AGROBACTERIUM MEDIATED TRANSFORMATION OF ELITE CULTIVARS OF INDIAN MUSTARD (*BRASSICA JUNCEA* COSS. & CZERN.)

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ABSTRACT

An efficient *Agrobacterium*-mediated transformation protocol has been developed in elite cultivars of Indian mustard (*Brassica juncea*) namely RH-30, RH-8812 (Laxmi) and Pusa Bold. The highest frequency of regeneration was observed from cotyledons excised from 5 day old seedlings on MS medium containing 0.2 mg/l IAA and 2.0mg/l BAP out of many combinations tried. During transformation, various factors were worked out. Cotyledon and hypocotyl explants excised from 5 day old *in vitro* grown seedlings were co-cultivated with the overnight culture of *Agrobacterium* strain EHA 105 carrying the plasmid pCAMBIA 1301. This is a binary vector having the hygromycin as a selectable marker gene and GUS as a reporter gene which has intron in it which only allows the GUS expression in the transformed cells and not in the *Agrobacterium* strain. The GUS expression was observed at the cut ends of the explants and response of cotyledons was higher than the hypocotyls. The freshly cut and two day precultured explants were used and frequency of GUS expression in pre-cultured cotyledons was higher than in freshly cut cotyledons. Effect of co-cultivation time (24h, 48h and 72h) on transient GUS expression was worked out. The best co-cultivation was 72h in all the cultivars where the frequency of transient GUS expression was more than 80%. After co-cultivation, the cotyledons and hypocotyls were transferred to the selection medium (MS basal + 0.2 mg/l IAA + 2.0 mg/l BAP +25 mg/l hygromycin + 250 mg/l carbenicillin). Plants regenerated within 20 days on the selection medium. Histochemical GUS assay was carried out and leaves and roots of the plants turned blue when incubated with X-gluc at 37^0 C. The protocol so developed is being used for producing the transgenic plants with value added genes for biotic and abiotic stresses.

Key words: *Agrobacterium*, *Brassica juncea*, Indian mustard, Transformation

INTRODUCTION

The oilseed *Brassicas* including *B.juncea*, *B.napus* and *B.rapa* are the world's third most important source of oilseeds and edible oil. *Brassica juncea* (Coss.& Czern.) is a major oilseed crop of Indian subcontinent, is cultivated as a winter crop in about 6 million hectares of land in rain- fed areas of Northern India. World production has been expanding at a rapid rate in several countries, largely in response to the continuous increase in the edible oil demand. However, compared to cereal crops, the increase per hectare productivity through breeding efforts in oilseed *Brassica* is not enough. Because the breeding methodology has been confined to simple selection procedures, operating on naturally occurring variability, success in improving seed yield has not been achieved. Also, the oilseed *Brassicas* are highly susceptible to fungal diseases, aphid attack and other environmental stresses. The lack of genetic variability present in the base population has been considered to be major limiting factor for progress both in qualitative and quantitative improvement of these crops. The desired goal can be achieved by incorporating additional genetic variability in existing germplasm.

Recent advances in biotechnology and molecular Biology has given a new face to research in designing oleiferous *Brassica* that overcome the hurdle of achieving conventional objectives of raising genetic production potential, creation of genetic variability, incorporating resistance to diseases and insect/pests and abiotic stresses. With the advent of recombinant DNA technology, increasing attention is now being given to the genetic manipulation for an accelerated improvement of oilseed *Brassicas*.

Brassica juncea (2n = 36) is the major oilseed crop of Northern India and contributes nearly 27% of the edible oilseed pool of the country. The productivity of *Brassica juncea* is affected by several biotic and abiotic factors. Fungal diseases are the main biotic stresses in oilseeds which result in considerable yield and economic losses in India as well as around the globe. *Alternaria* leaf blight caused by *Alternaria brassicae* and *A. brassicicola* is a serious disease of Indian mustard (*Brassica juncea*) and results in 30-50% yield losses alone in the country. There is no resistant source in *Brassica juncea* for *Alternaria* blight. Plant genetic engineering can significantly accelerate the crop improvement programmes, by offering unique opportunities for genetic modification of a plant that permits access to transfer the desirable genes from one species to another in a precise manner. There are number of Resistance (R) genes like *chitinases*, β-1,3-*glucanases* and *thaumatin* like proteins which have been cloned and introduced in plant species successfully for improved disease resistance. In the present study an efficient transformation protocol for *Brassica juncea* is described.

MATERIAL & METHODS

Seeds of *Brassica juncea* cvs. RH-30, RH 8812 and Pusa Bold were surface sterilized and cultured in MS basal medium without growth regulators for seed germination and the explants *i.e* cotyledons and hypocotyls were excised from 5 or 6 day old *in vitro* grown seedlings. The excised explants were pre-cultured on MS (Murashige and Skoog;1962) basal medium supplemented with 0.2 mg/l IAA and 2.0 mg/l BAP prior to Agroinfection .

The genetic transformation of seedling explants of *B. juncea* was carried out by using oncogenic *Agrobacterium tumefaciens* strain EHA105 harbouring a binary vector pCAMBIA 1301 containing hygromycin as a selectable marker and ß- *glucuronidase* (GUS)as a reporter gene. The explants were treated with overnight grown *Agrobacterium* culture for about 10 minutes and then blotted dry on sterilized filter paper and co-cultivated for 48-96 h. After 3 days of co- cultivation, the explants were studied for GUS expression by histochemical assay following the procedure by Jefferson *et al*. (1987). Percent explants exhibiting GUS activity were assessed. Then the explants were transferred to selection medium (MS basal medium supplemented with 0.2 mg /l IAA, 2.0 mg /l BAP, 25mg/l hygromycin and 250 mg/l cefotaxime). The regenerated shoots were sub-cultured on rooting medium (MS basal supplemented with 2.0 mg /l IBA and 250 mg/l cefotaxime) after 15 days. Regenerated shoots and leaves were also subjected to histochemical GUS assays in order to confirm the transgenic nature of plants.

RESULTS AND DISCUSSION

Five to Six day old explants (Cotyledons and hypocotyls) both freshly cut and pre-cultured on regeneration medium were treated with *Agrobacterium* strain EHA 105 carrying the binary vector pCambia 1301 having GUS as a reporter gene and subsequently assayed histochemically for GUS expression. To study the effect of pre-culturing both fresh and pre-cultured explants were treated with *Agrobacterium* strain. Pre-culturing of explants on regeneration medium for a few days was believed to prepare the cells receptive for transformation by increasing the rate of cell division and thus increasing the rate of regenerating competent cells at the wound site (An, 1985). It was found that the fresh explants showed less transformation efficiency as compared to pre-cultured explants (Figure 1). The petiolar region of the cotyledons consists of specialized cells competent to differentiate into shoots and was found to be very susceptible to *Agroinfection* (Bhojwani and Sharma, 1989) and is indicated by the fact that blue colour spots of GUS during transient expression was at the petiolar end. So pre-culturing was reported as essential requirement to have efficient transformation (Barfield and Pua, 1991; Metz et al, 1995; Babic et al ,1998; Soma- Paul and Sikadar,1999; Mehra et al. 2000 and Chakrabarty et al. 2002). Cotyledon explants were found to be superior to hypocotyls for genetic transformation. Co-cultivation for 72h was the best for transient GUS assay. The histochemical GUS assay was also done on callus arising from cotyledons and hypocotyls when they were placed on selection medium. To confirm the transgenic nature of plant the leaves from regenerated plant were GUS assayed. Roots showing blue colour also confirmed the transgenic nature of the plants. The protocol developed is being used for transferring value added genes in *Brassica juncea*.

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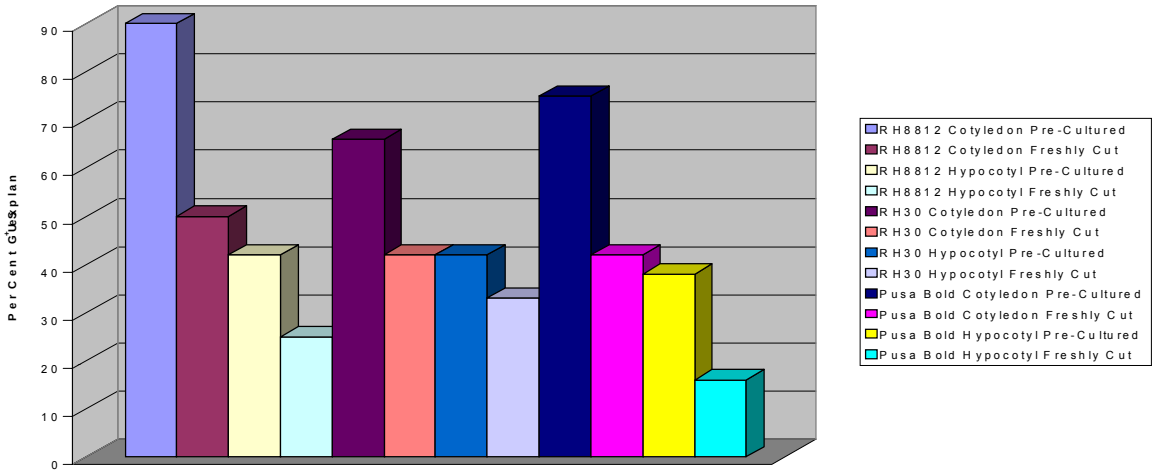
Figure 1:Effect of Pre-Cultured explants on GUS expression in *Brassica juncea* L.



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