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REVIEW

Cotton Biotechnology: An Efficient Gene Transfer Protocol via *Agrobacterium tumefaciens* for a Greater Transgenic Recovery

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ABSTRACT

Due to its economic worth, cotton (*Gossypium hirsutum* L.) is grown in almost 70 countries and provides income for more than 250 million people. Therefore, producing cotton with having some desired characteristics that includes extended biotic and abiotic stress tolerance, improved fiber quality, promoted nutritional content and increased yield is the main objective for cotton biotechnology. To achieve this goal, many tissue culture and gene transfer techniques are being developed and used throughout the years. As applications for the gene transfer, the *Agrobacterium*-mediated, particle bombardment and pollen tube pathway-mediated methods are most successfully used and in conjunction with this, meristematic shoot tips as explants are efficiently utilized in gene transfer methods. In this study, the main objective was to report an efficient protocol for a greater recovery of transgenic cotton plant using *Agrobacterium tumefaciens*-mediated transformation. For this, one of the cotton strains (Cukurova 1518) cultivated widely in Turkey was chosen and meristematic shoot tips as explant sources, and *GFP* and *NPTII* genes as reporter and marker genes were used, respectively. The effective post co-cultivation conditions were provided via using the selection regime *in vitro*. Finally, the current results showed highly reproducible protocol developed could be used to produce transgenic cotton plants expressing desired traits or can be utilized as a model system to study the expression of particular genes.

摘要

由于其经济价值, 棉花 (*Gossypium hirsutum* L.) 在近70个国家种植, 为2.5亿多人提供收入. 因此, 棉花生物技术的主要目标是生产具有某些期望特性的棉花, 包括延长生物和非生物胁迫耐受性、改善纤维品质、提高营养含量和增加产量. 为了实现这一目标, 多年来, 许多组织培养和基因转移技术被开发和使用. 作为基因转移的应用, 农杆菌介导、粒子轰击和花粉管通道介导的方法被最成功地使用, 与此相结合, 分生茎尖作为外植体被有效地用于基因转移方法. 在这项研究中, 主要目的是报告一种利用根癌农杆菌介导的转化更有效地恢复转基因棉花植株的方案. 为此, 选择在土耳其广泛种植的棉花品系之- (Cukurova 1518), 分生茎尖作为外植体来源, *GFP*和*NPTII*基因分别作为报告基因和标记基因. 通过体外筛选机制提供了有效的共培养后条件. 最后, 目前的结果表明, 所开发的高重复性方案可用于生产表达所需性状的转基因棉花植株, 或用作研究特定基因表达的模型系统.

KEYWORDS

Gossypium hirsutum L.; gene transfer; meristematic shoot; blotting techniques

关键词

基因转移; 分生芽; 印迹技术

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Introduction

Cotton (*Gossypium hirsutum* L.) belonging to genus *Gossypium* within Malvaceae family is the source of natural fiber and is an oilseed crop in the world (Hocaoglu-Ozyigit et al. 2020). While the cotton fiber is used for production of woven fabrics, the oil from its seeds can be used in cooking and in producing other foodstuffs as well as cosmetics and soap (Tokel 2021). The fatty acid composition of cottonseed oil is used as one of the most important criteria in determination of the industrial applications and nutritional contents (Gao et al. 2020). Nano-fibrillated cellulose materials that have special properties including low density, high specific surface area and strength, dimensional stability, chemical functionality, thermal stability, and good optical transparency produced from cotton fibers and linters using advance nano-technological applications are used in different fields (Bharimalla et al. 2017; Yi et al. 2020).

Cotton economy is of having great importance for cotton producing countries in terms of providing contributions to their GDPs and due to its economic impacts, its cultivation is so widespread all over the world (Khan et al. 2020a; Tokel 2021). According to the latest worldwide cotton production report published by the USDA in October 2021, the cotton productions of the countries (in thousand metric tons) are as of following: China-5,987, India-6,423, USA-3,473, Brazil-2,613, Pakistan-980 and Uzbekistan-762 (USDA 2021). Additionally, the total global transgenic crop cultivation area are 25.7 million hectares and albeit with challenges, the worldwide increase in the plantation rate continues and the top are as: the U.S. with 71.5 million hectares, Brazil with 52.8 million hectares, Argentina with 24 million hectares, Canada with 12.5 million hectares, India with 11.9 million hectares, and the rest of 24 countries with 17.7 million hectares, respectively and transgenic cotton has a large share in it (ISAAA 2021).

Among the GMOs, transgenic cotton is one of the first GMOs, commercially developed and used. The adaptation years by farmers were in the middle of the 1990s, and since then, transgenic cotton has been globally adopted for plantation (Tokel, Genc, and Ozyigit 2021; Zhang 2019). Nevertheless, today it is known that considering of using transgenic cotton for the plantation bears great disadvantages, mostly related with the high cost for the seed and technology fee (Blaise et al. 2020).

Painstaking process for commercialization approval for a biotech product is unavoidable due to requirements enforced by the regulatory authorities. Considering paying fee for the genes developed by a legal entity is a limiting factor countries, especially developing ones, adoption of this technology (Chaudhary and Singh 2019; Tabashnik, Carrière and Gassmann 2019).

Abiotic (drought, salinity, high temperature, and pollutants-especially heavy metals, polyaromatic hydrocarbons, herbicides and insecticides) and biotic (insect predation, viral and fungal infections) effect cotton yield in the fields (Hocaoglu-Ozyigit et al. 2020). Over the years, the limited achievements have been gained by the breeding practices for cotton through using conventional methods but the progress has been slow and time-consuming. Thus, biotechnological applications have been used rather than classical applications (Singh 2018; Tokel, Genc, and Ozyigit 2021).

Improving resistance to insects and tolerance to herbicides via putting transgenic technologies have particularly provided significant contributions to cotton agronomic performance (Tokel, Genc, and Ozyigit 2021; Zhang 2019). Thus, considerable pay attention is given for the development of tissue culture and gene transfer technology for cotton (Kesiraju et al. 2020).

In this study, a rapid and an efficient protocol for *Agrobacterium*-mediated transformation is presented. For the transformation, a widely cultivated cotton variety (Çukurova 1518) was employed along with plasmid pBI121 bearing the *GUS* reporter and *NPTII* marker genes as well as nos terminator. The *GUS* reporter and *NPTII* marker genes were controlled by the CaMV 35S and nos promoters, respectively.

History of cotton tissue culture and gene transfer studies

The first regeneration systems for cotton were established in the middle of the 80s. First successful plant regeneration was performed by Davidonis and Hamilton in 1983 using two-year-old calli from *G. hirsutum* L. cv. Coker 310 via somatic embryogenesis (Davidonis and Hamilton 1983). In following

years, the rate of regeneration success was extended through other works with using other commercial cottons including Acala and Pima cultivars, of which have high fiber quality, and a broad range of additional Mid-South upland cotton varieties (Rangan and Rajasekaran 1997; Rangan, Zavala, and Ip 1984; Sakhanokho and Rajasekaran 2016; Sakhanokho et al. 2001; Shoemaker, Couche, and Galbraith 1986; Trolinder and Goodin 1987).

Having as disadvantages including genotype dependent regeneration, prolonged culture period, low conversion rate somatic embryos in generating plantlets, high frequency abnormal embryo development, lack of shoot elongation and poor rooting, the somatic embryogenesis and direct organogenesis are the two main methods in cotton tissue culture practices (Kumria et al. 2003; Ouma, Young, and Reichert 2004; Ozyigit and Gozukirmizi 2009). Also, another problem in producing cotton tissue culture is phenolic oxidation dependent explant browning affecting tissue culture success (Ozyigit 2008; Ozyigit, Kahraman, and Ercan 2007). The *in vitro* regeneration of cotton is performed under suitable conditions that are depended on the explant type and factors including the types and composition of media hormones or other physical and environmental parameters surrounding the cultures (Ozyigit and Gozukirmizi 2009; Sakhanokho and Rajasekaran 2016).

Albeit, the difficulties arisen in tissue culturing of cotton, the first successful *Agrobacterium*-mediated transformations for cotton were reported in the year of 1987 (Firoozabady et al. 1987; Umbeck et al. 1987). Hypocotyl and cotyledon explants of Coker 210 cultivar were used for somatic embryogenesis and following co-cultivations were conducted with *A. tumefaciens* bearing neomycin phosphotransferase II (*NPTII*) gene that provides resistance to plant for kanamycin. Since then, genetically engineered cottons bearing insect- and herbicide-resistant genes were successfully created in the beginning of the 1990s and commercially available transgenic cotton cultivars were started to be used for planting in the year of 1995 (Hussain, Husnain, and Riazuddin 2007; Ozyigit and Gozukirmizi 2008; Song et al. 2000). *Agrobacterium*-mediated, biolistic particle delivery and pollen tube pathway-mediated genetic transformation approaches are three main applications used for introducing a gene to cotton. And, almost all transgenic cotton plants are currently being generated through using these three applications (Ahmed et al. 2020; Rajasekaran 2019; Wang et al. 2019).

As an alternative transformation technique, the biolistic particle delivery system is also used to create transgenic cotton (Ozyigit and Yucebilgili Kurtoglu 2020; Rajasekaran 2019). The gene gun delivery system that works by throwing gold beads coated with DNA at high velocity was utilized for the direct delivery of foreign genes into the meristematic tissue of excised cotton embryonic axes (McCabe and Martinell 1993). Also, a successful gene transformation to cotton using pollen tube pathway-mediated transformation approach was realized by Zhou et al. (1983). As a tissue culture (or genotype)-independent transformation approach, the pollen tube pathway-mediated transformation does not require expensive instruments. Since, being affordable it can be performed easily (Wang et al. 2019).

By the middle of the 1990s, the transgenic Bt cotton having resistance against insects was planted by the farmers. And since then, transgenic insect-resistant Bt cotton was adopted by the farmers quickly and planted globally (Mahmood and Hussain 2020). Currently, there are two kinds of transgenic Bt cotton that are planted in the fields, one of which is insect-resistant and, the other of which is herbicide-tolerant. The transgenic insect-resistant Bt cotton was first approved in 1993 for field trials for production in the USA and was started to be used commercially in 1995. The approved transgenic insect-resistant Bt cotton was used by China in 1997 and thereafter 2002, the other countries including India and Mexico, Argentina, Australia and South Africa, Brazil, Burkina Faso and Colombia started to the cultivation practices of transgenic insect-resistant Bt cotton (Kranthi and Stone 2020; Tokel, Genc, and Ozyigit 2021). The field trials proved that using Bt cotton having resistance to insects, the damages caused by bollworm and leaf-fed Lepidoptera were taken down to some extends. It was as much as 93–100% for the damage caused by bollworm. Related with this, according to a published report, pesticide application could be reduced by up to 70% via planting transgenic Bt cotton (Zhang 2019). Bt cotton having tolerance to herbicides is another success story in transgenic plants generated. Currently, a number of countries including the USA, Canada and Australia widely use herbicide

tolerant Bt cotton in agricultural practices (Ceccon et al. 2020). Its practices provide significant economic and societal benefits to the cotton farmers and can be considered as an excellent weed control method adopted by the cotton farmers (Iqbal, Khaliq, and Cheema 2020; Tokel, Genc, and Ozyigit 2021). Currently, the CRISPR/Cas9-mediated genome editing system has been reported for being used successfully as an effective gene-targeting tool in wide range of organisms as well as in cotton (Khan et al. 2021; Ozyigit, Can, and Dogan 2020; Wang et al. 2020).

Other than providing resistance against insects and tolerance to herbicides, biotechnology is also employed to produce solutions for abiotic (drought, salinity, water logging, erratic light, extreme temperatures and mineral imbalance) and biotic (insect predation and viral-fungal infections) stresses as well as cellulose biosynthesis and fiber quality through modifying cotton (Hocaoglu-Ozyigit et al. 2020; Mandal et al. 2020; Morello et al. 2020; Zhao et al. 2020). Numerous genes and microRNAs (as transcription factors) are proven to be having critical roles in cotton fiber initiation and development as well as response to biotic and abiotic stresses (Chen et al. 2020; Salih et al. 2020; Wang et al. 2017).

Agrobacterium-mediated genetic transformation is the most widely used and is most successful transformation technique, particularly for dicot plant species (Zhang 2019). The main challenge in *Agrobacterium*-mediated transformation is recalcitrancy in achieving of the production of the tissue culturing of commercially important cotton varieties (Emani 2016; Kesiraju et al. 2020). Looking at the history of cotton along with the usage of *Agrobacterium*, Coker and Acala in which have high regeneration potential were suitable cultivars for genetic transformation (Katageri et al. 2007; Mishra et al. 2003; Rajasekaran et al. 1996). Another problem for cotton is related with genotype dependent transformation capacity (Ozyigit and Gozukirmizi 2009; Ozyigit, Kahraman, and Ercan 2007). After the introduction of the regenerative competence from Coker to selected recalcitrant lines using conventional breeding, a Coker 310FR cultivar was generated and used for genetic transformation (Kumar, Sharma, and Pental 1998). However, transformation of the transgene from the Coker 310FR carried out using conventional breeding could cause introgression of undesirable characters from Coker 310FR. Thus, the transformation for the selected genotypes is worthy (Katageri et al. 2007). Also, other successful efforts using alternative methods for directly transforming selected genotypes have been reported (Gould and Magallanes-Cedeno 1998; Zapata et al. 1999). As examples, the genetic transformations for two Indian cotton genotypes were accomplished using shoot apices (Satyavathi et al. 2002) and a transformation protocol with minor modifications described by Gould et al. (1991) used for a selected genotype of Indian cotton was carried out using regenerated shoot apical meristems treated with *Agrobacterium* (Katageri et al. 2007). Using shoot apex (Gould and Magallanes-Cedeno 1998; Zapata et al. 1999) and apical meristem explants (Nandeshwar et al. 2009) for *Agrobacterium*-mediated transformation was a strategy for including different cotton cultivars (Emani 2016). The frequency of occurrence of genetic mutations and somaclonal variations in plants regenerated from shoot apices of explants due to the absence of tissue differentiation phases including callus induction or somatic embryo formations are low (Ozyigit and Gozukirmizi 2009). Thus, in the many recent studies, meristematic shoot apices have been used. Also, the tissue culture independent systems and/or genome editing tools have been recently employed for successful gene transformations. As examples: A synthetic gene providing tolerance for herbicides has been recently introduced to cotton using *Agrobacterium* strain LBA4404. In the study, the tissue culture independent in-planta transformation system was employed for integrating of the synthetic gene into cotton plants at three different stages in flowering time-range after pollination (Nazir, Iqbal, and Ullah 2020). An efficient and reproducible *Agrobacterium tumefaciens*-mediated transformation method for cotton using a shoot apex explant with the combination of microinjection and sonication was developed by Gurusaravanan, Vinoth, and Jayabalan (2020). Because of the recalcitrant nature of cotton, a genotype-independent non-tissue culture-based apical meristem-targeted in planta transformation approach was employed for developing of transformants (Karthik et al. 2020). An efficient *Agrobacterium*-mediated genetic transformation procedure using embryonic axis in cotton was established by Ahmed et al. (2020). In the procedure, the binary plasmid p35S-GUS-INT containing *NPTII* and *β-GUS* genes, of which are controlled by nos and CaMV 35S promoters, respectively, was utilized. In order to provide resistancy to cotton against leaf curl virus

disease, an *Agrobacterium*-mediated delivery of multiplex CRISPR/Cas9 system was constructed by Javed (2020). PCR results of this work showed that the CRISPR/Cas9-3gRNAs complex was completely transformed into the cotton plants through *Agrobacterium*, and the plants having the CRISPR/Cas9 constructs did not show the symptoms of the cotton leaf curl virus disease. A combined approach including usage of both CRISPR/Cas9 and *Agrobacterium* for forming resistancy against cotton leaf curl virus was developed by Khan et al. (2020b). And, a heat-inducible CRISPR/Cas12b (C2c1) genome editing system was applied by Wang et al. (2020) in tetraploid cotton plants in order to generate the mutants of upland cotton and following *Agrobacterium*-mediated genetic transformation under a range of temperatures, successful results were obtained. As proved by the latest studies given above as examples, even though alternative methods and technologies are also used, *Agrobacterium*-mediated genetic transformation in cotton biotechnology involving gene transfer still keeps its importance.

The tissue culture and gene transfer protocol presented in this review article was prepared using the related literature (Ozyigit 2012; Dogan, Ozyigit, and Demir 2012; Ozyigit and Gozukirmizi 2009; Ozyigit and Gozukirmizi 2008; Ozyigit, Kahraman, and Ercan 2007; Ozyigit, Gozukirmizi, and Semiz 2006) and it is believed that that this highly reproducible protocol could be used as a model system to produce transgenic cotton plants expressing beneficial traits.

Gene transfer to cotton via *Agrobacterium tumefaciens*

Culture media used for A. tumefaciens *production*

1 LB solid media

The media contains 10 g/L Bacto-tryptone, 5 g/L yeast extract, and 10 g/L NaCl (Sigma-Aldrich). They are dissolved in a beaker using a magnetic stir bar and the pH is adjusted to 7.5 with 1N NaOH before autoclaving. Final volume is 1 L. 15 g/L of Bacto-agar (Becton Dickinson) is added and after sterilization (autoclaving 25 min under standard conditions), media is dispensed into standard sterile petri plates (90 mm × 15 mm) (Jacobsen et al. 2006).

LB liquid media

10 g/L Bacto-tryptone, 5 g/L yeast extract, and 10 g/L NaCl (Sigma-Aldrich) are dissolved in a beaker using a magnetic stir bar and the pH is adjusted to 7.5 with 1N NaOH. Final volume is 1 L. The medium is sterilized by autoclaving 25 min under standard conditions. Before incubation with *A. tumefaciens*, the media is supplemented with 58.8 mg/L acetosyringone and is poured into 15 mm culture tubes (Sigma-Aldrich) (Taylor et al. 2006).

Kanamycin monosulfate

For the preparation of 50 mg/mL kanamycin monosulfate stock, 500 mg kanamycin monosulfate (Sigma-Aldrich) is dissolved in 8 mL ddH_2O. A magnetic stir bar is used when it is necessary. The final volume is adjusted to be 10 mL by adding ddH_2O. After filter-sterilization, the solution is divided into 1-mL aliquots and then stored at −20°C (up to 6 months) (Gasic and Korban 2006).

Acetosyringone

For the preparation of 100 mM acetosyringone stock, 0.392 g acetosyringone (3', 5', dimethoxy-4′hydroxy-acetophenone, Sigma-Aldrich) is dissolved in 10 mL of dimethyl sulfoxide (DMSO, Sigma-Aldrich) and then diluted to 1:1 using ddH_2O. A magnetic stir bar is used when it is necessary. The final volume is to be a 20 mM solution. After filter-sterilization, the solution can be stored as 0.5-mL aliquots in Eppendorf tubes at −20°C (Frame, Paque, and Wang 2006).

Cotton explant culture media

Sterilization of media used is done by autoclaving in 500-mL flasks at 121°C, 1.2 bars for 15 min. Growth regulators, antibiotics and vitamins were filter sterilized, and are added to the autoclaved media when the ambient temperature of autoclaved media is about 50–55°C. Each petri plate (90 mm × 15 mm) or culture tube (15 mm) (Sigma-Aldrich) containing 20 mL of solid media is prepared under aseptic conditions by using the flow hood and stored at 4–8°C (Dogan, Ozyigit, and Demir 2012; Ozyigit 2009).

MS (Murashigi and Skoog) medium

The medium contains 4.3 g basal salt mixture, 30 g sucrose, 2.2 g phytagel, and 1 mL MS vitamin solution (Sigma-Aldrich) (Table 1). Basal salt mixture and sucrose are dissolved in a beaker using a magnetic stir bar. After adjusting the pH of the media to 5.8 with 1 M NaOH (Merck), agar is added to the media before autoclaving. Following sterilization by autoclaving, the media is supplemented with 1 mL filter-sterilized MS vitamin solution (Sigma-Aldrich) when the ambient temperature of autoclaved media is about 50–55°C. Final volume is 1 L (Dogan, Ozyigit, and Demir 2012; Murashige and Skoog 1962; Ozyigit, Gozukirmizi, and Semiz 2006).

WPM (Woody plant medium)

The composition of the culture medium includes macro- and micro-elements, vitamins, glycine (Table 1) as free flowing powder (Himedia) and additional 30 g sucrose and 2.2 g phytagel (Sigma-Aldrich). In order to bring the medium completely dissolved by constant gentle stirring, the required

Table 1. Compositions and differences of MS (Murashige and Skoog 1962) and WPM (McCown and Lloyd 1981).

Media Type		MS	WPM
Composition	Molecular formula	Concentration (mg L^{-1})	Concentration (mg L^{-1})
Macronutrients			
Ammonium nitrate	NH_4NO_3	1650.00	400.00
Ammonium phosphate	$NH_4H_2PO_4$	-	-
Potassium nitrate	KNO_3	1900.00	-
Calcium chloride	$CaCl_2{\cdot}2H_2O$	440.00	-
Calcium chloride	$CaCl_2$	-	72.50
Calcium nitrate	$Ca(NO_3)_2{\cdot}4H_2O$	-	386.00
Potassium di hydrogen orthophosphate	KH_2PO_4	170.00	170.00
Potassium sulfate	K_2SO_4	-	990.00
Magnesium sulfate	$MgSO_4{\cdot}7H_2O$	370.00	180.70
Micronutrients			
Sodium EDTA	Na_2EDTA	37.30	37.30
Ferrous sulfate	$FeSO_4{\cdot}7H_2O$	27.80	27.80
Boric acid	H_3BO_3	6.20	6.20
Manganese sulfate	$MnSO_4{\cdot}4H_2O$	22.30	22.30
Potassium iodide	KI	0.83	-
Zinc sulfate	$ZnSO_4{\cdot}7H_2O$	8.60	8.60
Sodium molybdate	$Na_2MoO_4{\cdot}2H_2O$	0.25	0.25
Copper sulfate	$CuSO_4{\cdot}5H_2O$	0.025	0.25
Cobalt chloride	$CoCl_2{\cdot}6H_2O$	0.025	-
Vitamins			
Myo-inositol	$C_6H_{12}O_6$	100.00	100.00
Thiamine HCl	$C_{12}H_{17}ClN_4OS{\cdot}HCl$	0.10	0.50
Nicotinic acid	$C_6H_5NO_2$	0.50	0.025
Pyridoxine HCl	$C_6H_{11}NO_3{\cdot}HCl$	0.50	0.025
Amino acid			
Glycine	$C_2H_5NO_2$	2.00	1
Sugar			
Sucrose	$C_{12}H_{22}O_{11}$	30000.00	30000.00

quantity of powder (in 2/3 of total volume) is added. Before autoclaving, heat stable supplements are added to the medium and the final volume is made up using distilled water. The pH of the medium is adjusted to 5.75 ± 0.5 using 1N NaOH or HCl. After adding phytagel, the medium is heated to boiling point for dissolving of phytagel completely and following dissolving of phytagel, sterilization is done by autoclaving at 15 lbs and 121°C for 15 min. Then, the autoclaved medium is cooled down to about 45°C and the supplements feeble against heat are added aseptically under a laminar airflow unit using sterile culture vessels for preparation of the desired amount of medium (McCown and Lloyd 1981; Ozyigit and Gozukirmizi 2009; Ozyigit, Kahraman, and Ercan 2007).

Compositions and differences of MS and WPM are given below

Kinetin (KIN)

1 mg/mL stock solution are prepared by dissolving of 100 mg kinetin in 1 N NaOH and the final volume is brought to 100 mL with deionized distilled water. After filter-sterilization (0.22-µm pore size), it is stored at 4°C or frozen in aliquots at −20°C (Déchamp et al. 2015).

Naphtalene acetic acid (NAA)

After weighing of 20 mg NAA (Sigma-Aldrich), it is dissolved in 2 mL 1 N NaOH (Merck) for preparation of 1 mg/mL stock. To make 20 mL of final volume, ddH_2O is added and then stored at 4°C (up to 6 months). Filter-sterilization is performed by using 0.22-µm pore sized filters before utilization of it (Hervé and Kayano 2006).

Indole-3-butyric acid (IBA)

For preparation of 1 mg/mL stock, 10 mg IBA (Sigma-Aldrich) is weighed and then dissolved in 2 mL 1 N NaOH (Merck). A magnetic stir bar is used if it is necessary. A final volume of 10 mL was achieved by adding ddH_2O and it is stored at 4°C for up to 6 months. Before utilization of it, sterilization is performed by using 0.22-µm pore sized filters (Gasic and Korban 2006).

Plant watering solution

Hoagland solution

For preparation of stock solution, the steps shown below are followed.

a) 1.00 M $NH_4H_2PO_4$ is used as being at 1 mL/L in Hoagland solution.

b) 1.00 M KNO_3 is used as being at 6 mL/L in Hoagland solution.

c) 1.00 M $Ca(NO_3)_2$ is used as being at 4 mL/L in Hoagland solution.

d) 1.00 M $MgSO_4$ is used as being at 1 mL/L in Hoagland solution.

* Preparation of micronutrient stock. The microelements below are diluted in a total volume of one liter of ddH_2O. 1 mL/L of this stock mixture is used along with the above stocks (a–d).

2.86 g H_3BO_3, 1.81 g $MnCl_2.4H_2O$, 0.22 g $ZnSO_4.7H_2O$, 0.08 g $CuSO_4.5H_2O$, 0.02 g $H_2MoO_4.H_2O$ (Assaying 85% MoO_3)

*Preparation of iron stock: For making of iron stock, 26.1 g EDTA is weighed and dissolved in 286 mL of ddH_2O consisting ~19 g KOH. After that, 24.9 $FeSO_4$ $7H_2O$ is dissolved in ~500 mL ddH_2O. And then, 0.25 ml of iron stock is added to the above 5 stocks for 1 L of Hoagland solution.

The iron sulfate solution is mixed with the potassium EDTA. This solution is stirred slowly and aerated using an aquarium pomp overnight. The pH rises to about 7.1 and the color of solution looks like wine red. During the process, very little precipitation occurs. The final volume is 1 L. These solutions can be stored in bottles covered with foil for providing dark conditions at the room temperature (better if stored at +4°C) (Dogan, Ozyigit, and Demir 2014; Hoagland and Arnon 1950; Ozyigit et al. 2017).

Methodology

Seed disinfection

Fifty seeds are utilized for each treatment and the seed coats of the seeds are subjected to be removed using sterile scalpel and forceps in sterile petri plates before the application of surface sterilization. Using the laminar flow hood, the seeds are immersed in 70% ethanol (Sigma-Aldrich) for 3 min and then stirred in 20% commercial bleach (ACE Lever Co.) for 20 min in a 50 mL autoclaved glass bottle. The surface sterilized seeds are rinsed 3 times using sterile distilled water for 5 min. Later on, they are dried on sterile filter papers in a sterile petri plate (Figure 1a).

Seed germination

Sterilized and dried seeds having no seed coats are placed in upside down position in (76 × 76 × 102 mm) Magenta vessels (Sigma-Aldrich) containing 40 mL MS media supplemented with no hormones at upside down orientation using the laminar flow hood (Figure 1b). For each vessel, five seeds are germinated. The seeds are kept for germination in a growth chamber with a photoperiod of 16 h light (40 μE/m^2/s) and 8-hours dark, at 24°C for one week (Figure 1c).

Culture for A. tumefaciens

Prior to inoculation, *A. tumefaciens* from a permanent glycerol stock is streaked out onto an LB agar plate containing 100 g/L kanamycin and incubated at 28°C for 2 days. Following of the appearance of the colonies, only a single colony adequate for the application is transferred into 15 mm sterile culture tube or Schott Glass Bottle (Sigma-Aldrich) containing 50 mL of liquid LB medium with 100 mg/L kanamycin and 58.8 mg/L Acetosyringone from the agar plate and the colony is incubated for overnight in a temperature controlled incubator-shaker under continuous shaking mode at 175 rpm and 28°C (Figure 1d). Once again, *A. tumefaciens* culture grown overnight is transferred into a sterile culture tube (Sigma-Aldrich) containing 50 mL liquid LB medium with an appropriate antibiotic, for selection of *A. tumefaciens* carrying a specific vector pBI121 together with β-glucuronidase (*GUS*) gene with an intron and a selectable marker neomycinphosphotranferase (*NPTII*) gene (Figure 1e), used in the application, and grown until a period of reaching 1.3–1.5 OD_{600} reading in an incubator under continuous shaking mode at 250 rpm and 28°C. Before the inoculation of cotton explants, the *A. tumefaciens* culture is centrifuged at 6000 g and 24°C for 5 min. Then, the inoculum is prepared by adjusting bacterial suspension OD_{600} readout to be 0.4–0.5 using liquid MS media.

Preparation and inoculation of cotton explants

The procedures used in applications are undertaken using a laminar flow hood. After a 7 day of germination period, young plantlets are gently removed and laid the plants down on a sterile petri plate. Meristematic shoot tips are dissected out using sterilized scalpels and micro-forceps and then, the explants are transferred to sterile petri plates or other sterile containers (Figure 1f).

The meristematic shoot tips are placed into a Schott Glass Bottle having adjusted bacterial suspension OD_{600} readout to be 0.4–0.5 using liquid MS media and incubated on a rotary shaker with an agitation speed of 30 rpm at room temperature for 20 min (Figure 1g). Almost 50 explants are used for each treatment. Gene transfer occurred in this step (Figure 1h). The sterile filter papers are placed onto petri plates. MS media is pipetted off along with *A. tumefaciens* and explants are placed onto these sterile filter papers for removing excess liquid having *Agrobacterium*. Then, the explants are dried. After this step, the dried explants are transferred into standard sterile petri plates containing solid MS media supplemented with no hormones. The cultures are kept in a growth chamber at 24°C in dark for 2 days (Figure 1i).

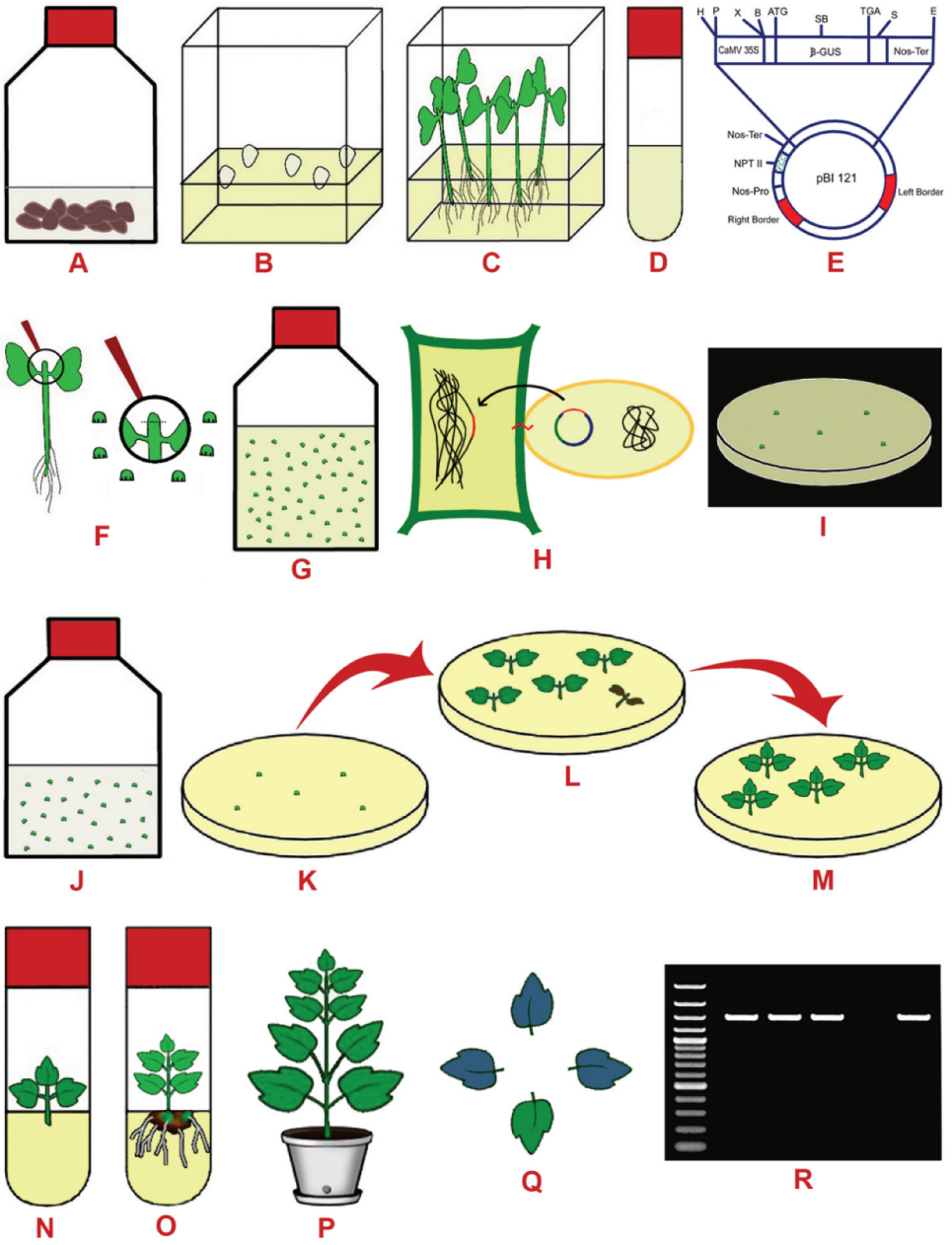

Figure 1. A modified *Agrobacterium*-mediated gene transfer protocol for cotton, (a) Surface sterilization, (b) Planting seeds, (c) 1-week-old young plantlets, (d) Culturing *A. tumefaciens*, (e) Ti plasmid, (f) Isolation of meristematic shoots, (g) Inoculation of *A. tumefaciens*, (h) Gene transfer to cotton meristematic shoots, (i) Culturing explants in darkness, (j) Removing *A. tumefaciens* by washing with cefotaxime, (k–m) Culturing explants on selective media, (n–o) Rooting, (p) Adaptation to the soil, (q) Histochemical *GUS* assay, (r) Molecular analysis (Modified from Ozyigit 2012; Ozyigit and Gozukirmizi 2008; Ozyigit, Gozukirmizi, and Semiz 2006).

Regeneration, selection and rooting of cotton explants

After co-cultivation, the explants are removed from the petri plates. In following step, they are rinsed for 30 minutes using half-strength MS liquid media consisting of 250 mg/L cefotaxime and dried on sterile filter paper (Figure 1j). Then, the explants are transferred onto MS media supplemented with 1 g/L Polyvinylpyrrolidone (Sigma-Aldrich) (for preventing the browning of the explants with

phenolic oxidation) 0.1 mg/L KIN + 2 mg/L NAA + (for regeneration) and 50 mg/L Kanamycin (for selection) and kept in a growth chamber with a photoperiod of 16 h light (40 μE/m^2/s) and 8-hours dark, at 24°C for 10 days (Figure 1k).

Shoot regeneration appears after a 15 day of culturing period on MS + 0.1 mg/L KIN + 2 mg/L NAA + 1 g/L PVP (Figure 1l). Regenerated plantlets are subcultured on the same media (Figure 1m). One week later, thin and weak roots are obtained (Figure 1n). After that, plantlets transferred into culture tubes containing Woody Plant Medium (WPM) supplemented with 1 mg/L indole-3-butyric acid (IBA). After a week, the basal parts of explants became very thick and then strong and tick roots are obtained for all plantlets (Figure 1o).

Transfer to the soil

The well-rooted plantlets are transferred into small pots containing sterile soil mix (1:1:1 of soil, peat, and perlite) and covered with a nylon bag for retaining humidity. They are kept in growth chamber under the same conditions applied for regeneration of the explants for 2 weeks. The bag is progressively opened by making 1 cm hole in a day during the next 5 days. After 2 weeks, the plants are transferred into bigger pots having a mixture of soil/sand (3:1, v/v) (Figure 1p). The plants are watered with full strength Hoagland solution in both growth chamber and greenhouse at three to four times in a week.

Histochemical GUS assays

The leaves from two weeks old plantlets are used for the histochemical *GUS* assay. After washing them for 30 min using 50 mM phosphate buffer (Sigma-Aldrich) (pH 7.0), they are placed in a fixation solution (0.3% formaldehyde, 10 mM MES, 0.3 M mannitol, Sigma-Aldrich) for 10 min. Then, they are subjected to a solution containing1 mM X-gluc (5-bromo-4-choloro-3-indolyl-b-glucuronic acid, Sigma-Aldrich) and left overnight at 37°C for the development of blue color. *GUS* activity is also obtained by histochemical assays using overnight cultures of *Agrobacterium* carrying pBI121 and pGUS-Int (Figure 1q), whereas control plants did not develop blue color.

PCR analyzes

For genomic DNA isolation, 2-month-old plant leaves are used. Specific forward ("*GUS* 1" 5' GGT GGG AAA GCG CGT TAC AAG 3') and reverse ("*GUS* 2" 5' GTT TAC GCG TTG CTT CCG CCA 3') primers of the *GUS* gene are utilized in PCR run driven by thermocycler for 60 s at 95°C, 60 s at 36°C and 90 s at 72°C for 35 cycles and the PCR is completed for 10 min at 72°C (Jefferson, Burgess, and Hirsh 1986). PCR products of DNA fragments are visualized on agarose gel electrophoresis (Figure 1r).

Hybridization by Southern blotting

Approximately, 8 μg of genomic DNA digested with XbaI (Sigma-Aldrich) is used for carrying out the transformation experiments and following assays related with the detection of the *GUS* gene integrated into plant genome and estimation of the number of insertions. The XbaI digested DNA is run on a 1% agarose gel and transferred to nylon membrane for the observation of DNA fragments after fixation by UV crosslinking. Hybridization analysis is conducted by using the nonradioactive digoxigenin (DIG) hybridization system (Sigma-Aldrich). The product generated by amplification of the *GUS* gene by PCR is labeled with DIG-dUTP and used as a probe for Southern blotting hybridization conducted at 68°C. The steps in accordance with Manufacturer's instructions were followed for immunological detection.

Notes

1. In case of difficulty for removing of the seed coats before sterilization, seeds can be kept for 1 hour under running tap water in a beaker covered with gauze, and then soaked to sterile distilled water in a container for 5 minutes for 3 times. In final step, humidified seed coats can be removed easily with sterile scalpel under laminar hood.
2. *Agrobacterium* strains, including plasmid pBI121 that consists of the *GUS* reporter and *NPTII* marker genes under the control of CaMV 35S and nos promoters are efficient transformation agents for utilization in the defined protocol.
3. Creating growth curve (i.e., inoculum quantity needed for reaching desired OD) and arranging growing conditions (i.e., shaker speed and incubator temperature) for *Agrobacterium* as important parameters for each strain used for transformation are crucial.
4. A very effective two-step rooting procedure (obtaining weak roots in MS + 0.1 mg/L KIN + 2 mg/L NAA + 1 g/L PVP in the beginning and at last, generating strong roots together with callus like structures in WPM + 1 mg/L IBA after subculturing) is utilized for producing young plantlets that rooted well being used under the soil conditions defined.
5. The procedure developed is very efficient for obtaining transgenic plants in high numbers. Also, generating transgenic plants via using meristematic tissues as direct way of organogenesis (without any callus phase and/or somatic embryos) reduces the somaclonal variation and enabling to develop uniform transgenic plants.

Conclusion

As known by the tissue culture and gene transfer research previously performed in cotton, prolonged culture period, low conversion rate of plantlets, lack of shoot elongation, poor rooting, browning and blackening were the problems seen in explants due to phenolic oxidation. Nevertheless, these problems encountered previously were not observed in our experimental period because of the protocol we applied (Kumria et al. 2003; Ouma, Young, and Reichert 2004; Ozyigit 2008; Ozyigit and Gozukirmizi 2009; Ozyigit, Kahraman, and Ercan 2007; Sakhanokho and Rajasekaran 2016). These problems are the causes, negatively affecting not only success in the generating tissue culture but also the yield for the transgenic plant. The use of meristematic tissues, adding PVP to the nutrient medium, and applying a two-stage rooting system had important contributions for the results we had. As a result, highly healthy and uniform full transgenic plants were obtained in our research. Bt cotton is of great importance in worldwide today and the cotton plant is not only exposed to insect attacks but also to other stress conditions, both biotic (viral and fungal infections) and abiotic (drought, salinity, high temperature and pollutants – especially heavy metals, polyaromatic hydrocarbons and herbicides). As an important tool for transferring desired characteristics into the cotton using different gene transfer methods for obtaining tolerant/resistant agricultural cotton lines, genetic engineering is employed frequently. Though successful alternative transformation approaches including tissue culture independent systems (Karthik et al. 2020; Nazir, Iqbal, and Ullah 2020) and/or genome editing tools like CRISPR/Cas have been recently utilized (Javed 2020; Wang et al. 2020), *Agrobacterium*-mediated gene transformation and combined gene transfer systems comprising usage of *Agrobacterium* still keep their importance due to the successes when they are employed (Ahmed et al. 2020; Gurusaravanan, Vinoth, and Jayabalan 2020; Khan et al. 2020b). Thus, this highly reproducible protocol could be used to produce transgenic cotton plants expressing useful traits or can be utilized as model system to study the expression of particular genes.

Disclosure statement

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