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Chapter

Gamma Radiation Effect on *Agrobacterium tumefaciens*-Mediated Gene Transfer in Potato (*Solanum tuberosum* L.)

Murat Aycan, Muhammet Cagri Oguz, Yasin Ozgen, Burak Onol and Mustafa Yildiz

Abstract

Potato (*Solanum tuberosum* L.) is one of the major crops of the world. Significant improvements can be achieved in terms of yield and quality by the determination of efficient transformation methods. On the other hand, low transformation frequency seriously limits the application of molecular techniques in obtaining transgenic crops. In the present study, the effect of gamma radiation on *Agrobacterium tumefaciens*-mediated transformation to the potato was firstly investigated. Sterile seedlings of potato cv. 'Marabel', which was grown on Gamborg's B5 medium in Magenta vessels, were irradiated with different gamma radiation doses (0-control, 40, 80, 120 Gy ^{60}Co). Stem parts having axillary meristems were excised from irradiated seedlings and inoculated by *A. tumefaciens* (GV2260), which harbors the binary plasmid p35S GUS-INT contains and *GUS* (β-glucuronidase) gene controlled by 35S promoter (CaMV) and *nptII* (neomycin phosphotransferase II) gene driven by NOS (nopaline synthase) promoter). Inoculated stem parts having axillary meristems explants were then directly transported to a selection medium containing duocid (500 mg l^{-1}), and kanamycin (100 mg l^{-1}), 4 mg l^{-1} gibberellic acid, 1 mg l^{-1} BAP and 0.1 mg l^{-1} NAA. The adult transgenic plants were detected by polymerase chain reaction (PCR) analysis. According to the number of transgenic plants determined by PCR analysis, results obtained from explants treated with 40 Gy gamma gave the best results compared to the control (0 Gy) application. The doses over 40 Gy were also found statistically significant compared to the control (0 Gy). It is expected that the protocol described in this study make the transformation studies easier by skipping the stages of 'co-cultivation', 'culturing explants on selection medium' and 'recovery of transgenic shoots on selection medium' not only for potato but also for other crop plants. This study was supported by a grant from the Scientific and Technological Research Council of Turkey (TUBİTAK) (Grant number 113O280 to Prof. Dr. Mustafa YILDIZ).

Keywords: transformation efficiency, gamma radiation, potato, *A. tumefaciens*

1. Introduction

Genetic transformation technologies developed with recombinant DNA technology and *in vitro* regeneration methods are successfully used to overcome the species differences and taxonomic obstacles encountered in traditional breeding programs.

Agrobacterium-mediated transformation method is the most proficient and commonly used plant transformation method among different gene transfer techniques. However, there are a number of variables that affect the success of *Agrobacterium*-mediated genetic transformation [1]. The success of genetic transfer efficiency with *A. tumefaciens* depends on, *Agrobacterium* strain used, bacteria concentration, antibiotic types and concentration used for *in vitro* selection, inoculation time, and temperature [2]. Besides, the type of the target plant, plant explants, hormone combinations used in *in vitro* regeneration, pH, etc., are among the factors affecting the recovery of transgenic plants [3]. Increasing the transformation efficiency by reducing the limiting factors in genetic transformation studies will significantly contribute to the success rate [4].

Gamma radiation treatments are innovative biotechnological interventions used to increase yield and quality. Gamma radiation technique is successfully applied in plant breeding programs to increase genetic diversity and biotic/abiotic stress tolerance [5]. With the use of this method, thousands of mutant varieties have been obtained from approximately 200 plant species [6]. Gamma radiation is considered a physical mutagen that has significant effects on cytological, biochemical, molecular, physiological and morphological processes in plants [7–9]. The biological effect of gamma radiation is due to its interaction with atoms and water molecules in the cell [10]. As a result of the interaction of gamma rays with atoms, free radicals are produced at the cellular level. These radicals affect physiological and metabolic activities in plants [11].

Low doses of gamma have positive effects on cell proliferation; cell and tissue growth, germination percentage, enzyme activity, chlorophyll content, biotic and abiotic stress tolerance and crop yield [12–15]. On the other hand, high doses of gamma particles cause damage to protein synthesis, enzyme activity hormone balance, water exchange and leaf gas exchange [16].

The adverse effects of gamma rays are divided into two as direct and indirect. While its direct impact is realized by the interaction between radiation and target living molecules, its indirect effect arises from the formation of free radicals [17]. These free radicals are called radiation hormones and inhibit the growth of the plant [18]. Besides, the effect of gamma-ray on the plant depends on the source and dose of gamma radiation, exposure time, target plant species and variety, plant tissue and the plant's growth period [19].

New approaches are needed to increase the low success rate in genetic transformation studies. The positive effect of a reduced dose of gamma radiation on genetic transformation efficiency has been investigated in a few studies [4, 20]. Determining the effect of gamma radiation on genetic transformation frequency in different plant species will contribute to genetic transformation and molecular assisted cultivar development studies. This study was aimed to determine the effect of gamma radiation to increase the genetic transformation frequency in potato and to create a repeatable successful protocol.

2. Materials and methods

2.1 Plant material

Potato (*S. tuberosum* L.) tubers of cv. 'Marabel' were used in the study.

2.2 Explant material

Stem parts having axillary meristems isolated from irradiated sterile seedlings were used as explant (**Figure 1**).

2.3 Radiation source

0.8 kGy h^{-1} of ^{60}Co γ ray source at the Sarayköy Nuclear Research and Training Center, Turkish Atomic Energy Authority, Sarayköy, Ankara.

2.4 *A. tumefaciens* strain

The GV2260 including p35S GUS-INT plasmid of *A. tumefaciens* strain was utilized for inoculation. The characteristic of p35S GUS-INT binary plasmid described Yildiz et al. [20]. GV2260 strain (OD = 0.6) was incubated overnight in a liquid medium (Nutrient Broth) including rifampicin (50 mg l^{-1}) and kanamycin (50 mg l^{-1}) in an incubator (rotary shaker) at 180 rpm under 28°C and used for transformation studies.

2.5 Irradiation of seedlings

One-month-old sterile seedlings were irradiated with different doses (0-control, 40, 80 and 120 Gy) of ^{60}Co γ source. Fricke and alanine dosimeters were used for dose mapping and determination of dose rates of gamma source. Seedlings were irradiated along with a dosimeter for each dose to be sure that ionization was uniform.

2.6 Culture conditions

Seedlings were grown on the Gamborg's B5 medium containing the mineral salts and vitamins, sucrose (3%, w/v) and agar (0.7%, w/v). The pH of the medium was adjusted to 5.8 before autoclaving. All cultures were grown at 25 ± 1°C under cool white fluorescent light (27 μmol m^{-2} s^{-1}) with a 16/8 h day/night photoperiod in the growth chamber.

2.7 Transformation procedure

A. tumefaciens GV2260 carrying p35S GUS-INT plasmid was incubated overnight and diluted with a liquid medium to 1X10^8 cell/ml. Stem parts having

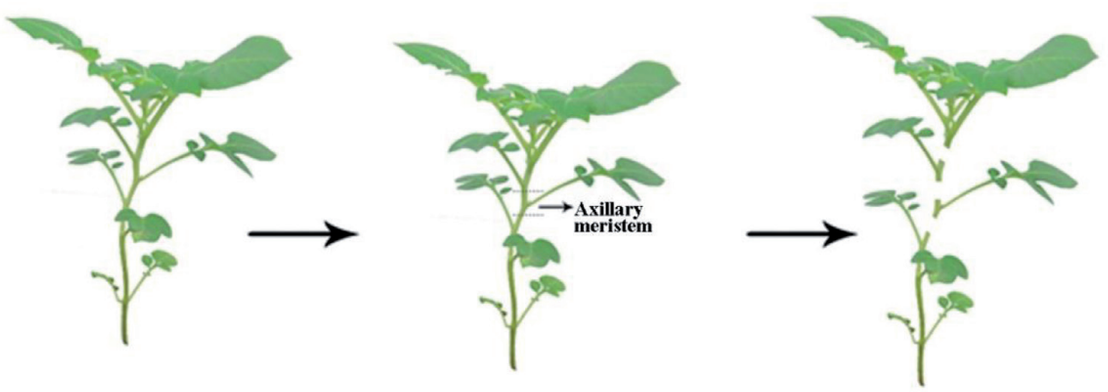

Figure 1.
Stem parts having axillary meristems excised from irradiated sterile seedlings.

axillary meristems excised from irradiated sterile seedlings were inoculated in a liquid regeneration medium containing 4 mg l^{-1} gibberellic acid, 1 mg l^{-1} BAP and 0.1 mg l^{-1} NAA for 20 min. After inoculation, stem parts having axillary meristems were directly transferred to selection medium bypassing co-culture stage in Magenta vessels containing 4 mg l^{-1} gibberellic acid, 1 mg l^{-1} BAP and 0.1 mg l^{-1} NAA, supplemented with 100 mg l^{-1} kanamycin and 500 mg l^{-1} duocid for 2 weeks.

2.8 Recovery of putative transgenic plants

Seedlings were planted to pots having commercial soil in a growth chamber for 3 weeks where temperature (24 ± 1°C), light (27 μmol m^{-2} s^{-1}) and humidity were controlled. To keep the humidity high, the pots were covered with a thin nylon transparent bag and placed in the growth chamber. The humidity was gradually reduced by making small holes in the bags every 2-3 days. After 10 days, the bags were completely removed. By this way, humidity was reduced gradually from 100–40%. Candidate transgenic plants were irrigated with 50 ml water including kanamycin (100 mg l^{-1}) at 2 day-intervals during 14 days for further selection.

2.9 gDNA (genomic DNA) extraction

The gDNA was extracted from fresh leaves of putative transgenic plants and from control (non-transformed) plants with slight modification of the protocol described by [21].

2.10 Polymerase chain reaction (PCR)

PCR amplification was performed to detect the *npt-II* gene with the following designed specific primer sets Forward: 5′-TTGCTCCTGCCGAGAAAG-3′ and Reverse: 5′-GAAGGCGATAGAAGGCGA-3′. PCR amplification of the chromosomal virulence gene (*chv*) was carried with the following primer sets Forward: 5′-CGAACCGCTGTTCGGCCTGTGG-3′ and Reverse: 5′-GTTCAGGAGGCCGGCATCCTGG-3′ for determine of *A. tumefaciens* contamination in putative transgenic plants.

The PCR was conducted in 2 μL containing 100 ng of DNA, 10 pmol of each forward and reverse primers, 0.25 μM dNTP, 2 mM $MgCl_2$, 1× PCR buffer, and 0.625 U of DreamTaq DNA polymerase enzyme (Thermo Scientific, Waltham, Massachusetts, USA). The PCR was run with an initial denaturation of the DNA template at 95°C for 5 min followed by 36 cycles, each consisting of 95°C for 1 min, 58°C for 1 min and 72°C for 1 min, and final extension at 72°C for 5 min in a Prime G Gradient Thermal Cycler (Techne, Staffordshire, UK). Amplified PCR products were electrophoresed on a 1% agarose in TAE (tris-acetate EDTA) buffer. The bands were stained with ethidium bromide staining and visualized with UV light.

2.11 Observations

Number of explants cultured on selection medium, number of plants growing on selection medium, number of putative transgenic plants transferred to soil, number of PCR positive (+) plants, number of PCR (+) plants after *chv* gene analysis and transformation efficiency were determined.

2.12 Statistical analysis

Five replicates of rooted plants in the pots were tested and considered the units of replication. One-way Analysis of Variance (ANOVA) was used to test the effect of gamma radiation on gene transformation efficiency. All experiments were repeated two times. Data were statistically analyzed by "IBM SPSS Statistics 22" computer program. Duncan's multiple range test was used to compare the means [22].

3. Results and discussion

Results of gene transformation to stem parts having axillary meristems of irradiated seedlings were given in **Table 1**. In the current study, inoculation was performed to 30 stem parts having axillary meristems at each of the gamma doses (0-control, 40, 80 and 120 Gy). Only 14 plants were grown in control treatment where gamma radiation was not applied. As the result of PCR analysis, band of *npt-II* gene was detected in only 5 putative transgenic plants. However, after *chv* gene analysis, it was determined that none of 5 putative transgenic plants was real transgenic which meant band of *npt-II* gene detected in 5 putative transgenic plants came from bacteria being on the plants in control treatment. In all gamma treatments, increases were observed in the number of plants growing on selection medium. In all the parameters examined, the highest values were recorded in plants grown from stem parts having axillary meristems to which 40 Gy gamma dose was applied. At 40 Gy gamma dose, 28 out of 30 inoculated stem parts having axillary meristems of irradiated seedlings were successfully grown in soil. Thirty three out of 28 putative transgenic plants were found PCR(+) (**Table 1**, **Figure 2**). The presence of *chv* gene was checked in 33 putative transgenic plants, and consequently, 28 plants were confirmed as real transgenic without bacterial contamination. Transformation efficiency was calculated as 100% (**Table 1**, **Figure 3**).

Results showed positive effects of gamma radiation on transformation at 40 Gy as compared to control. Higher gamma doses over 40 Gy, transformation hindered significantly. PCR analysis confirmed that 28 plants out of 33 were transgenic at 40 Gy gamma treatment (**Table 1**).

A. tumefaciens, a plant pathogen, is commonly used as a vector for genetic transformation to plants [23, 24]. The genetic transformation prosperity of *A. tumefaciens* method is limited in plant species largely, because the mechanism of plant's resistance will be active when pathogen attacks. That is why, genetic manipulations of the plant, physical conditions and bacteria have been applied to increase the virulence of bacteria and to increase the transformation efficiency [25, 26].

Before inoculation, pre-culturing explants [25, 27], alteration of temperature [25, 28] and medium pH [28, 29], addition chemicals to inoculation medium such as acetosyringone [25, 26, 28, 30–32], altering bacterial density and co-cultivation time [27, 29, 31] and vacuum infiltration [33–35] have been reported to increase transformation.

Possible molecular effects of gamma radiation in plants include activations of RNA and protein synthesis, acceleration of cell division, and direct or indirect activation of genes [36, 37]. Ionizing radiation causes a single strand break and replication inhibition at high doses, while at low doses it causes only minor replication blockade [38]. Gamma radiation cause chromosome strand breaks and consequently integration of genes transferred from extracellular to DNA. Köhler et al. [39] reported an increase in the frequency of transgenic plants regenerated from protoplasts exposed to gamma radiation. It has been reported that this occurred as a

Gamma radiation dose (Gy)	Number of stem parts cultured on selection medium	Number of plants growing on selection medium	Number of putative transgenic plants transferred to soil	Number of PCR (+) plants	Number of PCR(+) plants after *chv* gene analysis	Transformation efficiency (%)*
0	30.00	14.00 b	14.00 b	5.00 c	0.00 c	0.00 d
40	30.00	28.00 a	28.00 a	33.00 a	28.00 a	100.00 a
80	30.00	26.00 ab	26.00 ab	15.00 b	14.00 b	53.85 c
120	30.00	25.00 ab	25.00 ab	17.00 b	17.00 b	68.00 b

Values in a column followed by different letters are significantly different at the 0.01 level.
**Transformation efficiency = (Number of PCR (+) plants after chv gene analysis/Number of putative transgenic plants transferred to soil) x 100.*
The reason why number of PCR (+) plants is higher than number of putative transgenic plants transferred to soil at 40 Gy gamma treatment is the development of new shoots from buds on tuber.

Table 1.
Results of PCR analysis in plants grown from stem parts having axillary meristems of irradiated seedlings inoculated by Agrobacterium tumefaciens.

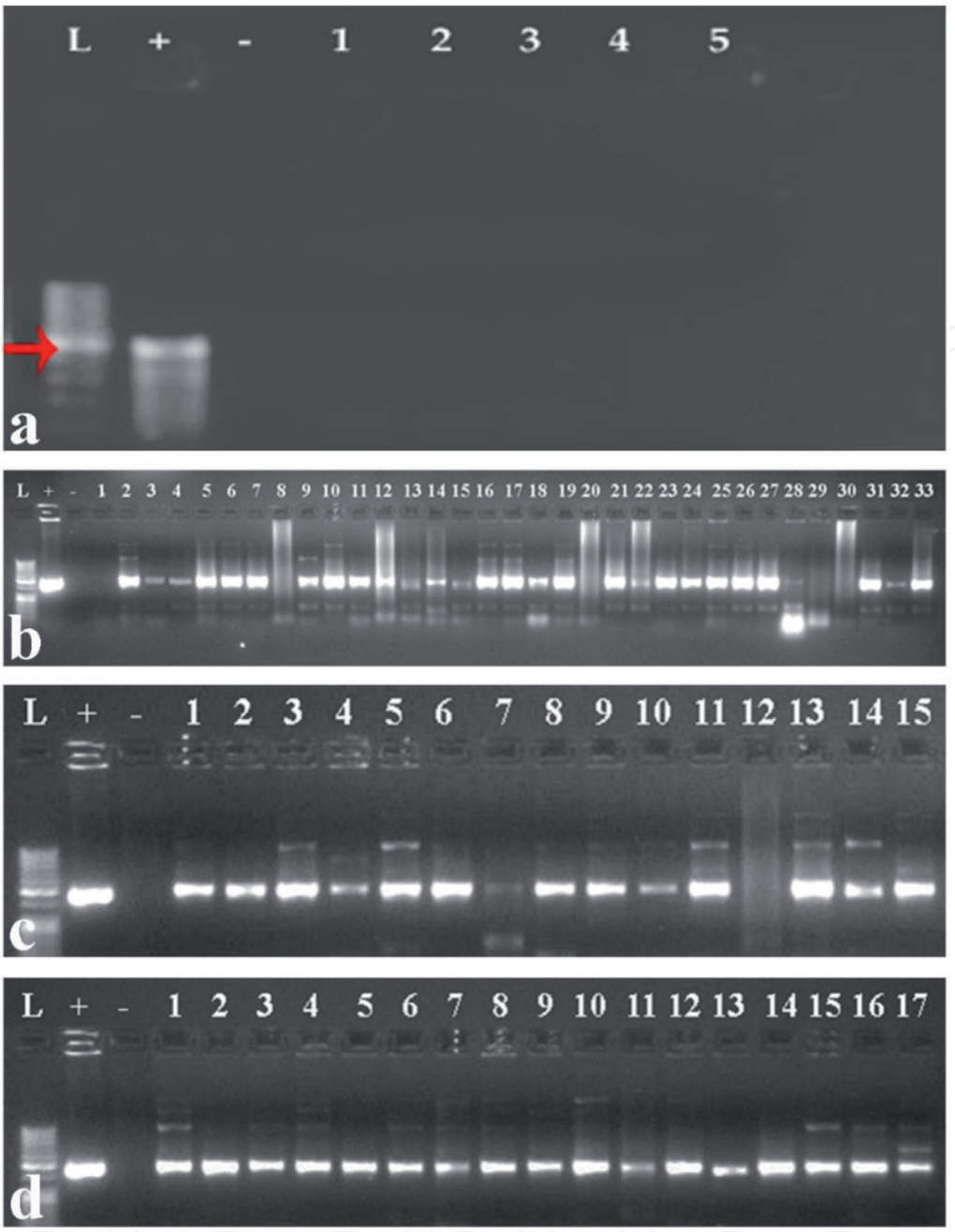

Figure 2.
PCR analysis of genomic DNA of putative transgenic plants grown from stem parts having axillary meristems of irradiated seedlings for the amplification of npt-II gene. L-DNA ladder, + positive control, – negative control. a. 0 Gy, b. 40 Gy, c. 80 Gy, d. 120 Gy.

result of the increased recombination mechanism in the irradiated cells, resulting in an increased number of transformed colonies with high integration rates. Similarly, in our study, the positive effect of low dose gamma dose on potato transformation efficiency was determined. Another possible effect of gamma radiation on genetic transformation efficiency may be related to the process of radiation of the target plant. It was reported that protoplast radiation one hour before transformation increased the success rate, whereas radiation performed one hour after transformation had no effect on the transformation efficiency [39]. In our study, gamma irradiation was applied before the gene transfer stage. The results obtained from our study coincide with the results stated above.

From the results of the current study, in the gene transformation to potato stem parts having axillary meristems by *A. tumefaciens*, it was observed that 40 Gy

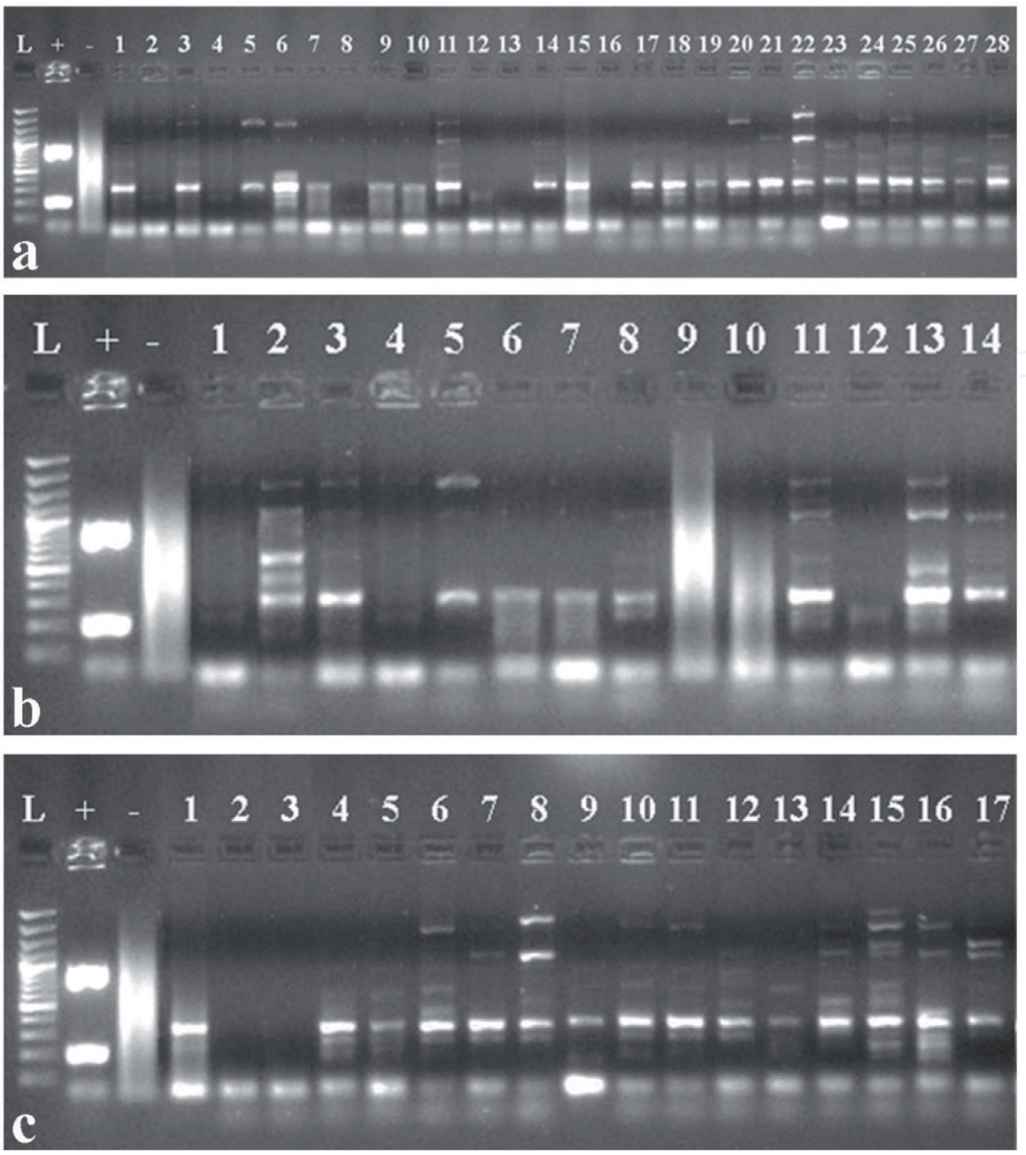

Figure 3.
PCR analysis of genomic DNA of putative transgenic plants grown from stem parts having axillary meristems of irradiated seedlings for the amplification of chv gene. L-DNA ladder, + positive control, – negative control. a. 40 Gy, b. 80 Gy, c. 120 Gy.

gamma dose significantly increased the transgenic plant frequency compared to control in which no gamma was used. To our knowledge, this was the first study revealing gene transformation to stem parts having axillary meristems via *A. tumefaciens* in potato.

DOI: http://dx.doi.org/10.5772/intechopen.99878

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