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A Study on RNA Interference for Generation of Transgenic Black Gram Plants

Pratibha Pundir[1] Dr. Arun Vats[2]

[1]Research Scholar of Singhania University, Rajasthan

[2]Lecturer in Botany, Sri Ganganagar

Abstract – Black gram is native to India (Vavilov, 1926). The progenitor of black gram is believed to be Vigna mungo var. Silvestre's, which grows wild in India (Lukoki et al., 1980). There is a mention of black gram in Vedic texts such as Kautilya's "Arthashasthra" and "Charak Samhita". The ancient Sanskrit name of black gram was masha. Even today in Punjab, black gram is called mash and in West Bengal, it is called mash kalaya. In all other Indian languages, the name urd is used, which seems to have originated from the Tamil word ulundu. Vigna mungo is the Latin name of black gram (Nene, 2006).

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INTRODUCTION

Black gram is one of the most highly prized pulse crop, cultivated in almost all parts of India. It has inevitably marked itself as the most popular pulse and can be most appropriately referred to as the "king of the pulses" due to its mouthwatering taste and numerous other nutritional qualities. Whether it be the very special "Dal makhni" of Punjab or the "Vada Sambhar" of South India, the taste rules the hearts of one and all alike. Indian immigrants have popularized the taste worldwide as well. In Japan, the health conscious people eat these seeds by soaking them in water overnight and then serving them as fresh bean sprout salad which is highly nutritious. Blackgram is perfect combination of all nutrients, which includes proteins (25-26%), carbohydrates (60%), fat (1.5%), minerals, amino acids and vitamins (Karamany, 2006). It stands next to soybean in its dietary protein content. It is rich in vitamin A, B1, B3 and has small amount of thiamine, riboflavin, niacin and vitamin C in it. It contains 78% to 80% nitrogen in the form of albumin and globulin (Das *et al*., 1998). The dry seeds are good source of phosphorus. It also has very high calorie content. 100 gm of blackgram has 347 calories. Therefore, blackgram is the cheapest available source of protein for the poor and vegetarians (Tharanathan and Mahadevamma, 2003). The combination of dal-chawal (pulse-rice) or dal-roti (pulse-wheat bread) is an important ingredient in the average Indian diet. The biological value improves greatly, when wheat or rice is combined with blackgram because of the complementary relationship of the essential amino acids such as lysine and sulphur containing amino acids methionine and cysteine. In addition, being an important source of human food, it is also used as nutritive fodder, especially for milch animals. Blackgram also has medicinal properties, like curing diabetes, sexual dysfunction, nervous disorder, hair disorders, digestive system disorders and rheumatic afflictions. It is valued for its high digestibility and freedom from flatulence effect (Fary, 2002).

Being a proper leguminous crop, it is itself a mini-fertilizer factory, as it has unique characteristics of maintaining and restoring soil fertility through fixing atmospheric nitrogen in symbiotic association with *Rhizobium* bacteria, present in the root nodules (Ahmad *et al.,* 2001). It proves to be a great rotation crop enhancing the yield of main crop as well. It is mainly cultivated in a cereal-pulse cropping system primarily to conserve soil nutrients and utilize the left over soil moisture particularly after rice cultivation. It is short duration pulse crop (Delic *et al.,* 2009), usually flowering within 30-60 days of sowing and maturing within 60-90 days. It is generally cultivated as kharif crop but also does well in summer season as a catch crop.

Blackgram is annual trailing or erect plant with a height of 30-90 cm and profuse branching. The stem is slightly ridged and covered with brown hairs. The leaves are large, trifoliate and hairy generally with a purplish tinge. The flowers are axillary, racemose, complete, self-pollinated and yellow in color. The inflorescence consists of cluster of 5-6 flowers at the top of long hairy peduncle. Pods are short, erect to suberect, 4-6 cm long, brown to black in color and hairy containing about 6-10 seeds. The seeds are generally black or dark brown with smooth seed coat and protruding hilum.

REVIEW OF LITERATURE:

Blackgram [*Vigna mungo* (L.) Hepper] is an important leguminous source of protein for a large segment of the vegetarian population in the developing countries of South East Asia. A number of biotic (fungal and

viral diseases) and abiotic stresses are the major hurdles for full realization of the yield potential of the crop. Among disease, viral disease mainly yellow mosaic disease caused by *mungbean yellow mosaic virus* (MYMV) leads to maximum yield loss. Therefore, there is an urgent need to use transgenic technologies to fight against the virus. RNA interference has emerged as a new technology which holds good promise for generating resistance against the viruses. However, genetic transformation requires a reliable procedure for *in vitro* plant regeneration. Therefore, the current status of *in vitro* plant regeneration , genetic transformation and the use of RNA interference technology for mungbean yellow mosaic virus resistance is reviewed with a view to identify the constraints of these techniques and to suggest strategies for their development and improvement. Regeneration in *V. mungo* has been reported via shoot organogenesis (direct shoot organogenesis or organogenesis from callus) and somatic embryogenesis from various seedling explants.

DIRECT SHOOT ORGANOGENESIS

Multiple shoot induction from cotyledonary node explants depends on the number and size of cotyledons attached to the cotyledonary node, type and concentration of cytokinin, presence or absence of shoot tip and genotype. Cotyledonary node explants with or without cotyledon developed multiple shoots on MS (Murashige and Skoog, 1962) or MSB (MS salts and B5 vitamins) medium supplemented with different concentrations of cytokinins (Gill *et al*., 1987b; Ignacimuthu *et al*., 1997; Sen and Mukherjee, 1998; Franklin and Ignacimuthu, 2000; Saini *et al*., 2003; Mony *et al*., 2010).

MATERIALS AND METHODS

Plant material

Seeds of four commercially grown blackgram cultivars, T-9, PS-1, PS-2, T-27 and PU-19 were obtained from the National Bureau of Plant Genetic Resources and the Pulse Research Laboratory, Division of Genetics, Indian Agriculture Research Institute, New Delhi - 110012, India. The widely grown cultivar, PS-1 was used for detailed studies.

Seed sterilization and germination

Healthy and uniform mature seeds were rinsed with 70% alcohol (v/v) for 1 min and then surface sterilized in 0.2% (w/v) aqueous solution of mercuric chloride for 5 min. The seeds were subsequently rinsed with autoclaved distilled water, 4-5 times in a laminar air flow cabinet. Sterilized seeds were germinated on MSB medium [MS (Murashige and Skoog, 1962) salts, B5 (Gamborg *et al.*, 1968) vitamins and 3% sucrose] supplemented with 10.0.

Preparation of explants

The cotyledonary node without cotyledons and the primary leaf with petiole with half lamina were used as explants until and unless otherwise mentioned. They were excised from 4-d-old seedlings raised *in vitro* on MSB medium containing 10 µM BAP. The cotyledonary node explants (5 mm in size) without cotyledons were excised from the seedling by removing cotyledons and cutting both epicotyls and hypocotyls approximately 2 mm above and below the nodal region. The entire primary leaves with petioles were cut at the node region from the *in vitro* - raised seedlings.

Multiple shoots regeneration

Primary leaf petiole and cotyledonary node explants without cotyledons were cultured on MSB medium (MS salts, B5 vitamins and 3% sucrose) containing different concentrations of BAP (0–10.0 µM) for multiple shoot induction. The cotyledonary node explants were cultured in a vertical upright position with cut end slightly embedded in medium. The primary leaf explants with petiole were cultured with petiolar cut end slightly embedded in the medium and abaxial surface of lamina close to the medium. The pH of the medium was adjusted to 5.8 with 1 N NaOH and 1 N HCl prior to autoclaving at 121°C under a pressure of 15 psi for 20 min.

Effect of different cytokinins

To compare the shoot multiplication response of BAP with other cytokinins, the explants were cultured on MSB basal medium containing kinetin, zeatin, 2-ip and TDZ at 1.0 µM concentration.

Effect of different auxins

To study the effect of auxins (IAA or NAA) on shoot regeneration response of BAP, the explants were cultured on MSB basal medium containing either 0.5 or 1.0 µM of IAA or NAA with 1.0 µM BAP.

Type of explants

The effect of the presence or absence of lamina on the primary leaf petiole (excised at node) was studied by culturing the primary leaf petiole with whole lamina or without lamina or transverse proximal or longitudinal half of lamina on MSB basal medium supplemented with 1.0 µM BAP.

Age of explant

The effect of the age of explant on multiple shoot induction was determined by culturing the cotyledonary node and primary leaf explants excised from 16h, 2 to 10-d-old seedlings.

Effect of genotype

In order to study the effect of genotype on shoot regeneration, explants were excised from seedlings of

different cultivars, T9, PS-1, PS-2, T27, PU-19 and cultured on MSB medium supplemented with 1.0□.

Rooting of shoots

Well-developed shoots (2-3 cm) were excised from the proliferating explants and transferred to medium containing half-strength MS salts (Murashige and Skoog, 1962), full-strength B5 vitamins (Gamborg *et al.,* 1968), 3% sucrose and different concentrations of IBA

SIGNIFICANCE OF THE STUDY:

The primary requirement for an efficient plant regeneration system is the selection of an appropriate explant. Various seedling explants, cotyledon, Cotyledonary node, hypocotyl and primary leaf have been used for shoot regeneration in *Vigna* species (Jaiwal and Singh, 2003; Sahoo and Jaiwal, 2007; Chaudhary *et al*., 2007). In the present study, the seedling (primary leaf and Cotyledonary node) explants which are easily available throughout the year and are relatively free from microbial contamination have been used for *in vitro* plant regeneration. These explants were excised from 4-d-old seedlings raised *in vitro* on 10 µM BAP and were assessed for their regeneration potential. Cotyledonary node explants have been widely used for direct shoot organogenesis via multiple shoot regeneration in various leguminous species including pea (Mallick and Rashid, 1989; Jackson and Hobbs, 1990), soybean (Kothari *et al*.,1991), pigeonpea (Shiv Prakash *et al*.,1994; Geetha *et al*., 1999; Lawrence *et al.* 2000; Thu *et al.*, 2003), chickpea (Polisetty *et al*.,1997) black gram (Saini *et al.,* 2003; Muruganantham *et al*., 2007) and mungbean (Sonia *et al*., 2007). The Cotyledonary node explants have pre-existing meristem in the axil of cotyledon which gives rise to multiple shoots on induction by cytokines. Primary leaf explants have been induced to directly regenerate shoots in a few legumes, e.g. mungbean (Mahalakshmi *et al*., 2006) and soybean (Wright *et al*., 1987). In black gram, regeneration from the primary leaf explants with petiole via direct organogenesis has not been achieved so far. However, primary leaves via indirect organogenesis through callus formation regenerated limited number of shoots with low regeneration frequency (Srivastava and Pandey, 2011). The regeneration protocols via direct shoot organogenesis are fast and regenerates are not associated with somaclonal variations. Seeds germinated on 10 µM BAP medium (pre-conditioning) has beneficial effect in increasing shoot regeneration efficiency of different explants in the legumes, *Glycine max* (Thorne *et al.*, 1995), *Cajanus cajan* (Shiv Prakash *et al.*, 1994), *Phaseolus* species (Santalla *et al.*, 1998) and *Vigna mungo* (Saini *et al.*, 2005).

CONCLUSION:

Blackgram (*Vigna mungo* L. Hepper), is one of the important pulse crops which is grown as a source of income and nutrition to billions of people in South East Asia. It has very important role in human diet, as it contains vegetable proteins and supplement to cereal based diet. It contains about 26% protein and other minerals and vitamins. Besides, it is also used as nutritive fodder, especially for milch animals. Being a leguminous crop, it has all the essential nutrients which make it to turn in to a good fertilizer. With its ability to fix atmospheric nitrogen, it restores soil fertility as well. The production of blackgram is mostly confined to Asian countries, out of which India is the largest producer followed by Myanmar and Thailand. Despite being an important pulse crop, blackgram production is continuously decreasing resulting in protein-calorie malnutrition and escalation in its market price. So in order to meet the demand of increasing population, the yield is to be enhanced in the areas where it is exposed to numerous abiotic and biotic stresses. Major hurdle in achieving maximum production includes its susceptibility to several diseases (fungal and viral) and pests. Out of different constraints, viral diseases mainly yellow mosaic disease caused by mungbean yellow mosaic virus (MYMV) is the major threat for huge economical losses in the Indian subcontinent. The virus also infects other grain legumes, French bean, pigeon pea, soybean and mungbean. It can cause even 100% yield loss if infection occurs at the seedling stage. In India, the yield losses per annum due to MYMV in mungbean, blackgram and soybean have been estimated to be US $ 300 million. Eradication of the virus through chemical control of vector, avoidance of infection source and breeding for virus resistance has been proved to be inefficient. Therefore, the only option left is to use genetic engineering techniques to transfer desirable genes to fight against the virus. Pathogen-derived resistance (PDR) is a very effective genetic engineering approach to control plant viruses. PDR is a strategy in which resistance imparting genes are taken from the virus for which resistance is to be developed. Out of different viral genes used for PDR, invert repeat construct of MYMV-Vig *rep* gene to express ds RNA for RNA interference approach holds promise for developing geminivirus resistance. RNA interference is triggered by ds RNA to suppress the target gene expression. Therefore, the present investigation was undertaken with the major objective to generate blackgram transgenic plants for MYMV resistance using *Agrobacterium tumefaciens* strain EHA 105 containing binary plasmid pGD3 harboring hp RNA of MYMV Vig-*rep* gene employing the optimized protocols. The results obtained in the present investigation are summarized as follow-

1. A fast, efficient and reproducible *in vitro* multiple shoot induction system from primary leaf petiole and cotyledonary node explants have been developed in blackgram. The factors affecting *in vitro* regeneration

of multiple shoots were optimized. The genotype, age and size of the explant, type and concentration of cytokinin and combinations of cytokinin with auxins influenced the frequency of shoot formation and the number of shoots per explant. Explants cultured on MS basal medium produced a single or two shoots whereas those cultured on medium containing 6-benzylaminopurine induced multiple shoots. This indicates that cytokinin (BAP) is essential for multiple shoot induction. The age of explant also affects the multiple shoot forming response. The cotyledonary node and primary leaf explants excised from 4-d-old seedlings developed a maximum of 4.5 and 10 shoots per explant in 65% and 95% of the cultures respectively, on medium containing 1.0 µM BAP as a sole growth regulator. Genotype was found to exert a pronounced effect on the regeneration frequency as well as on the number of shoots per explant. Out of five cultivars checked, PS-1 produced the maximum number of shoots from both the explants. Shoots originated directly from the nodal regions and cut ends of the cotyledonary node and primary leaf petiole explants without callus formation.

2. The shoots regenerated from both explants were rooted on medium containing IBA (2.5 µM). Shoots with well-developed roots (plantlets) were established in soil where 80-100% of them survived and developed into morphological normal plants which subsequently produced flowers and pods with viable seeds.

3. Identification of an efficient selection agent and its suitable threshold concentration is a very important parameter for transformation system. The sensitivity of the primary leaf explants to selective agent, kanamycin was checked to determine its optimal concentration for the selection of transformed shoots.

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