ISSN (Online) 2393-8021
ISSN (Print) 2394-1588
International Advanced Research Journal in Science, Engineering and Technology
Vol. 2, Issue 6, June 2015

An Efficient Method of Isolation and Transformation of Protoplasts from Tomato Leaf Mesophyll Tissue using the Binary vector pCambia 1302

Sarmistha Ray[1], Suvanwita Lahiri[1], Madhushree Halder[1], Monimala Mondal[1], Tathagata Ray Choudhuri[2], Surekha Kundu[1]

Molecular and Applied Mycology and Plant Pathology Laboratory, Dept of Botany,

University of Calcutta, Kolkata, India[1]

Asutosh College, Kolkata, India[2]

Abstract: This is a new method of isolation and transformation of protoplasts from tomato leaf mesophyll tissue using polyethylene glycol. The binary vector pCambia 1302 carrying green fluorescence protein sequence as reporter was used and the transformed protoplasts were selected using hygromycin. Important parameters which are critical for the quality of protoplast and efficiency of transformation, such as molecular weight of polyethylene glycol, kinds of osmoticum used and period of incubation, were standardized. The transformed protoplasts were screened by the selectable marker and further confirmed with PCR. The protoplasts were analyzed with propidium iodide and fluorescence microscopy. The transformation efficiency was studied with the assay of the green fluorescence protein. This is the first protoplast isolation protocol for the Pusa Ruby variety of tomato and also the first protocol to use hygromycin as the selectable marker for tomato.

Key words: Tomato, protoplast, mesophyll, pCambia, PEG, fluorescence, GFP, hygromycin.

I. INTRODUCTION

A common approach to studying sub-cellular localization of protein, promoter activities or formation of protein complexes in vivo is by means of transient expression [1]. Transient expression assays allow rapid and high-throughput analysis of genes in plants and thus have become widely used for characterization of gene function. In Arabidopsis, maize, tobacco, rice, and tomato, protoplasts are commonly used for transient assays in cells gene expression, protein sub-cellular localization, protein-protein interaction and protein activity studies [2]. Protoplast transient expression is used as a tool to dissect the functions of cis-elements and trans-factors in many essential processes and signaling pathways [3]. The standard approaches for transient transformation include biolistic approach, Agrobacterium tumefaciens-mediated transient transformation of leaves and protoplast transfection. Each method has its limitations. For example many plants are recalcitrant to Agrobacterium mediated transformation. Moreover Agro bacterium causes alteration in protein expression in protoplasts due to pathogenic stress [4]. Cell bombardment on the other hand causes severe tissue damage, is expensive, and has low transformation efficiency often resulting in chimeras [5]. Protoplasts are thus better as a starting material for transformation because they are tot potent, which allows transgenic plants to be regenerated from single cells Thereby avoiding formation of chimeras and allows production of stable transgenic lines [6].

Polyethylene glycol-mediated (PEG-mediated) protoplast transformation is a superior option among other means [7]. Earlier reports showed that protoplasts isolated from diverse tissues of different plants retained physiological activities and unaltered regulation of different biological processes. For example, freshly isolated mesophyll protoplasts perform active photosynthesis and respiration [8]. In barley (Hordeum vulgare) aleurone protoplasts, the endogenous α-amylase gene is regulated by ABA and GA in parallel to what is observed in seeds [9]. In more recent times genetic transformation of protoplast is being done for diverse plant species, including those belonging to Brassicaceae, Solanaceae and some ornamental plant families [2][10][7].

However, protoplast isolation as well as subsequent downstream analyses is often hindered by a number of factors. In order to work with these highly fragile single cells, the first and most important step is the isolation of viable protoplasts. Several parameters, particularly the source tissue, culture medium, and environmental factors, influence the ability of protoplasts and protoplast-derived cells to express their totipotency in order to develop into fertile plants [1]. For cell culture-derived protoplasts, the plant cell cultures need to be established which is time consuming, cost intensive and requires specific laboratory equipments. In addition, there always persists a perpetual risk of contamination [4]. These problems faced during isolation of plant

ISSN (Online) 2393-8021
ISSN (Print) 2394-1588
International Advanced Research Journal in Science, Engineering and Technology
Vol. 2, Issue 6, June 2015

protoplasts are partially circumvented when using mesophyll-derived protoplasts.

Many mesophyll protoplast isolation procedures involve the cutting of leaves, followed by enzymatic lysis of the cell wall and separation of released protoplasts from non protoplast tissue debris. Uptake of isolated DNA into protoplasts provides the basis for transient and stable nuclear transformation, and also organelle transformation to generate transplastomic plants [2]. Protoplast transformation has the advantage of quick assay of gene expression and characterization of gene function. It also provides opportunity of insertion of multiple genes within a short period of time [3].

These protoplasts can be transformed together and then separated into batches for different studies keeping the original protoplast uniform thus giving rise to uniform experimental sets.

In this study we describe a new protocol for obtaining and transforming protoplasts from leaf mesophyll cells of tomato leaves using hygromycin as the selectable marker. Critical standardizations were made considering the important aspects of PEG- mediated protoplast transformation. Tomato, being a highly studied model crop plant was used as the source of mesophyll cells. In this study a popular Indian variety of tomato (Pusa Ruby or PR) and the plasmid vector pCambia1302 harboring GFP coding region were used. Two different osmoticums were used for protoplast isolation, different concentration of polyethylene glycol, optimization of the incubatory period were made for optimizing the steps of stable transformed protoplast and its integrity. Various confirmations and assay parameters were made for the stable transformed protoplast.

II. MATERIAL AND METHODS

A. Plant material

Seeds of the popular tomato variety, Pusa Ruby (PR) (Sutton Pvt. Ltd., India) were surface sterilized with 3% sodium hypochlorite solution supplemented with Tween 20 (commercially available Polysorbate surfactant) for 30 min followed by washing with sterile distilled water 6 times. The seeds were sown in soilrite (mixture of horticulture grade perlite, Irish peat moss and exfoliated vermiculite in 1:1:1 ratio) and grown in convirons set at 28±1ºC, 16:8h light/dark photoperiod [11].

B. Protoplast isolation

Green leaves from 3-4 weeks plantlets were collected and incubated overnight, at room temperature on a horizontal shaker (40rpm) in enzyme solution (1% cellulase, with different osmoticums used separately 400mM mannitol or 0.4M Tris-HCl, 10mM $CaCl_2$, 20mM KCl, 0.1% BSA, 20mM MES pH5.7). On the following day the protoplast suspension were filtered to separate out the debris and finally transferred to 2ml plastic tubes and pelleted for 3mins at 200 rpm. The cells were washed twice (2min, 100rpm) with W5 (154mM NaCl, 125mM $CaCl_2$, 5mM KCl, 2mM MES pH5.7) and finally resuspended in 500µl W5solution. After a 30mins incubation on ice, protoplast were counted, centrifuged (2mins, 100 rpm) and resuspended in MMg (400mM mannitol, 15mM $MgCl_2$, 4mM MES) to a final density of $2x10^5$-$1x10^5$ cells/ml. [12].

C. Transformation of E.coli and Agrobacterium with GFP plasmid

E coli and Agrobacterium were cultured and transformed using standard protocol with modification [13]. The antibiotic resistant E. coli and Agrobacterium were selected and cultured on appropriate selection media and cultured at 37°C and 28°C respectively.

D. Plasmid extraction by alkaline lysis method

Transformed in E.coli DH5a strain carrying the binary vector pCambia 1302 containing GFP coding regions with CaMV35S promoter and nos as terminator was transformed in E.coli DH5a strain. Then the plasmid was isolated by alkaline lysis method [14].

E. Transformation of protoplast using GFP construct and microscopy

100µl of protoplast ($2x10^5$-$1x10^5$ cells/ml) were added to 1µg plasmid DNA in 2ml microfuge tubes. After mixing gently, 150µl PEG (40 % PEG 4000or/ PEG 6000, 100mM $CaCl_2$, 200mM mannitol) was added. Samples were mixed until it appeared homogenous. The samples were incubated at room temperature, 500µl W5 was added and protoplasts were centrifuged at 600rpm, for 6mins at room temperature in dark. Finally the protoplasts were incubated overnight at room temperature for stable transformation [1].

F. Fluorescence microscopy and GFP fluorescence assay

Transformation efficiency was determined through fluorescence microscopy (Leica). Samples were prepared according to our previously published protocol [15][16]. The fluorescence was quantified with microplate reader (Thermofischer, Germany) using standard protocol.

G. Assessment of cell viability

Propidium iodide was added to the transformed protoplast suspension in W5 solution to a final concentration of 0.04%. Following 24hrs, 48hrs and 72hrs incubation at room temperature in dark these were subjected to microscopy and micro plate reading.

III. RESULTS AND DISCUSSIONS

A. Binary vector, bacterial transformation and plasmid isolation

The binary vector used in this study was pCambia 1302 carrying the reporter green fluorescent protein (GFP) (Fig. 1). The vector was used to first transform E. coli. The transformed E. coli was selected on selection media containing Kanamycin (Fig. 1 b,c). This E. coli was used to isolate plasmid. The plasmid was run on agarose gel electrophoresis to ascertain the quality and quantity of plasmid. (Fig.1d).

ISSN (Online) 2393-8021
ISSN (Print) 2394-1588
International Advanced Research Journal in Science, Engineering and Technology
Vol. 2, Issue 6, June 2015

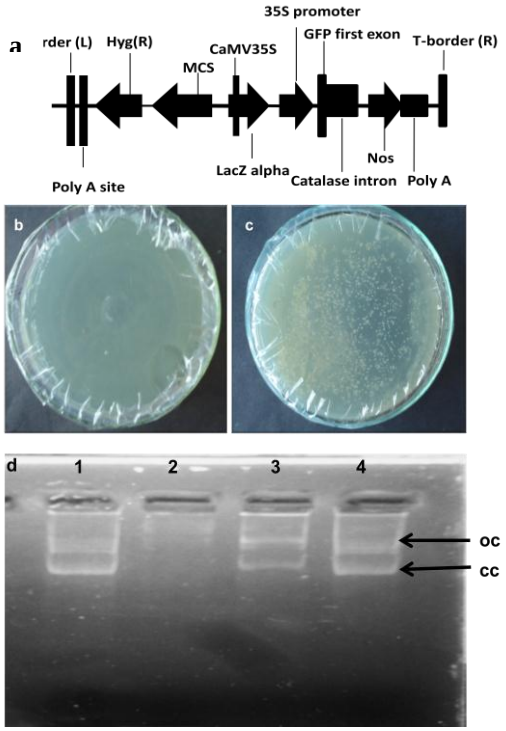

Fig 1. Plasmid construct and bacterial transformation. a. Vector constructs harboring GFP used for protoplast transformation. b-c.

Bacterial transformation with pCambia 1302 in E.coli (DH5α). Plate with kanamycin (50mg/l) showing no growth of untransformed E. coli (DH5α) (b) Plate containing single colonies of E.coli (DH5α) harboring pCambia 1302 screened by kanamycin (50mg/l) (c). d. Isolation of plasmid by alkaline lysis method Lanes 1-4 plasmid with both closed circular, open circular forms.

B. Standardization isolation of protoplasts with two osmoticums

Our first aim was to isolate protoplasts from young expanding leaves of healthy tomato plants by mechanical shearing in presence of Mannitol or Tris-HCl, followed by enzymatic digestion in standardized buffer containing 1% cellulase. The extraction conditions used were 0.4M Tris-HCl in one set (Fig.2a-d) and in another set (Fig.2e-h), the isolation condition was 8% mannitol. The concentrations of protoplasts of each set were calculated using a haemocytometer. It was found that Tris-HCl yielded a higher number of intact protoplasts. Intact leaf mesophyll protoplasts of tomato had a spherical shape. The protoplast concentration was approximately 210cells/µl for Tris-HCl and approximately 150 cells/µl for mannitol (Fig. 2i).

C. Standardization of use of Polyethelene Glycol during transformation of the protoplasts

The measure of transformation efficiencies of PEG-4000 and PEG-6000 was done by Relative Fluorescence Units (RFU) of GFP obtained from the GFP-tagged protoplasts in each case. Maximum Relative fluorescence intensity value was obtained from protoplasts that had been transformed with PEG-4000 showing intact round protoplast (Fig. 3a-d) while that with PEG 6000 was less in number (Fig 3e-h). This was reflected in the RFU values with PEG 4000 showing RF intensity of 24% while that of PEG 6000 was 18% (Fig. 3i). This suggests that PEG-4000 is a better choice to carry out tomato protoplast transformation under these experimental conditions.

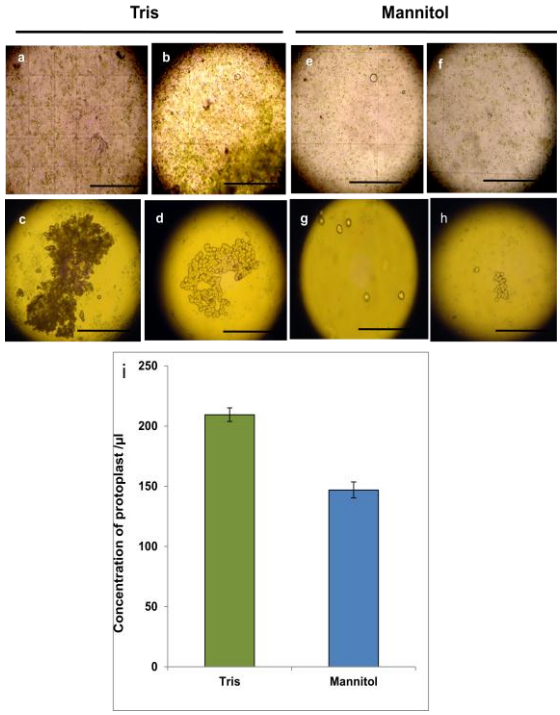

Fig 2. Isolation of intact protoplast from tomato plantlets, using two different osmoticum. a-d. shows isolation with 0.4M Tris; .e-h. shows isolation with mannitol.i. Graphical representation of protoplast concentration /µl quantified by haemocytometer. Bar=100µm.

Since PEG- mediated transformation is a standard method for gene transfer to protoplasts that allows for rapid analysis of transient reporter gene expression, this method was considered as a first step in the development of an efficient transformation protocol [17]. In normal transformation approaches, selectable markers are required to allow the propagation of the rare, stably-transformed cells while killing or suppressing the large excess of non-transformed or transiently-transformed cells in the target tissue [18]. Here GFP was used as a visual marker for recognition of transient expression. GFP was also used as a parameter for assaying efficiency of transformation o protoplast (Fig. 3. a-i).

D. Determination of viability of protoplasts by propidium iodide uptake assay

A viable protoplast must have an intact cell membrane. So when the protoplast suspensions were stained with propidium iodide and incubated for 24 hours, 48 hours and 72 hours respectively, only the damaged protoplasts with broken cell membrane took up the stain (Fig. 4a-i). Viability of such protoplasts was determined by calculating the relative fluorescence emitted by propidium iodide i.e., the relative fluorescence intensity (%) of propidium iodide denote the percentage of non-

ISSN (Online) 2393-8021
ISSN (Print) 2394-1588
International Advanced Research Journal in Science, Engineering and Technology
Vol. 2, Issue 6, June 2015

viable protoplasts. Fluorescence microscopy shows protoplasts transformed using PEG 4000 (MW) expressing GFP e-h. shows protoplasts transformed using PEG 6000 (MW) expressing less GFP. i. Graphical representation of relative fluorescence unit for the two methods of isolation of protoplast. Bar=100µm.

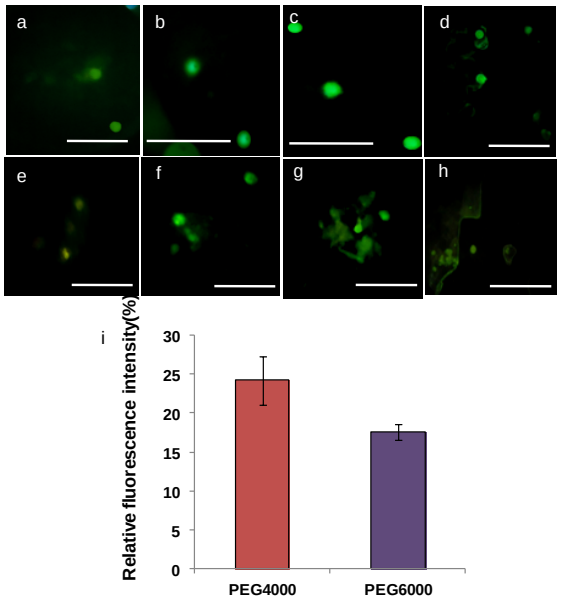

Fig 3 Quantification of transformation efficiency using two different PEG (MW) concentrations by measurinf GFP fluorescence. a-d.

It was observed that the protoplasts degenerated with elapsed time of incubation. The protoplasts incubated for only 24 hours showed the least RFU value of 6%, intermediate Relative Fluorescence Intensity value of 28% for protoplasts incubated for 48 hours, and the highest RF intensity of 49% for the ones incubated for 72 hours. In other words, maximum viability was retained in protoplasts incubated for 24 hours and a little more than 1/3 retained viability after 72 hours (Fig. 4j).

E. PCR confirmation of transformed protoplast

PCR was done using primers specific to GFP sequence to detect the integration of the GFP transgene. In the case of DNA isolated from non-transformed protoplasts, amplification of the target gene sequence was not observed, while the DNA from transformed protoplasts showed a band corresponding to the target sequence (Fig. 5). This observation implies successful transformation of the tomato mesophyll protoplasts.

IV. CONCLUSION

In this study isolation of mesophyll protoplast from Pusa Ruby variety of tomato and transformation of these protoplasts were standardized using the binary vector pCambia 1302. Protoplasts were isolated with two different osmoticums, and the frequency of viable protoplasts was maximum with 0.4M Tris buffer. For transformation of the protoplast, two different concentrations of PEG were used viz. PEG 4000 and PEG 6000. Use of PEG 4000 resulted in more number of viable protoplasts which was verified by cell viability assays. Fluorescence emitted by expressed GFP sequence and that emitted by propidium iodide. Incubation period was found to be critical for obtaining intact, high number of protoplasts, and four hours incubation proved to be optimal.

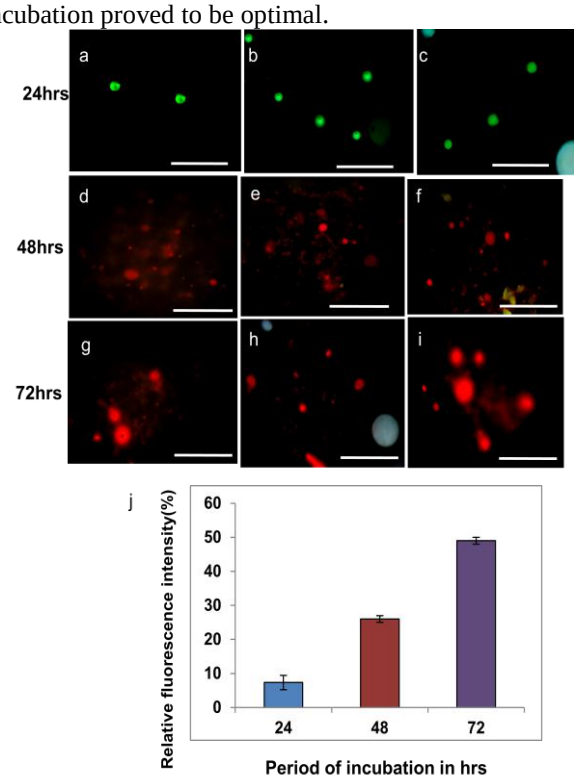

Fig 4 Quantification of protoplast viability with propidium iodide (PI) after different incubation periods. a-c. 24hrs of incubation, d-f. 48hrs of incubation g-j. 72hrs of incubation. i. Graph representing relative fluorescence intensity (%)of protoplasts with PI which were measured using plate reader. Bar=100µm.

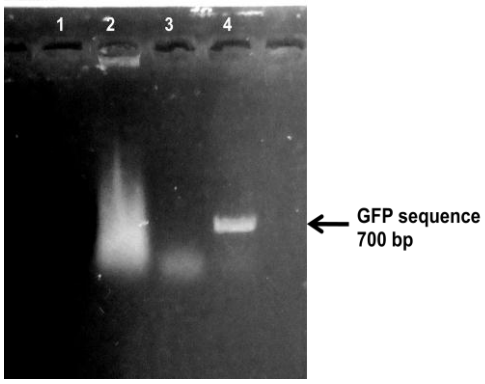

Fig 5 Confirmation of integration of GFP sequence with PCR using primers specific to GFP. Lane 1: dye, Lane 2: Genomic DNA from protoplast, Lane 3: Untransformed protoplast, Lane 4: Transformed protoplast.

All transformed protoplasts were screened by plant selectable marker. The integration of transgene was confirmed by PCR using primers corresponding to GFP sequence. This is the first protoplast isolation protocol

ISSN (Online) 2393-8021
ISSN (Print) 2394-1588
International Advanced Research Journal in Science, Engineering and Technology
Vol. 2, Issue 6, June 2015

for PR variety of tomato and also the first protocol to use hygromycin as the selectable marker for tomato. The present protocol is reproducible and involves minimum handling of equipments and also is cost effective resulting in stable, viable protoplasts.

V. ACKNOWLEDGEMENT

This work was partially supported by Department of Biotechnology, Ministry of Science and Technology, Government of India (DBT). **S. Ray** is thankful to Department of Science and Technology, Govt. of India (DST) for Inspire fellowship. **M. Mondal** is thankful to University Grants Commission (UGC) for his fellowship.

REFERENCES

[1] Mathur J, Koncz C. PEG-mediated protoplast transformation with naked DNA. Methods Mol Biol., 1998, 82:267–276.
[2] Davey MR, Anthony P, Power JB, Lowe KC. Plant protoplasts: status and biotechnological perspectives. Biotechnol Adv., 2005, 23(2):131-71.
[3] Qiudeng Q, Elumalai S, Li X, Zhong H, Nalapalli S, Schweiner M, Fei X, Nuccio M, Kelliher T, Gu W, Chen Z and Chilton M. Maize transformation technology development for commercial event generation. Frontier in plant science review article, 2014.
[4] Pitzschke A, Hirt H. New insights into an old story. Agrobacterium induced tumour formation in plants by plant transformation. EMBO J., 2010, 29:1021–1032.
[5] Masani MYA, Noll G, Parveez GKA, Sambanthamurthi R. (2010). Regeneration of viable oil palm plants from protoplasts by optimizing media components, growth regulators and cultivation procedures. Plant Sci, 2010, 21: 118–127.
[6] Pitzschke A. and Persak H. Poinsettia protoplasts -A simple, robust and efficient system for transient gene expression studies Plant Method., 2012, 8: 14.
[7] Masani MY, Noll GA, Parveez GK, Sambanthamurthi R, Prüfer D. Efficient transformation of oil palm protoplasts by PEG-mediated transfection and DNA microinjection. PLoS One, 2014, 12; 9(5):96831.
[8] Podibelkowska M, Zarska-Maciejewska B, Kacperska- Palacz A. Morphology of protoplast as affected by an inhibition of respiration. Protoplasma, 1975, 83: 201–208.
[9] Jacobsen JV, Beach LR. (1985) Control of transcription of α-amylase and rRNA genes in barley aleurone protoplasts by gibberellin and abscisic acid. Nature, 1985, 316:275–277.
[10] Shen J, Fu J, Ma J, Wang X, Gao C, Zhuang C, Wan J, Jiang L. Isolation, culture, and transient transformation of plant protoplasts. Curr Protoc Cell Biol., 2014, 3; 63: 1-17
[11] Ray S, Mondal S, Chowdhury S, Kundu S. Differential responses of resistant and susceptible tomato varieties to inoculation with *Alternaria solani*. *Physiological and Molecular Plant Pathology*, *2015*, 90, 78–88.
[12] Wu FH, Shen SC, Lee LY, Lee SH, Chan MT, Lin CS. Tape-Arabidopsis Sandwich - a simpler Arabidopsis protoplast isolation method. Plant Methods, 2009, 5:16.
[13] Ray S, Sarkar S and Kundu S. Extracellular Biosynthesis of silver nanoparticles using the mycorrhizal Mushroom Tricholoma crassum (Berk).Sacc. It antimicrobial activity against pathogenic bacteria and fungus including multi-drug resistant plant and human bacteria. Digest Journal of Nanomaterials and Biostructures, 2011, 1289-1299.
[14] Chowdhury S, Basu A and Kundu S. Green synthesis of protein capped silver nanoparticles from phytopathogenic fungus Macrophomina phaseolina (Tassi) Goid with antimicrobial properties against multidrug-resistant bacteria. Nanoscale Research Letters, 2014b, 9:365
[15] Chowdhury S, Basu A, and Kundu S. In-vitro characterization of the behaviour of Macrophomina phaseolina (Tassi) Goid at the rhizosphere and during early infection of roots of resistant and susceptible varieties of sesame. European Journal of Plant Pathology, 2013, 361-375.
[16] Chowdhury S, Basu A and Kundu S. A new high frequency Agrobacterium mediated transformation technique for Sesame indicum L Using de-embryonated cotyledon explants. Protoplasma, 2014a, 625-704.
[17] Miao Y, Jiang L. Transient expression of fluorescent fusion proteins in protoplasts of suspension cultured cells. Nat Protoc., 2007, (10):2348-53.
[18] Twyman RM, Stoger E, Kohli A, Capell T, Christou P. Selectable and screenable markers for rice transformation. Molecular Methods of Plant Analysis, 2002, 1-11.