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AN INSIGHT INTO *Agrobacterium tumafaciens*-MEDIATED GENETIC TRANSFORMATION STUDIES IN MUNGBEAN (*Vigna radiata* L. WILCZEK)

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Mungbean or greengram (*Vigna radiata* L. Wilczek) is an important legume of East Asia, Southeast Asia and Indian subcontinent due to its high nutritional value. The yield of this important crop is decreasing due to facing different biotic and abiotic stresses. Traditional breeding program in mungbean has limited success due to limited mungbean germplasm and less genetic compatibility with its wild relatives. The problem of bottlenecks in mungbean breeding can be achieved by applying modern biotechnological tools and genetic engineering. Mungbean is recalcitrant in nature to in vitro regeneration and genetic transformation like other food legumes. First successful report on genetic transformation in mungbean was reported in 2001 by using *Agrobacterium tumafaciens*. Thereafter, *A. tumafaciens* mediated genetic transformation in this recalcitrant crop by incorporating different genes of interest ranged from marker/selectable to genes for increasing resistance against pathogens, insects and salinity stress were reported by many researchers. In these studies, different techniques, protocols and explants were tested on medium supplemented with different plant growth regulators (PGRs) and selective agents in order to induce putative transgenic plants with high genetic transformation efficiency. Putative transgenic shoots were rooted and acclimatized in the pots containing different substrates. Molecular techniques like PCR, RT-PCR, hybridization techniques, ELISA, Histochemical analysis and leaf bioassay were performed in order to confirm genetic transformation and efficiency.
Keywords: A. *tumafaciens*, genes, genetic transformation, mungbean, molecular techniques.

INTRODUCTION

Mungbean or greengram (*Vigna radiata* L. Wilczek) is an important edible grain legume and good source of vegetable protein for poor people (Tazeen and Bushra, 2004; Yadav *et al.*, 2017). The mungbean seeds and sprouts are used as food or vegetable in some Asian countries including India, Pakistan and China (Tang *et al.*, 2014). It is cultivated in different eco-geographical regions (Ebert, 2014) with major consumption in Subcontinent (Sahoo *et al.*, 2016) due to containing more folate and iron compared to other legumes (Keatinge *et al.*, 2011). However, India is the major mungbean producer with 2/3rd of world's cultivated area with almost world's half production (Singh and Singh, 2011). The average yield in India is not high due to facing biotic (Sahoo and Jaiwal, 2008; Vijayan and Kirti, 2012), abiotic stresses like salinity problem (Sehrawat *et al.*, 2013) and complicated mungbean production technology (Dungarwal *et al.*, 2003; Ghosh *et al.*, 2015) and less availability of resistance varieties that can sustain in harsh environmental conditions (Pratap et al., 2018).

The use of mungbean dated back to over 2000 years in China for its detoxification activities and for alleviating heat stroke, regulation of gastrointestinal upset and skin moisturization (Min, 2001). The dried mungbean seeds are using in cuisine as whole or splitted into two with or without husk, fermented or as flour (Tamooka, 2002). Furthermore, certain products like alcoholic beverages, confections, curries, porridge and soups are also made from mungbean (Lambrides, 2007). Mung beans sprouts are popularly used as a fresh salad vegetable (Tamooka, 2002). Due to its high nutritional value, mungbean shows anti-inflammatory, antimicrobial, antioxidant and antitumor activities due to containing amino acids, proteins, oligosaccharides, and polyphenols (Randhir *et al.*, 2004; Vanamala *et al.*, 2006; Anjum *et al.*, 2011; Kanatt *et al.*, 2011). The mungbean is also useful for fixing atmospheric nitrogen and enhance the organic matter in agriculture land/soil (Mekala *et al.*, 2016), It can be use as green manure that can improve soil health and can utilize as green forage for animal (Vijayan and Kirti, 2012).

Recent advances in genetic engineering techniques enables the researchers to insert desirable gene/s of interest to achieve new elite traits in the target crop/plant (Karami, 2008). Major techniques used for genetic transformation are *Agrobacterium*-mediated transformation, particle bombardment/biolistic or electroporation (Anayol et al., 2016, Aasim *et al.*, 2018a). Besides that, number of other techniques like use of nanofibres, liposme mediated transformation and in plant transformation (*Agrobacterium*-mediated vaccum infiltration) are also employed. However, the efficiency of each system is dependant on variable factors like genetic transformation techniques, genotype/cultivar, explant, plant growth regulators, wounding or provision of chemicals, co-cultivation period, selection medium and

concentration, *Agrobacterium* strains and cell density etc. Furthermore, techniques employed for the confirmation of gene insertion, integration and expression are also significant in different progenies (Aasim *et al*., 2018a).

Agrobacterium-mediated genetic transformation is considered to be more efficient transformation technology (Karami, 2008) for dicot plants especially legumes. This system results in stable integration of foreign DNA into plant genome but with limitation of low copy numbers (Shou *et al.,* 2004) but allows the stable transgene expression in next progeny (Hu *et al.,* 2003).

Biotic and abiotic factors limits the mungbean production by hindering the plant growth and yield by affecting the physiological process (Hasanuzzaman *et al.,* 2013). On the other hand, limited mungbean germplasm and compatibility with wild relatives affects the breeding program especially for salinity tolerance (Sahoo and Jaiwal, 2008). Advances in genetic engineering enable the researchers to overcome the bottlenecks in mungbean breeding at cellular level (Mishra *et al.,* 2014) and allow to incorporate desirable gene in the crop. In mungbean, *Agrobacterium*-mediated genetic transformation technique is the most widely used dated back in early 2001 followed by several reports thereafter (Yadav *et al.,* 2017). These studies reflects the successful use of different *A. tumefaciens* strains, plasmids, gene of interest and marker genes. Similarly, different expalnts were used for putative transgenic mungbean plant selection on medium enriched with different selective agents and plant growth regulators. After successful putative plant selection, these transgenes were confirmed by employing varaible technique in different progenies. In mungbean, the most widely used technique for genetic transformation is *A. tumafaciens*-mediated genetic transformation harbouring variable genes of interest but with very low genetic transformation efficiency (Baloda *et al.,* 2017). This study enlightens the use of *A. tumefaciens* for optimization of genetic transformation in mungbean from different point of view including explant, PGRs, selection medium, genes incorporated and gene expression.

***A. tumafaciens-mediated genetic transformation*:** Legumes are dicotyledenous plants and most of them are considered as recalcitrant plants due to variable factors under *in vitro* conditions (Somers *et al.,* 2003; Varshney *et al.,* 2009). The recalcitrant nature of legumes are based on factors like low regeneration response and other physiological factors followed problems related to rooting and adaptation (Aasim, 2012; Barpete *et al.,* 2015). Therefore, successful establishment of reproduceable whole plant regeneration is basic requirement for developing successful genetic transformation protocol (Jaiwal *et al.,* 2001). *Agrobacterium*-mediated genetic transformation techniques is widely used in mungbean. Moreover, there is no report on the use of other genetic transformation techniques like biolistic or electropoaration in mungbean. The genetic transformation studies on mungbean reveal the use of different *Agrobacterium* stratins carrying different plasmids. In mungbean, the most widely used *Agrobacterium* strains for genetic transformation were EHA105 (Suraninpong *et al.,* 2004, Saini *et al.,* 2007; Jaiwal *et al.,* 2016; Kumar *et al.,* 2016), LBA4404 (Islam and Islam, 2010; Vijayan and Kirti, 2012; Yadav *et al.,* 2012; Jaiwal *et al.,* 2016; Mekala *et al.,* 2016) and C58C1 (Tazeen and Mirza, 2004; Jaiwal *et al.,* 2016). Whereas, other starins like EHA101 (Baloda *et al.,* 2017), GV2260 (Vijayan and Kirti, 2012), K599 (Suraninpong *et al.,* 2004) and C-58 (Mahalakshmi *et al.,* 2006) were also reported by researchers.

Proper selection of culture explant for genetic transformation study affects the gene/s transfer efficiency due to susceptibility to *Agrobacterium.* Explants that lead to axillary or apical shoot regeneration are considered more potent than explants generating adventitious shoots regeneration (Aasim *et al*., 2018b). For mungbean, different explants were used for *Agrobacterium*- mediated genetic transformation, which include cotyledonary node (Tazeen and Mirza, 2004; Suraninpong *et al.,* 2004; Saini *et al.,* 2007; Vijayan and Kirti, 2012; Yadav *et al.,* 2012; Kumar *et al.,* 2016; Baloda *et al.,* 2017), contylednory node preconditioned with TDZ or BAP (Sahoo *et al.,* 2016), cotyledon attached with embryonal axis (CAEA) (Islam and Islam, 2010; Jaiwal *et al.,* 2016) and shoot tips (Mekala *et al.,* 2016). On the other hand, explants which lead to adventitous shoot regeneration were also tested like hypocotyl (Tazeen and Mirza, 2004; Jaiwal *et al.,* 2016), roots (Tazeen and Mirza, 2004), primary leaves (Mahalakshmi *et al.,* 2006; Jaiwal *et al.,* 2016), primary leaves with petioles (Tazeen and Mirza, 2004) or cotyledonary leaves (Islam and Islam, 2010) for genetic transformation studies in mungbean.

Application of proper PGRs at appropriate concentration also play a critical role especially for recalcitrant plants (Villemont *et al.,*1997). PGRs in the culture medium enhanced the cell division of infected cells at particular stage to develope transgenic shoots (Chateau *et al.,* 2000; Karami, 2008). The work on mungbean reflected the use of BAP either single (Tazeen and Mirza, 2004; Mahalakshmi *et al.,* 2006; Suraninpong *et al.,* 2004; Saini *et al.,* 2007; Islam and Islam, 2010; Vijayan and Kirti, 2012; Kumar *et al.,* 2016; Sahoo *et al.,* 2016; Baloda *et al.,* 2017) or in combination with other auxins (Tazeen and Mirza, 2004; Yadav *et al.,*2012; Jaiwal *et al.,* 2016; Mekala *et al.,*2016) as PGR for *Agrobacterium*-mediated genetic transformation. On the other hand, Tazeen and Mirza (2004) used Kinetin, TDZ or different combinations of BAP+Kin, 2,4-D+Kin for genetic transformation in mungbean.

Selection of explants using selective agents (antibiotics, herbicides etc.) in the culture medium to generate putative transgenic plants plays significant role in genetic transformation. Therefore, proper selection agent (type) and its concentration must be optimized prior to genetic

transformation based on genotype, explant or marker gene used in the study (Aasim *et al*., 2018a,b). The genetic transformation studies reflected the application of different antibiotics in combination like 50 mg/L kanamycin+500 mg/L cefotaxime (Tazeen and Mirza, 2004; Jaiwal *et al.,* 2016), 75 mg/L kanamycin+500 mg/L cefotaxime (Baloda *et al.,* 2017), 100 mg/L kanamycin+500 mg/L cefotaxime (Sahoo *et al.,* 2016), 100 mg/L kanamycin+250 mg/L cefotaxime (Vijayan and Kirti, 2012; Mekala *et al.,* 2016), 50 mg/L kanamycin+200 mg/L cefotaxime (Islam and Islam, 2010) and 25 mg/L hygromycin+500 mg/L timentin (Suraninpong *et al.,* 2004). Apart from these, some studies revealed the use of hygromycin at different concentrations (Suraninpong *et al.,* 2004, Mahalakshmi *et al.,* 2006) or 100 mg/L kanamycin (Yadav *et al.,* 2012).

Besides of antibiotics, ppt (Saini *et al.,* 2007) or 5 µM PPT+500 mg/L cefotaxime (Kumar *et al.,* 2016) was also used as selective agent in some studies. Islam and Islam (2010) gradually used kanamycin as selective agnet by increasing the kanamycin concentration from 50 mg/L to 200 mg/L in the selection medium. Vijayan and Kirti (2012) also tested L-cysteine at the rate of 200-1000 mg/L for enhancing genetic transformation efficiency. On the other hand, some studies also reported the use of acetosyringone (Saini *et al.,* 2007; Kumar *et al.,* 2016; Mekala *et al.,*2016; Yadav *et al.,*2012) at different concentrations during co-cultivation.

The ultimate goal of genetic transformation is the incorporation of genes to obtain elite traits with elite characteristics. The earlier studies on genetic transformation were based on the use of reporter/selectable marker genes like uidA/GusA (Islam and Islam, 2010; Yadav *et al.,* 2012; Jaiwal *et al.,* 2016), nptII (Tazeen and Mirza, 2004; Islam and Islam, 2010; Vijayan and Kirti, 2012; Yadav *et al.,* 2012,; Jaiwal *et al.,* 2016; Baloda *et al.,* 2017) or hpt gene (Mahalakshmi *et al.,* 2006; Jaiwal *et al.,* 2016; Baloda *et al.,* 2017). However, incorporation of genes related to desirable agronomical traits or resistance to insect/pest are the ultimate goal of genetic transformation. In mungbean, different genes like CodA which control the Cod pahway that help to change choline into glycine betaine (Baloda *et al.,* 2017), genes used against salt stress like Na^+/H^+ antiporter (AtNHX1) (Kumar *et al.,* 2016; Sahoo *et al.,* 2016) or Annexin gene-AnnBj1 which are Ca2+ and phospho-lipid binding proteins (Yadav *et al.,* 2012; Mekala *et al.,* 2016) were also incorporated successfully. Some studies also revealed the use of non-expressor of pathogenesis related gene-1 (BjNPRI) (Vijayan and Kirti, 2012), or genes related to insect resistance like cholesterol oxidase (choA) (Suraninpong *et al.,* 2004) or α-amylase inhibitor-1 gene (αAI-1) (Saini *et al*., 2007). There are some previous reports that highlighted the use of bar gene (Saini *et al.,* 2007; Kumar *et al.,* 2016) for producing herbicide resistance mungbean.

The previous studies reported that the genes incorporated were regulated or driven by constitutive or non constitutive promotors. Constitutive promotors like CAMV35S (Suraninpong *et al.,* 2004; Mahalakshmi *et al.,* 2006; Tazeen and Mirza 2004; Saini *et al.,* 2007; Vijayan and Kirti, 2012; Yadav *et al.,* 2012; Jaiwal *et al.,* 2016; Mekala *et al.,* 2016; Sahoo *et al.,*2016) was the mostly used for incorpoartaion of genes of interest (marker/reporter/agronomic). However, other promoters like stress-inducible rd29A (Kumar *et al*., 2016), NOS (Jaiwal *et al.,* 2001,Tazeen and Mirza, 2004), and phytohemagglutinin PHA (Saini *et al.,* 2007) were also used. Similarly, different terminators used in these studies were polyadenylation (Jaiwal *et al.,* 2001), NOS (Tazeen and Mirza, 2004; Mekala *et al.,* 2016), nos poly-A or CaMV 35 S poly-A Mahalakshmi *et al.,* 2006) and phytohemagglutinin (Saini *et al.,* 2007).

Integration and expression of desired genes in transgenes at different progenies expressed the level of transformation efficiency. For mungbean transformation, researchers employed different techniques for the confirmation of gene transferred. These studies revealed the use of DNA based PCR (Mahalakshmi *et al.,*2006; Saini *et al.,*2007; Vijayan and Kirti, 2012; Kumar *et al.,* 2016; Mekala *et al.,* 2016; Sahoo *et al.,* 2016; Baloda *et al.,* 2017) or Real-Time PCR Analysis (Vijayan and Kirti, 2012; Kumar *et al.,* 2016; Sahoo *et al.,* 2016). Some studies revealed the use of other techniques like Western (Baloda *et al.,* 2017) or Southern hybridization (Jaiwal *et al*., 2001, Mahalakshmi *et al.,* 2006; Saini *et al.,* 2007; Vijayan and Kirti, 2012; Yadav *et al.,* 2012; Kumar *et al.,* 2016; Sahoo *et al.,* 2016), ELISA (Baloda *et al.,* 2017) or Dot-blot analysis (Jaiwal *et al*., 2001; Baloda *et al.,* 2017). Whereas, different bioassays like leaf senescence assay (Kumar *et al.,* 2016). leaf necrosis test, chlorophenol red assay (Saini *et al.,* 2007), leaf-antifungal bioassay and seeding rot bioassay (Vijayan and Kirti 2012) or leaf disc assay (Jaiwal *et al.,* 2001; Kumar *et al*., 2016) were also used in these studies. Histochemical GUS assay was most performed assay (Tazeen and Mirza, 2004; Suraninpong *et al.,* 2004; Mahalakshmi *et al.,* 2006; Islam and Islam, 2010; Yadav *et al.,* 2012; Mekala *et al.,* 2016; Sahoo *et al.,* 2016) due to use of GUS reporter gene in most of the studies.

In vitro rooting of putative transgenic plants is important step for obtaining seeds to test next progeny. Rooting of *in vitro* regenerated shoots of most legume plants is considered to be relatively difficult due to recalcitrant nature (Aasim *et al.,* 2009) which results in low response to PGRs (auxins), callus induction or multiple shoot induction (Aasim *et al.,* 2012). The studies on mungbean genetic transformation revealed the successful rooting by using either IBA (Mahalakshmi *et al.,* 2006; Islam and Islam, 2010; Mekala *et al.,* 2016; Baloda *et al.,* 2017) or different concentrations of NAA or IAA (Mekala *et al.,* 2016). Whereas, some studies highlighted the use of selective agnet alone like use of 500 mg/L cefotaxime (Sahoo *et al.,* 2016) or 250 mg/L cefotaxime (Vijayan and Kirti, 2012) or selective agent alongwith IBA like 2.5 µmIBA, 12.5 mg/L kanamycin and 350 mg/L cefotaxime (Baloda *et al.,*

2017), 0.5 mg/L IBA maintaining selection pressure with 200 mg/L kanamycin (Islam and Islam, 2010) and 5µM IBA+500 mg/L cefotaxime (Kumar *et al*., 2016) in the rooting medium for further selection. After successful rooting, researchers also acclimatized these plants in pots filled with different substrates like peat moss or soil:sand ratio.

***Conclusion*:** Mungbean is considered as edible nutritional food legume plant for both human and animals. However, the avearge yield of this plant is declining due to adverse effects of biotic and abiotic factors. Researchers tried to induce different genes from the start of this millenium to produce plants with desired agronomic characteristics. The results of this study revealed that only *A. tumefaciens*- mediated genetic transformation has been used by researchers and there is no report on other techniques like biolistic or electroporation techniques. Although, genes related to pathogens, saline stress and insects resistance genes have been reported, there is still need to introduce more novel genes to improve the local germplasm for better yield management as well as future breeding strategy in mungbean.

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