency in most plant species. We hope that transformation protocols described in this chapter may help researchers to increase the success of transformation studies via *A. tumefaciens*.

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