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IN-PLANTA GENETIC TRANSFORMATION OF SYNTHETIC HERBICIDE TOLERANT GENE IN UPLAND COTTON

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Herbicides are the chemicals used to kill unwanted plants or weeds which can compete with main crop for light, water and other useful nutrients. Two types of herbicides i.e. selective and non-selective are being used but the later one is more efficient and it cannot differentiate between the crops and weeds. Hence, development of herbicide tolerant (*ht*) plant is an ultimate solution to cope with the challenge of non-selective ones. Nucleotide sequence of *ht* bacterial gene encoding 5-enolpyruvylshikimate-3-phosphate (*EPSP*) synthase was modified according to crop codon usage. The synthetic herbicide tolerant gene was cloned in a gateway compatible plant expression vector and introduced into an *Agrobacterium* strain LBA4404. Tissue culture independent *in-planta* transformation system was used to integrate the transgene into cotton plants at three different stages of flower after pollination. Flower shedding was the main problem observed during inoculation. Maximum inoculated bolls were developed at 16 hours after pollination and plantlets were screened using different doses of roundup spray having glyphosate as an active ingredient. Successful transgene integration was verified using PCR and Southern blot analysis in plants surviving at @ 600 mL/acre roundup herbicide. Whereas on higher dose of herbicide transgenic plant did not manage to survive. These results showed that the cotton is successfully transformed with *ht* gene but the expression of transgene is low or it may have been gone in silent mode. Gene silencing is a natural phenomenon which could be avoided by using more efficient regulatory elements or by introducing the transgene into other genetic compartments of plant. In addition, development of more transformation events could result in transgenic cotton plants with higher transgene expression.
Keywords: Glyphosate Herbicide; Pollen tube pathway; Transgenics; Synthetic EPSPS; Cotton

INTRODUCTION

Weeds are the major constraint to crop production as they compete for light, nutrients, and moisture, promote insects/diseases attack and may reduce yield considerably. Some weeds release toxic chemicals which may adversely effects on the crop growth. Cotton is a tropical perennial plant which is grown in warm temperate regions as a summer annual, and is a poor competitor against weeds. During the initial two months of crop (Douti, 1997; Latif *et al.,* 2015), weed-free growth is very important to attain maximum yield. The control of weeds is a principal consumer of manpower and increases cost of production to great extent. Deep ploughing and other mechanical/manual control of weeds has now been replaced with chemical control. More than half of the pesticides used worldwide are for weed control. Herbicides are the chemicals which have been developed to kill unwanted plants. Most of the herbicides, used for the control of weeds are non-selective ones i.e. they cannot discriminate between main crop and weed (Chen *et al.,* 2006; Dong *et al.,* 2017). So, we have to develop a resistance/tolerance mechanism in our crops against these herbicides. The recent advances in molecular biology and genetic engineering have provided mankind with unprecedented power to manipulate and develop novel crop genotypes. By adopting genetic engineering techniques, we can transfer the beneficial information from any source e.g. plants, bacteria, viruses, animals etc. into our target crop through genetic transformation. Several potential genes such as herbicide resistance, insect resistance, increased nutritional value and shelf life, stress resistance, disease resistance, male sterility and bio-farming etc. have been used to develop transgenics in different crop plants (Rani and Usha, 2013). By using this we can create a new face of our exiting crop which can fight better against currently prevailing threats especially weeds. Currently, the most widely used methods for transferring genes into plants are *Agrobacterium*-mediated transformation (Chilton *et al.,* 1977) and the biolistic gene delivery (Klein *et al.,* 1987). Other methods, such as polyethylene glycol (PEG)-mediated transformation (Datta *et al.,* 1990) and electroporation (Fromm *et al.,* 1985) have also been used to transfer genes into plants.

In-planta genetic transformation via pollen tube pathway (PTP) is a competent, fast and tissue culture independent method for crop improvement. This system is very useful for those plants which show poor response to *in-vitro* regeneration like cotton (Keshamma *et al.,* 2008; Jan *et al.,* 2016). PTP method of transformation via *Agrobacterium*

tumefaciens has been reported to be successful in several crop species including cotton (Liu *et al.,* 2007; Bibi *et al.,* 2013). In this method, exogenous DNA is transferred by cutting the stigma following pollination and applying the *Agrobacterium* inoculum. DNA presumably reaches the ovary by flowing down the pollen tube and then integrates into the just fertilized but undivided zygotic cells (Zhou *et al.,* 1983). PTP method with no tissue culture system have proved to be a nice alternative for genetic transformation of cotton. Hence, objective of this study was to develop the herbicide tolerant (*ht*) transgenic cotton via PTP mediated genetic transformation technology. Furthermore, molecular screening and herbicide assay of putative transformants was also performed.

MATERIALS AND METHODS

***In-silico analysis and synthesis of herbicide tolerant gene*:** The nucleotide sequence of herbicide tolerant CP4-*EPSPS* gene (1368 bp) was retrieved from NCBI and subjected to codon optimization with the help of geneious online software https://www.geneious.com/ (geneious biologics, New Zealand). The secondary structures of mRNA were analyzed using the CLC Main Workbench 5.0.1. The optimized *ht* gene sequence was got synthesized commercially from GeneLink Company, USA.

***Cloning and construction of Agrobacterium compatible plant transformation vector*:** *S*ynthetic *ht* gene was cloned in a gateway compatible binary vector i.e. pB7WG2D,1 (Karimi *et al.,* 2002). The ligation mixture was transformed into Top-10 strain of *E. coli* competent cells and grown on solid LB plates having spectinomycin @ 75 µg/mL at 37ºC for 12-14 hours. The appeared colonies were multiplied in liquid LB and plasmid DNA was isolated using GeneJET Plasmid miniprep kit (Thermo Scientific, Lithuania). The resultant recombinant DNA was confirmed with different restriction enzymes. Binary vector pB7WG2D,1 containing synthetic *ht* gene was transformed into *Agrobacterium* strain LBA4404 using ECM-2001 Electroporation System (BTX Harvard Apparatus, USA) and confirmed with different molecular biology techniques. *Agrobacterium* containing synthetic *ht* gene was grown on LB media supplemented with spectinomycin @ 75 µg/mL at 28ºC for 48 hours at 180 rpm in shaking incubator (Taitec, Japan).

***Plant material, inoculation and screening*:** Two different cotton genotypes MNH-886 and FH-142 were selected for *Agrobacterium* mediated *in-planta* genetic transformation (Bibi *et al.,* 2013). Plants were grown under controlled conditions in glass house/tunnel and regularly inspected for healthy growth. Three different flower stages i.e. 08, 16 and 24 hours after pollination were selected for *Agrobacterium* inoculation. Cotton buds were not emasculated but tied up with thread and cover with butter paper bag one day before opening to avoid cross pollination. After specific time period the petals and top of the pistils were removed to expose the ovary. Exogenous DNA containing target gene was injected into the exposed ovary, where DNA moved down to pollen tube to fertilized ovule. Inoculated flowers were treated with GA_3 @ 40 ppm to control the flower shedding (Daud *et al.,* 2009). 500 flowers from each selected stage were inoculated and developed bolls were picked and ginning was done separately for each boll. Boll-to-row progenies were grown in field after mixing the seed with *G. arborium* cotton seeds to ease and improve the germination percentage. Three different doses of roundup spray i.e. 600, 800 and 1000 mL/acre were planned to use for the screening of transformed plants.

***Molecular analysis of herbicide resistant cotton plants*:** Total genomic DNA was isolated from roundup resistant plants using CTAB method (Rogers and Bendich, 1985) and subjected to PCR (Eppendorf, Germany) for the verification of transgene integration. PCR profile was comprised of 40 cycles at 94ºC for 30 seconds, 56ºC for 45 seconds and 72ºC for 1.0 minute. The amplified product was resolved on 1.0% agarose gel and visualized under UV gel documentation system (Photonyx Ultra, UK). *Hind*III digested 10.0 µg of genomic DNA was resolved on 1.0% agarose gel and transferred onto pure nylon neutral transmembrane (AppliChem, Germany) using iBlot gel transfer system (Invitrogen, USA). The DNA was fixed on membrane with UV Cross Linker (CL-1000M, USA). *ht* gene specific probe was used in hybridization and detection was carried out using Biotin Chromogenic Detection Kit (Thermo Scientific, Lithuania) as described by Nazir and Khan (2012), Nazir and Khan (2013) and Khan *et al.* (2015). Strip test for the presence of EPSPS protein in PCR positive plants was also performed by using protein specific strips (Envirologix, USA).

RESULTS

***Modification and synthesis of herbicide tolerant gene*:** The original *EPSPS* gene sequence (1368 bp) of *Agrobacterium tumefaciens* strain CP4 was retrieved from NCBI and was modified according to cotton plant codon usage. This gene is responsible for decrease in binding affinity to glyphosate and confers tolerance against glyphosate herbicides (Funke *et al.,* 2006). During modification various sites of restriction enzymes were removed to facilitate the cloning and onward confirmation. After modification, sequence homology was also verified using online BLAST tools and maximum 77% homology was observed with already existing data base. Almost equal ratio of GC and AT contents was adjusted during modification of nucleotide sequence without changing its protein sequence. The modified sequence bearing minimum secondary structures was got synthesized commercially. A short sequence of adapters compatible for gateway cloning was also added at both ends of the synthetic *ht* gene to clone it in gateway fashion. The final synthetic *ht*

Table 1. Summary of flowers inoculated with *Agrobacterium* culture carrying synthetic *ht* gene and initial evaluation of harvested seeds.

Genotype	Flower stage (hours after pollination)	Flowers inoculated	Inoculated developed bolls	Developed bolls %age	Survived plants after spray	PCR positive plants	Strip test positive plants	Transformation efficiency (% of inoculated flowers)
FH-142	08	500	49	9.8	0	0	0	0
	16	500	67	13.4	2	2	0	0.4
	24	500	34	6.8	0	0	0	0
MNH-886	08	500	37	7.4	0	0	0	0
	16	500	43	8.6	0	0	0	0
	24	500	39	7.8	0	0	0	0
Total		**3000**	**269**	**8.9**	**2**	**2**	**0**	**0.06**

gene sequence was submitted to the GenBank and accession number i.e. KP212901 was awarded.

***Cloning of synthetic HT gene and development of plant transformation vector*:** An *Agrobacterium* compatible gateway plant transformation vector was developed by cloning the synthetic *ht* gene in between the border sequences of plasmid pB7WG2D,1. This vector was equipped with *bar* gene as antibiotic selection marker and *gfp* gene facilitating the visual identification of transformed cells. Both of these selection markers had their own regulatory elements. Furthermore, a *ccdB* gene cassette was also the part of this vector which was replaced with a 1368 bp fragment of synthetic *ht* gene. This replacement and ligation was carried out using BP clonase enzyme in a single reaction setup. The size of the resultant transformation vector was 12,258 bp and the synthetic *ht* gene was driven by P35S promoter and T35S terminator (Fig. 1). The successful ligation of *ht* gene in final plant transformation vector was verified using *Hind*III and *Bam*HI restriction enzymes as shown in Figure 2.

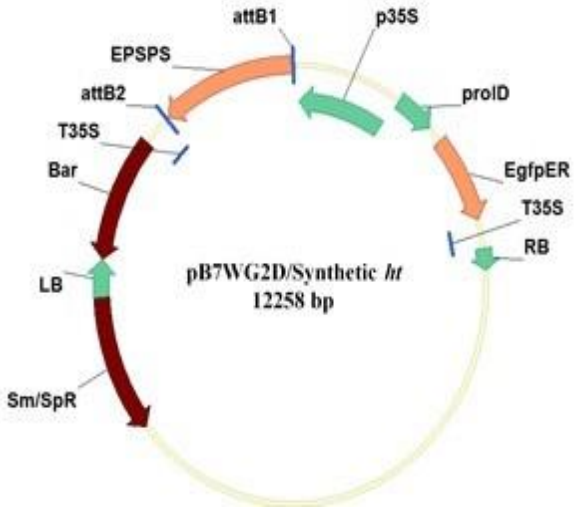

Figure 1. Physical map of *Agrobacterium* compatible plant transformation vector with synthetic *EPSPS* gene in combination with other marker genes.

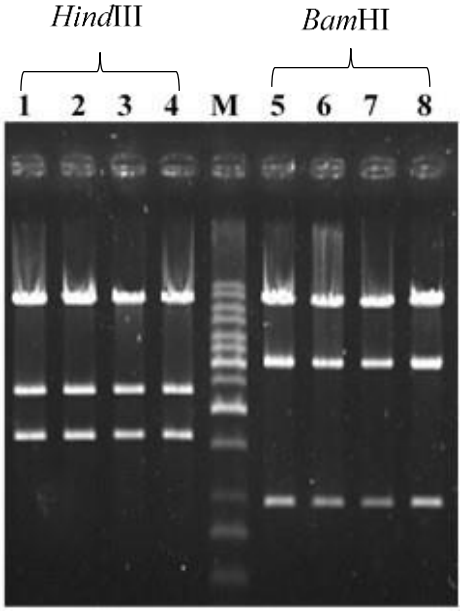

Figure 2. Enzymatic restriction confirmation of cloned synthetic *ht* gene in a gateway compatible plant transformation vector pB7WG2D,1. Lanes 1-4 restricted DNA with *Hind*III, Lane 5-8 restricted DNA with *Bam*HI and Lane M is 1 kb DNA ladder.

***Flower selection and inoculation with Agrobacterium*:** Flowers of cotton plants grown under controlled conditions were selected and sealed one day before opening. Flowers were selected at three different stages after pollination (Fig. 3A-C). One third portion of the stigma was removed and *Agrobacterium* culture having synthetic *ht* gene vector was injected with the help of 28 gauge BD syringe (Fig. 3D-F). The OD of the *Agrobacterium* culture was adjusted upto 0.6-0.8 at 600 nm. Flower shedding was one of the major problems observed during the inoculation work which could not be avoided even by the using 40 ppm solution of

GA_3.Very few inoculated flowers developed into mature bolls. The percentage of developed bolls and transformation efficiency was much low as shown in Table 1. Maximum 13.4% of survived bolls were observed in flower stage that was targeted at 16 hours after pollination of FH-142 genotype. Overall 0.06% genetic transformation efficiency was recorded using PTP mediated genetic transformation protocol in cotton.

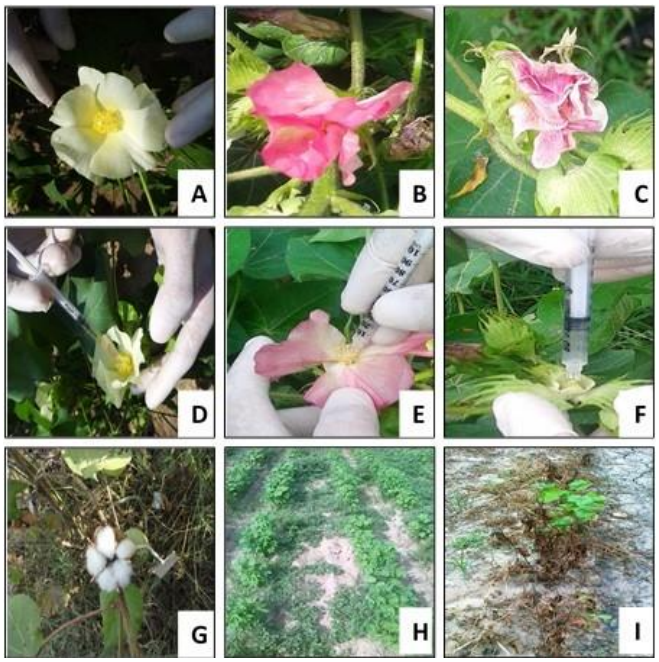

Figure 3. Cotton flower selection, inoculation and screening of herbicide tolerant plants. (A-C) three different stages of selected flowers for *Agrobacterium* inoculation (D-E) inoculation procedures for selected flowers (G) developed boll after inoculation (H) boll-to-row progenies sown in field (I) herbicide assay for screening of tolerant plants

***Herbicide assay of inoculated germinated cotton plants*:** Separate boll-to-row progenies were grown in the field by mixing a single inoculated seed with three seeds of *G. arborium* to facilitate the efficient germination of all inoculated seeds from each boll. At true leaf stage the herbicide assay was carried out using three different doses of roundup spray having glyphosate as an active ingredient. Plant survival and tolerance data was recorded regularly. After two weeks of spray, very few plants showed tolerance to roundup sprayed @ 600 mL/acre. One leaf from each herbicide tolerant plants was picked and preserved for onward molecular analysis. But when the dose of herbicide was increased upto 800 mL/acre, the survived plants showed wilting and unable to survive and third planned dose of spray could not be used. All the weeds and *G. arborium* plants displayed susceptible response and deceased showing the efficacy of the herbicide (Fig. 3I). These results showed that the plants were tolerant only at initial levels of herbicide and gave susceptible response when the dose was increased.

***Molecular analysis of herbicide tolerant plants*:** Transgenic cassette integrated into cotton plant with all transgenes information and their position is shown in Fig. 4A. Isolated genomic DNA for *ht* plants survived at 600 mL/acre of roundup spray was used to investigate the transgene integration. The marker gene specific primers were used in PCR and amplification of about 207 bp fragment verified the marker gene integration (Fig. 4B). Second primer set specific for synthetic *ht* gene was also tested and amplification of about 1000 bp fragment confirmed the transgene integration into two tested cotton plants (Fig. 4C).

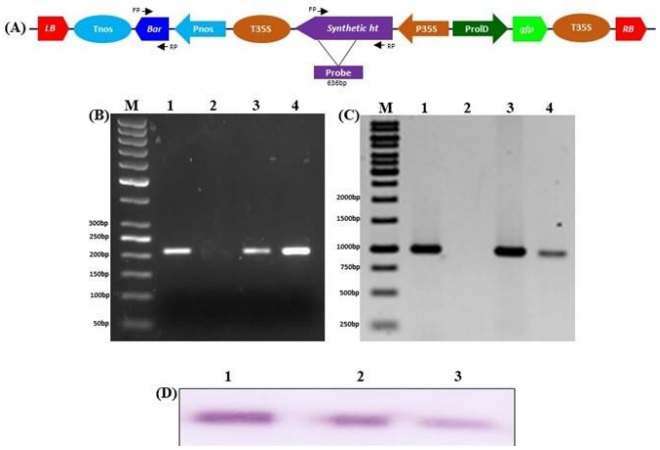

Figure 4. Molecular investigation of herbicide tolerant cotton plants (A) Physical map of transformed gene(s) cassette with primer position and selected probe for southern blotting (B) Selection marker gene specific PCR: lane M is 50 bp DNA ladder, lane 1 positive control with plasmid DNA, lane 2 untransformed plant's DNA, lanes 3-4 are inoculated plant DNA (C) Synthetic *ht* gene specific PCR: Lane M is 1 kb DNA ladder, lane 1 positive control with plasmid DNA, lane 2 untransformed plant's DNA, lanes 3-4 are inoculated plant DNA (D) southern hybridization signals on membrane, lane 1 positive control, lanes 2-3 PCR positive plant's DNA.

In this molecular assay, untransformed plant's DNA was also used as transformation negative control and plasmid DNA serving as a positive control to check the PCR conditions and working efficiency of all consumables. Southern hybridization was also carried out on PCR positive *ht* plants. PCR amplified 636 bp of synthetic *ht* gene specific probe (Fig. 4A) was used in hybridization experiment. *Hind*III restricted genomic DNA was shifted on nylon membrane and

hybridized with Biotin labelled synthetic *ht* gene specific probe. Appeared signals on the membrane were confirming successful hybridization of probe with its complementary sequence on the membrane (Fig. 4D). Both of above DNA based verification experiments confirmed the successful integration of transgene in cotton plants. Moreover, qualitative ELISA using EPSPS protein specific strips was also performed but it was unable to detected protein in PCR positive plants (data not shown). These molecular analyses showed that, both plants were positive for transgene integration but the expression level of synthetic *ht* gene was low because the protein specific strips were unable to identify the target protein.

DISCUSSION

Development of genetically modified (GM) plants against various biotic and abiotic threats is now a routine activity and being efficiently used in many crops. Most of the approaches used for the development of GM plants require an efficient tissue culture response of the target crop. Tissue culture based genetic transformation systems normally requires well-trained manpower and may leads to somaclonal variations which could affect phenotype and genotype of plant (Labra *et al.,* 2004). In comparison to this, *in-planta* genetic transformation is an easy, time saving and tissue culture independent method for crop improvement. By using this technique, one can produce a large number of transgenic plants in a very short time (Jan *et al.,* 2016). Pollen tube pathway (PTP) mediated *in-planta* genetic transformation via *Agrobacterium* is one of the most popular methods for the development of GM plants. This method is commonly used for the transfer of key genes in many agronomically important crops including cotton (Luo and Wu, 1988; Hu and Wang, 1999). In this technique, exogenous DNA is transferred by cutting the stigma and applying the *Agrobacterium* solution containing target DNA on served style. DNA presumably reaches the ovary by flowing down the pollen tube and then integrates into just fertilized but undivided zygotic cells (Song *et al.,* 2007). A synthetic glyphosate herbicide tolerant (*ht*) gene construct was developed and characterization analysis was carried out in model plant i.e. tobacco (unpublished). Glyphosate works by competitive inhibition of an enzyme in the aromatic amino acid biosynthetic pathway, 5-enolpyruvylshikimate-3-phosphate (EPSP) synthase (Peng *et al.,* 2012). Glyphosate is an active ingredients of the most non-selective herbicides widely and efficiently used as weed control throughout the world. Hence, glyphosate herbicide tolerant plant is the ultimate solution to address the challenge of these chemicals. To harvest the beauty of PTP system, cotton flowers were inoculated with *Agrobacterium* culture having synthetic *ht* gene. Three different flower stages were targeted and maximum success was found at 16 hour after pollination. Flower shedding is one of the main problems in cotton under stress conditions and it was also observed in case of inoculated flowers. To control the flower shedding hormones GA_3 was also applied, but no considerable success was achieved. A maximum of 13.4% of inoculated flowers matured into bolls. Boll-to-row progenies were grown and initial screening was carried out at 600 mL/acre of roundup ready spray having glyphosate as an active ingredient. Prior to this experiment, the dose of roundup spray was also optimized by using five different levels (100, 200, 400, 600 and 800 mL/acre) on non-transgenic cotton plants. It was found that maximum plants were wilted and died at 600 mL/acre, while no plant was survived beyond this level. Moreover, the age of the plant was also linked with survival rate because plants with 8-10 true leaves showed tolerance response towards roundup ready spray (unpublished). So, 600 mL/acre of roundup spray was selected as preliminary dose for screening the putative *ht* transgenic plants. Two survived plants after 15 days of spray were screened at molecular level using trasgene(s) specific primers in PCR. Successful integration of synthetic *ht* and *bar* marker gene was confirmed using isolated DNA from leaves. A more specific DNA based transgene identification protocol i.e. southern blotting also confirmed the incorporation of synthetic *ht* gene cassette. Low transformation efficiency i.e. 0.06% was observed using PTP method which is in accordance with other reports in different crops (Daud *et al.,* 2009; Han *et al.,* 2015). EPSPS protein specific strips were used to verify the presence of herbicide tolerant protein in leaves, but no results were found. Additionally, plants were wilted and unable to survive at second dose of roundup ready spray which was 800 mL/acre. From these results it is concluded that plants are transgenic for synthetic *ht* gene, but the expression is low or it may have been gone in silent mode because no protein was identified in strip test. The detection limit of strip test is 0.1-1.0%, whereas PCR is more sensitive with 0.01% limit of detection. Transcriptional or translational gene silencing is a natural phenomenon which is routinely observed in nuclear transformation work (Daniell *et al.,* 2002; Huntzinger *et al.,* 2011). Moreover, low expression or gene silencing could be avoided by using more effective regulatory elements (promoters and terminators) or by the introduction of synthetic *ht* gene in other cellular compartments like plastome, which facilitates pre-determined and precise insertions of transgene(s) with high level of expression (Nazir *et al.,* 2012).

***Conclusion*:** Nucleotide sequence of herbicide tolerant bacterial CP4-*EPSPS* gene was modified and cloned in gateway compatible plant expression vector. *Agrobacterium* mediated pollen tube pathway genetic transformation protocol was used to integrate the synthetic gene in cotton. Gene transfer using PTP method bypasses the laborious and time taking plant tissue culture system. Two PCR positive transgenic cotton plants were developed while protein was not

detected in both plants. Plants were tolerating minimal dose of herbicide which could be due to low expression of synthetic *ht* gene. Hence, it was concluded that transformation protocol could be modified and more transformation event should be developed with higher transgene expression.

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REFERENCES

Bibi, N., K. Fan, S. Yuan, M. Ni, I.M. Ahmed, W. Malik and X. Wang. 2013. An efficient and highly reproducible approach for the selection of upland transgenic cotton produced by pollen tube pathway method. Aust. J. Crop Sci. 7:1714-1722.

Chen, Y.S., H. Christopher, T. Minhtien, M.R. Amy, C.R. Eric, S. Doug and C. Claire. 2006. Expression of CP4 *EPSPS* in microspores and tapetum cells of cotton (*Gossypium hirsutum*) is critical for male reproductive development in response to late-stage glyphosate applications. Plant Biotechnol. J. 4:477-487.

Chilton, M.D., M.H. Drummond, D.J. Merlo, D. Sciaky, A.L. Montoya, M.P. Goprdon and E.W. Nester. 1977. Stable incorporation of plasmid DNA into higher plant cell: the molecular basis of crown gall tumorigenesis. Cell 11:263-271.

Datta, S.K., A. Peterhans, K. Datta and I. Potrykus. 1990. Genetically engineered fertile indica-rice recovered from protoplast. Nat. Biotechnol. 8:736-740.

Daniell, H., M.S. Khan and L. Allison. 2002. Milestone in chloroplast genetic engineering: a new environmentally friendly era in biotechnology. Trends Plant Sci.7:84-91.

Daud, M., M. Variath, S. Ali, M. Jamil, M.T. Khan, M. Shafi and Z. ShuiJin. 2009. Genetic transformation of bar gene and its inheritance and segregation behaviour in the resultant transgenic cotton germplasm (BR001). Pak. J. Bot. 41:2167-2178.

Dong, Y., J. Xi, T. Qiaoling, Z. Xin, Y. Jiangtao, L. Xiaojing, C. Junfeng, Z. Xiaobing, W. Xujing and W. Zhixing. 2017. Development and Event-specific Detection of Transgenic Glyphosate-resistant Rice Expressing the G2-EPSPS Gene. Front. Plant Sci. 8:885.

Douti, P. 1997. Cotton crops versus weeds: when is the competition period? Agric. Dév. (May special issue):11-16.

Fromm, M.C., L. Taylor and P.V. Walbot. 1985. Expression of genes transferred into monocot and dicot plant cell by electroporation. Proc. Natl. Acad. Sci. U. S. A. 82:5824-5828.

Funke, T., H. Han, L. Martha, F. Healy, M. Fischer and E. Schonbrunn. 2006. Molecular basis for the herbicide resistance of Roundup Ready crops. Proc. Natl. Acad. Sci. USA. 103:13010-13015.

Han, L.W., Y. Ge, L. Yao, C.S. Cui and S.P. Qu. 2015. Transformation of soft rot resistant aiiA gene into Chinese cabbage (*Brassica rapa* ssp. pekinensis) by the pollen-tube pathway. Inter. J. Agric. Biol. 17:407-409.

Hu, C.Y. and L.Z. Wang. 1999. In-planta soybean transformation technologies developed in China: Procedure, confirmation, and field performance. In Vitro Cell Develop. Biol. Plant 35:417-420.

Huntzinger, E. and E. Izaurralde. 2011. Gene silencing by microRNAs: contributions of translational repression and mRNA decay. Nat. Rev. Gen. 12:99-110.

Jan, S. A., Z. K. Shinwari, S.H. Shah, A. Shahzad, M.A. Zia and N. Ahmad. 2016. In-planta transformation: recent advances. Rom. Biotechnol. Lett. 21:11085-11091.

Karimi, M., D. Inzé and Depicker. 2002. Gateway vectors for *Agrobacterium*-mediated plant transformation. Trends Plant Sci. 7:193-195.

Keshamma, E., S. Rohini, K.S. Rao, B. Madhusudhan and M.U. Kumar. 2008. Tissue culture-independent *in-planta* trasformation strategy: an *Agrobacterium tumefaciens*-mediated gene transfer method to overcome recalcitrance in cotton (*Gossypium hirsutum* L.) J. Cott. Sci. 12:264-272.

Khan, M.S., B. Kanwal and S. Nazir. 2015. Metabolic engineering of the chloroplast genome reveals that the yeast ArDH gene confers enhanced tolerance to salinity and drought in plants. Front. Plant Sci. 6:725.

Klein, T. M., E.D. Wolf, R. Wu and C.J. Sanford. 1987. High velocity micro projectiles of delivering nucleic acids into living cells. Nat. 327:70-73.

Labra, M., C. Vannini, F. Grassi, M. Bracale, M. Balsemin, B. Basso and F. Sala. 2004. Genomic stability in Arabidopsis thaliana transgenic plants obtained by floral dip. Theoret. App. Gen. 109:1512-518.

Latif, A., A.Q. Rao, M.A.U. Khan, N. Shahid, K.S. Bajwa, M.A. Ashraf, M.A. Abbas, M. Azam, A.A. Shahid, I.A. Nasir and T. Husnain. 2015. Herbicide resistant cotton (*Gossypium hirsutum*) plants: an alternative way of manual weed removal. BMC Res. Notes. 8:453 1-8.

Liu, X., S. Herbert, Q. Zhang and A. Hashemi. 2007. Yield-density relation of glyphosate-resistant soya beans and their responses to light enrichment in North-eastern USA. J. Agron. Crop Sci. 193:55-62.

Luo, Z. and R. Wu. 1988. A simple method for the transformation of rice via the pollen-tube pathway. Plant Mol. Biol. Rep. 6:165-174.

Nazir, S. and M.S. Khan. 2013. Integration of novel chlorophyll genes from black pine into the chloroplast genome of tobacco. Pak. J. Bot. 45:595-600.

Nazir, S. and M.S. Khan. 2012. Chloroplast-encoded chlB gene from *Pinus thunbergii* promotes root and early chlorophyll pigment development in *Nicotiana tabaccum*. Mol. Biol. Rep. 39:10637-10646.

Peng, R.H., Y. S. Tian, A.S. Xiong, W. Zhao, X.Y. Fu, H.J. Han, C. Chen, X.F. Jin and Q.H. Yao. 2012. A Novel 5-Enolpyruvylshikimate-3-Phosphate Synthase from *Rahnella aquatilis* with Significantly Reduced Glyphosate Sensitivity. PLoS ONE. 7:e39579.

Rani, S.J. and R. Usha. 2013. Transgenic plants: Types, benefits, public concerns and future. J. Phar. Res. 6:879-883.

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Rogers, S.O. and A.J. Bendich. 1985. Extraction of DNA from milligram amounts of fresh, herbarium and mummified plant tissues. Plant Mol. Biol. 5:69-76.

Song, X., Y. Gu and G. Qin. 2007. Application of a transformation method via the pollen-tube pathway in agriculture molecular breeding. Life Sci. J. 4:77-79.

Zhou, G.Y., J. Weng, Y. Zeng, J. Huang, S. Qian and G. Liu. 1983. Introduction of exogenous DNA into cotton embryos. Methods Enzymol. 101:433-481.