Journal of Pharmacognosy and Phytochemistry
Available online at www.phytojournal.com

E-ISSN: 2278-4136
P-ISSN: 2349-8234
www.phytojournal.com
JPP 2021; 10(3): 357-359
Received: 14-02-2021
Accepted: 12-03-2021

Sumalatha Katta
[a] Agri biotech Foundation, Rajendranagar, Hyderabad, Telangana, India
[b] Centre for Biotechnology, JNTU college of Engineering, Hyderabad, Telangana, India

Uma Addepally
Centre for Biotechnology, JNTU college of Engineering, Hyderabad, Telangana, India

Mallikarjuna Garladinne
Agri biotech Foundation, Rajendranagar, Hyderabad, Telangana, India

Corresponding Author:
Mallikarjuna Garladinne
Agri biotech Foundation, Rajendranagar, Hyderabad, Telangana, India

An efficient and rapid kanamycin screening assay for identifying true transgenics in cotton

Sumalatha Katta, Uma Addepally and Mallikarjuna Garladinne

DOI: https://doi.org/10.22271/phyto.2021.v10.i3e.14096

Abstract
Genetic engineering is the best suitable approach for the generation of insect resistant cotton in private and public funded laboratories. *Neomycin Phosphotransferase-II* is the most commonly used selectable marker gene that can catabolize to kanamycin resistant protein which helps in screening and differentiation of resistant and sensitive plants. In current paper, the optimal concentration of kanamycin was determined as 200 mg/L in control plants. Based on this standardized a procedure for screening transformed plants by spraying 200 mg/L at seedling stage. The resistant plants were reconfirmed by PCR using *Npt* II specific primers. About 51% and 45% of kanamycin resistant plants were showed amplification of *Npt* II gene at T_1 and T_2 generation respectively. This method is simple and fast which can be exploited for screening large number of seedlings with low cost in effective manner.

Keywords: *Npt*-II, *Agrobacterium*, Rapid screening assay, PCR, selectable markers

Introduction
Cotton (*Gossypium hirsutum L.*) is a commercial fiber producing crop and cultivating particularly in tropical and sub-tropical countries. The cotton yield loss is occurring due to an incidence of boll worms and sap sucking pest. In several laboratories researchers are working on developing insect resistant cotton *via* genetic engineering approach. These transgenic crops are helpful in reduction of insecticide usage and improve the resistance against broad spectrum of insect pest that ultimately led to improvement in crop production. The calli mediated transformation of cotton is laborious and time consuming, involved several methodologies and tests to identify the true transformants. Alternatively, *in planta* method of transformation is developed. However, the plants generated through *Agrobacterium* mediated *in planta* transformation would produce large number of seeds. So screening such large number of seeds is cumbersome and difficult to identify the true transformants. Hence, effective screening approach is essential to identify the positive transformed plants at early stage.

In the plant expression vector, Selectable markers are allow to differentiate the transformed and untransformed tissue. The *Npt*-II (*neomycin phosho transferase*), *Hpt*-II (hygromycin phosho transferase) and BAR (Biolophos amino transferase/ phosphinothricin) are the selectable markers present in the plant binary vectors, which would useful for the identification of transformed tissues. Based on selectable marker, different concentrations of antibiotics are used in MS media for identification of putative transformed plants [16]. Kanamycin is an important aminoglycoside antibiotic which restrain the protein synthesis in green parts of plants [3, 14, 17, 20]. The *Npt*-II has an ability to inactivate the Kanamycin in the growth medium. Constitutive expression of *Npt-II* gene under 35S promoter would facilitate the continuous expression in all parts of the plant [1, 2]. The amino glycoside chain combine with ribosomal subunit in chloroplast and mitochondria where it disrupts the protein synthesis and resulted in etiolation and death of non-transformed plants [3, 13].

Among transformation methods, *Agrobacterium* mediated system is routine and most successful for the generation of transgenic crops. The concentration of Kanamycin used in the growth media is species specific, for instance the explants of *Cicer arietinum*, *Lycopersicum esculentum* [19], *Brasicca napus* [15] were selected on 15 to 100 mg/L concentration of Kanamycin, whereas *Beta vulgaris* was selected on high concentration of about 400 mg/L[11]. *Npt*-II gene product degrades the kanamycin in transgenic plants and keeps plant resistant and healthy. Different kind of tools ELISA, PCR and Lateral flow strip assay used for screening putative transgenic plants, but kanamycin screening assay is simple and easy to select even large population, which could perform at early stage of the plant [10]. The *Npt*-II or *neo* gene was identified and isolated from the transposon Tn5 of *Escherichia coli*. It encodes *Npt*-II (E.C.2.7.1.95) and recognised as amino glycoside 30 phosphotransferase II [8, 12]. The active amino glycoside inhibits the protein synthesis by binding the 30S subunit of the ribosome,

blocking the formation of initiation complexes and decreasing the fidelity of translation in prokaryotes.

In current study, we have standardized kanamycin resistant transgenic cotton *var*. Narasimha plants *via Agrobacterium* mediated transformation approach. The control seedlings were screened using kanamycin spray assay with different concentrations. Out of all, 200 mg/L concentration was optimal and shown highly effective, which is selected for further screening of all putative transformed plants. Further, true transgenic plants have grown healthy whereas non transformed plants become etiolated. Based on reaction pattern plantlets showing no symptoms were treated as kanamycin resistant plants. Then these plantlets were reconfirmed by PCR assay using *Npt*-II gene specific primers. This method can be applied for the screening of large population of putative transformed plants.

Methods and Materials

Narasimha (*cv* NA1325) cotton seeds were delinted using concentrated H_2SO_4 and carefully rinsed under running tap water for 10 min 2-3 times. Calcium carbonate ($CaCo_3$) was used for neutralisation and washed thoroughly using distilled water and dried at 40˚C for 24 hrs. These seeds were surface sterilised again using 0.1% $HgCl_2$ for 10 min and 70% alcohol for 2 min and thoroughly washed with distilled water 5-6 times. These seeds were soaked in distilled water and incubated for 6 hrs under dark conditions.

The embryo axis from the cotton seeds were excised and infected with *Agrobacterium* containing pMDC100 vector with triple *Bt* genes and kanamycin as selectable marker gene [13]. The transformed plants were generated by following the protocol described by Katta *et al*. (2020) [13]. The plants were allowed to mature in the glass house under controlled conditions. Then the T_1 seeds were collected from T_0 plants. To screen the putative transformed plants, the kanamycin spray assay was used. The T_1 cotton seeds and control seeds were surface sterilized and soaked in distilled water for overnight. These seedlings were placed in protrays. After 2 weeks, when 3rd and 4th leaves emerged from the plantlets, sprayed with 200 mg/L Kanamycin solutions for 3 alternative days (day 1, 3 and 5). The assay was performed in three biological replicates. Post 15 days of treatment, the plants were scored as kanamycin resistant and sensitive.

The genomic DNA was isolated from kanamycin resistant and control plants using C-TAB) method as described by Doyle *et al.* (1987) [6] with few modifications. The quality and quantity of DNA were measured by using Nano spectrophotometer (GE Healthcare Pvt Ltd). The DNA was diluted to 100 ng/L with milli Q grade water for PCR analysis. The PCR (Polymeric chain reaction) carried out using primers *Npt* –II specific primers. *Npt*-II FP 5'-CATGTGATGTCACGACGACC-3', *Npt*-II RP 5'-TCTCGTCCATACCTTCACCG-3'. Reaction was performed in 50µl of reaction mixture containing 10 X buffer, 200 ng of DNA, 10 mM dNTPs, 150 *ng* of each primer DNA and 5U of Taq DNA polymerase. PCR carried out under following conditions: 94˚C for 5 min for initial denaturation, then 30 cycles of 94˚C denaturing for 1 min, 58˚C annealing for 1 min and final extension 72˚C for 10 min. Amplified DNA was visualized on ethidium bromide staining and documented (G-Box-Syngene).

Results and discussion

The control cotton seeds were used for identifying the optimal concentration of kanamycin. The seedlings were sprayed with different concentration of kanamycin including 100, 200, 300, 400, 500 mg/L. About 95% of control leaves were bleached and etiolated at 200 mg/L, whereas 52 % of seedlings were bleached at 100 mg/L. At higher concentrations, none of the seedlings were grown and completely etiolated. Hence, 200 mg/L was chosen as optimum concentration for screening of putative transgenic plants. The mode of action of kanamycin is to inhibit the growth of plant cells by binding to the 30S ribosomal subunit and hindering the initiation of plastid translation.

In this method application of kanamycin directly showed effect on organs involved in photosynthesis, absorption and transpiration [9] and also affects leaf and root growth[5]. Several reports indicated that the reliability of the test can be used for the screening of transgenic plants. About 200 mg/L Kanamycin was the stringent enough to remove non transformed plants which were etiolated completely. Firoozabady and Umbeck *et al*. (1987) [7, 18] reported high concentration of kanamycin to be toxic for somatic embryogenesis and reduced level of Kanamycin led to embryo germination. In the present study, 200 mg/L is the efficient concentration for screening of transgenics (T_1 & T_2) *in vivo*. When the concentration of Kanamycin is less than the optimal, that would lead to false positive plants, whereas high concentration may cause mortality of true transgenic plants. A method for identifying transformed plants using kanamycin spray has been developed. Cotton T_1 seeds were obtained from T_0 plants. The putative transgenic T_1 and untransformed control seeds were allowed to grow in pro trays and sprayed kanamycin, 200 mg/L for 3 alternative days (Fig.1). Post 15 days of spray, the non transformed tissues and control plants were bleached and etiolated.

About 1545 T_1 seeds were germinated on portrays. The expanded leaves were sprayed with 200 mg/L Kanamycin solution for preliminary identification. From these, 248 plants with 16 % were growing healthy and green. These plants were considered as resistant, whereas the plants were showing yellow spots and not growing healthy are considered as sensitive. Further, these resistant plants were used for molecular analysis. Out of 248 resistant plants, 128 samples were showed amplification of *Npt*-II gene with an expected amplification product of 395 bp (Fig. 2). About 51% of the resistant plants are showed positive for PCR amplification. In the next generation, 781 T_2 seeds collected and sprayed kanamycin. Then 193 plants exhibited resistance to Kanamycin (Table 1). About 45% of resistant plants (87) showed PCR positive when used *Npt*-II gene specific primers.

Fig 1: Kanamycin spray assay for screening putative transgenic plants. A. Putative transgenic cotton plants grown in pro trays. B. Kanamycin resistant plants C. Kanamycin sensitive plants

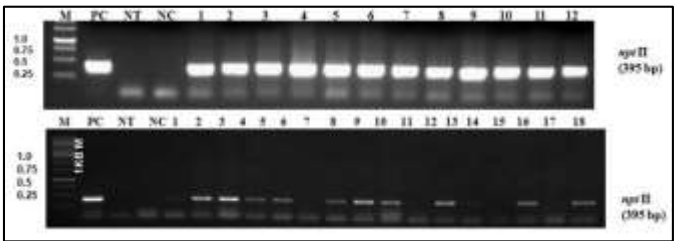

Fig 2: Molecular confirmation of kanamycin resistant plants: (A) Amplification of 395 bp region in *Npt*-II gene in T_1 palnts (B) T_2 plants. M-marker, PC-.positive control, NT-nontransgenic, NC-negative control, lane numbers 1to12 represented the plant samples.

Table 1: Comparison of percentage of kanamycin resistance with PCR results

Generation	No of seeds germinated	Kan resistant	%	PCR	%
T_1	1545	248	16	128	51
T_2	781	193	24.7	87	45

Conclusion

Kanamycin is a selection agent used in this study for screening of putative transgenic cotton plants. The Kanamycin selection method can be used as an easy method for screening large number of segregating population of transgenic plants. The 200 mg/L kanamycin concentration was determined as optimal concentration for screening putative transgenic plants (T_1 and T_2 generations). About 51% of T_1 and 45% T_2 kanamycin resistant plants showed positive when tested with PCR. This method can be applied for the screening large population of cotton plants

Acknowledgement

We would like to thank Prof G Pakki Reddy, Executive Director and Dr J S Bentur, Agri Biotech foundation for their suggestions and support Rashtriya Krishi Vikas Yojana (RKVY), Telangana, for funding of the project is duly acknowledged.

References

1. Abdel-Mawgood AL, Gassem MA, Alsadon AA, Alghamdi SS, Al-Doss AA. Monitoring of genetically modified food in Saudi Arabia. African Journal of Food Science 2010;4(8):536-40.
2. Anklam E, Gadani F, Heinze P, Pijnenburg H, Van Den Eede G. Analytical methods for detection and determination of genetically modified organisms in agricultural crops and plant-derived food products. European Food Research and Technology 2002;214(1):3-26.
3. Chen XB, Xu CB, Luo YL, Li BJ. Study on Effect of Kanamycin on germination of Aptenia cordifolia. Seed 2005;24(7):29-31.
4. Chen Y, Rivlin A, Lange A, Ye X, Vaghchhipawala Z, Eisinger E, Dersch E *et al*. High throughput Agrobacterium tumefaciens-mediated germline transformation of mechanically isolated meristem explants of cotton (*Gossypium hirsutum* L.). Plant cell reports 2014;33(1):153-64.
5. Duan H, Ding X, Song J, Duan Z, Zhou Y, Zhou C. Effects of kanamycin on growth and development of Arabidopsis thaliana seedling, cotyledon and leaf. Pakistan Journal. Bot 2009;41(4):1611-1618.
6. Doyle JJ, Doyle JL. A rapid *DNA* isolation procedure for small quantities of fresh leaf tissue (No. RESEARCH).
7. E. Firoozabady D, DeBoer D, Merlo E, Halk L, Amerson K.E, Rashka E E, Murray, Transformation of cotton (*Gossypium hirsutum* L.) by *Agrobacterium tumefaciens* and regeneration of transgenic plants, Plant Mol. Biol. 1987;10:105-116.
8. Fraley Robert T. *et al*. "Expression of bacterial genes in plant cells." Proceedings of the National Academy of Sciences 1983;80(15):4803-4807.
9. Franco AC, Matsubara S, Orthen B. Photo inhibition, carotenoid composition and the co-regulation of photochemical and non-photochemical quenching in neotropical savanna trees. Tree physiology 2007;27(5):717-25.
10. Freitas-Astua J. Characterization of resistance in transgenic tobacco plants expressing begomovirus genes. 2001.
11. Garcia JA, Hille J, Goldbach R. Transformation of cowpea Vigna unguiculata cells with an antibiotic resistance gene using a Ti-plasmid-derived vector. Plant Science 1986;44(1):37-46.
12. Herrera Estrella, L De Bloc M, Messens E, Hernalateen J P. Chimeric genes as dominant selectable markers in plant cells. *The EMBO journal* 1983;2(6):987-995.
13. Katta S, Talakayala A, Reddy MK, Addepally U, Garladinne M. Development of transgenic cotton (Narasimha) using triple gene Cry2Ab-Cry1F-Cry1Ac construct conferring resistance to lepidopteran pest. Journal of biosciences 2020;45(1):1-1.
14. Lu WX, Li MY, Pei Y, Jin ZP, Luo XY. Incorporating maize *chitinase* into rapeseed (*Brassica napus*) by *Agrobacterium tumefaciens*. Journal of Southwest Agricultural University 2001;23(2):130-3.
15. Moloney MM, Walker JM, Sharma KK. High efficiency transformation of Brassica napus using Agrobacterium vectors. Plant cell reports 1989;8(4):238-42.
16. Miki B, McHugh S. Selectable marker genes in transgenic plants: applications, alternatives and biosafety. Journal of biotechnology 2004;107(3):193-232.
17. Nap JP, Bijvoet J, Stiekema WJ. Biosafety of kanamycin-resistant transgenic plants. Transgenic research 1992;1(6):239-49.
18. Umbeck P, Johnson G, Barton K, Swain W. Genetically transformed cotton (*Gossypium hirsutum* L.) plants, *Biotechnology* 1987;(5):235-266.
19. Rajesh PN, Coyne C, Meksem K, Sharma KD, Gupta V, Muehlbauer FJ. Construction of a HindIII bacterial artificial chromosome library and its use in identification of clones associated with disease resistance in chickpea. Theoretical and Applied Genetics. 2004;108(4):663-9.b.
20. Zixuan W, Zili Y. The Application of Kanamycin in Transgenic Plants and the Biosafety Assessment of Kan~ r Gene. Progress in Biotechnology 2003, 6.