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Plant Regeneration and Expression of Beta-glucuronidase Gene in Hypocotyl Tissues of Chickpea (*Cicer arietinum* L.)

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Abstract: In order to develop an efficient protocol for inserting novel genes in chickpea plants, conditions of regeneration and transformation of tissues were optimized. In case of in vitro plant regeneration of *Cicer arietinum* variety 6153, the hypocotyl tissue showed somatic embryogenesis and regenerated shoots in two basal media with addition of NAA and BAP. Different auxins seemed to have different effects on the differentiation and maturation of embryos. The replacement of one auxin NAA with another IAA induced the germination of embryos. Elaborate rooting of regenerated shoots in two basal media containing IBA was obtained with a frequency of 80 percent. Conditions of biolistic transformation were also optimized for transient expression of marker gene using a homemade particle acceleration gun. The beta-glucuronidase gene was introduced into hypocotyl tissue for transient expression and upto 58 percent of hypocotyl showed transient expression. These results provide the basic information for the transformation of chickpea via somatic embryogenesis.

Key words: Beta-Glucuronidase, Biolistic Transformation, *Cicer arietinum* L., Transient Expression.

Introduction

Plant regeneration technique finds its use in many areas of biotechnology, such as the production of pathogen free plants, germplasm storage and recovery of improved plants from engineered and selected cells. In grain legumes, plant regeneration was reported in *Glycine max* (Myers *et al*., 1989; Ghazi *et al*., 1986) *Lathyrus sativus* (Gharyal and Maheshwari, 1983), pigeon pea (Kumar *et al*., 1983), *Lens culinaris* (Saxena and King, 1987), *Vigna aconitifolia* (Kumar *et al*., 1988) and chickpea (Kumar *et al*., 1994). The source material were protoplasts, cell suspension and immature embryos in soybean, callus culture in *L. sativus*, *L. culinaris* and pigeon pea, cell suspension in *V. aconitifolia* and leaf explants in chickpea. Although there are many reports of successful callus induction from various explants of chickpea including hypocotyl (Gosal and Bajaj, 1979; Riazuddin *et al*., 1988), but the limited success in regeneration from resultant calli has been reported. The present studies reports embryogenesis and subsequent regeneration of *Cicer arietinum*.L variety 6153 from hypocotyl explants.

Pod-borer (*Heliothus armigera*) and leaf weevil (sitona) are serious pests of chickpea. A toxin gene from *Bacillus thuringiensis* has shown to be effective against lepidopteran and coleopteran pests. We have collected Bt (*B. thuringiensis*) isolates from local environment and screened for their toxicity on pod-borer and relevant gene responsible for the toxicity has been identified. The toxin gene from *B. thuringiensis* is being inserted by particle bombardment and Agrobacterium mediated transformation of chickpea plants (Husnain *et al*., 1997).

Materials and Methods

The plasmid (pBI121) was obtained from Jefferson (1987) containing a kanamycin resistant gene and Beta-glucuronidase gene and 35S promoter from cauliflower masaic virus.

Chickpea (*Cicer arietinum* L.) varieties 6153 and CM72 were obtained from Nuclear Institute for Agriculture and Biology (NIAB), Faisalabad, Pakistan.

The seeds were thoroughly washed with 1 percent (v/v) liquid soap (Max National Detergent Limited, Pakistan) and surface sterilized by immersion of seeds in 10 percent (v/v) commercial sodium hypochlorite for 10 minutes. After removal of sodium hypochlorite by repeated washings with sterile distilled waster, the seeds were blotted dry and allowed to germinate in solid agar based B5 medium devoid of hormones and vitamins at $25 \pm 2^{\circ}C$ under 200-300 umol m^{-2} s^{-1} light intensity.

Explants for callus induction were prepared from *in vitro* grown seedlings of chickpea. Hypocotyl segments measuring 5-6 mm were excised above the root-hypocotyl transition region upto or above the cotyledonary nodal portion. These explants were incubated in culture tubes containing 20 ml of MS and B5 media with different combination of phytohormones.

Six different concentration of auxins IBA (Indole-3-butyric acid and NAA (Naphthalene acetic acid) in MS medium were used to study the root induction. Approximately 3cm long cutting were excised from the shoot and placed on rooting media to study initiation of adventitious roots.

Particle Bombardment: The 5-6 mm segments of hypocotyl of five-day-old seedlings were bombarded with plasmid DNA coated with tungsten particles by using a home made gun as described by Vain *et al*. (1993). The explants were placed on a filter paper in a petri dish containing 20 ml MS medium containing kinetin and 2, 4-D. Preparation of DNA with tungsten particles coated were carried out as reported by Husnain *et al*. (1997).

To optimize distance through which the with tungsten particles DNA coated travelled to hit the target explant, the plant tissues were placed at various distances. The bombarded explants were kept for two days at $26^{\circ}C$ under dark.

Beta-Glucuronidase Assay: The beta-glucuronidase (GUS) gene was used as a histochemical marker. Expression of Beta-glucuronidase activity was determined by the method of Jefferson (1987). Transformed cells were distinguished from non-transformed cells by blue coloration and blue foci per explant were counted and percentage was recorded. Transient expression was calculated as the ratio of the number of hypocotyl tissues showing blue colour to the total number of embryos used in the experiment.

Results

Callus Induction: Table 1 shows that effect of basal media and phytohormone on the frequency of callus induction from hypocotyl explants of chickpea variety 6153. Auxins, 2, 4-D (2,4-dichlorphenoxy acetic acid and NAA (were used alone or in

Table 1: Effect of basal media and phytohormones on days to callus initiation, frequency of callus formation and characteristics of callus derived from hypocotyl explants of 6153

Treatment	Freq. of callus formation after 10-days (%)	Colour	Compactness response (Root formation)	Morphogenetic
MS + 2 = 4-D*3	90	Light green	Compact	+
MS + 2 = 4-D3 + BAP1	84	Light green	Friable	-
MS + 2 = 4-D3 + BAP3	87	Light green	Friable	-
MS + 2 = 4-D3 + KN1	80	Brownish green	Friable	-
MS + NAA3 + BAP1	80	Dark green	Compact	+ + +
MS + NAA3 + BAP3	92	Light green	Friable	-
MS + NAA3 + KN1	89	Light green	Friable	+
B5 + 2 = 4-D3	88	Light green	Compact	+
B5 + 2 = 4-D3 + BAP1	80	Green	Compact	-
B5 + 2 = 4-D3 + BAP3	74	Brownish green	Friable	-
B5 + 2 = 4-D3 + KN1	76	Brownish green	Friable	-
B5 + NAA3 + BAP1	88	Light green	Compact	+ +
B5 + NAA3 + BAP3	91	Green	Friable	-
B5 + NAA3 + KN1	88	Light green	Compact	+ +

\+ 1-3 roots/callus + + 4-6 roots/callus + + + >7 roots/callus
\- No root growth * 2, 4-D3 means 2, 4-D 3uM and so on.

Table 2: Morphogenetic expression of calli derived from hypocotyl explants of 6153 after transferring to different shoot regeneration media

Treatment (Growth regulators in uM)	Changes induced in callus	
	Colouration	Morphogenetic activity
MS + BAP5	Dark green	None
MS + BAP10	Dark green to brown	None
MS + BAP5 + 2,4-D 0.5	Yellowish	Proliferated and became highly friable
MS + BAP5 + NAA 0.5	Dark green	Proliferating and horn shaped embyros were observed
MS + BAP5 + IAA 0.5	Dark green	Vigorous prolferation and embryos germinated
MS + BAP5 + IAA 0.5 + Adenine sulphate 0.5mM	Dark green	Embryos germinated and produced 10-15 multiple shoots

Table 3: Effect of auxins on number of root regenerated shoots from microcuttings of different genotypes of chickpea. Each value is a mean of 3 replicates with 3 culture tubes per replicate

Treatment (in uM)	Number of Regenerated Roots
MS + IBA 0.5	8.80
MS + IBA 1.0	8.00
MS + IBA 2.0	7.20
MS + NAA 0.5	7.23
MS + NAA 1.0	4.57
MS + NAA 2.0	5.37
MS + IBA 0.5 + NAA 0.5	5.70
MS + IBA 1.0 + NAA 0.5	7.77
1/2 MS + IBA 1.0	6.37
LSD at 5 % level of probability for Treatment (T)	= 0.77
LSD at 5 % level of probability for Genotype (G)	= 0.51
LSD at 5 % level of probability for GxT	= 1.54

combinations with cytokinins BAP (Benzyl amino purine and Kin (Kinetin) in two different basal media B5 and MS. The callus induction at a frequency of 74-92 percent was observed within 10 days. Both compact and friable calli in different range of colours, dark green, light green and brownish green were observed. The highest percentage of callus formation was observed in MS or B5 with NAA (3 μM) and BAP (3 μM). However, the calli obtained on MS basal medium were friable but that of B5 basal medium were compact. Other combinations of phytohormone gave 76-80 percent callus formation. A combination of 2,4-D (3 μM) and Kin (1 μM) resulted in brownish green friable calli in both basal media. While the NAA (3 μM) and BAP (1 μM) produced roots after callus formation from hypocotyl explants on the B5 and MS media.

Mosphogenetic Response: Rhizogenesis was observed in two combinations and other did not produce any shoot/root. These calli which did not produced roots were subcultured on eight combinations of phytohormones in MS media. Only one phytohormone combinations in MS medium containing 2, 4-D (3 μM) and Kin (1 μM) resulted in morphogenetic response. The

Table 4: Effect of cultivar on the formation of embryogenic calli from hypocotyl explants

Genotype	Age of Seedling	No. of Explant Culture	No. of Embryos Developed After 2-3 Months	No. of Plant Recovered
6153	5	134	17(12%)	2
	7	116	43(37%)	3
C44	5	24	4(16.6%)	0
	7	34	7(20.6%)	0

Table 5: Transient expression of beta-glucuronidase gene in hypocotyl tissue of chickpea cultivar 6153

Treatment No.	Distance Travelled by Particle	No. of Explant Bombarded	No. of GUS Expressing Tissues	Frequency of GUS Expressing Tissues	Total No. of Blue Spots
1.	17 cm	50	19	38±0.716	80
2.	21 cm	50	16	32±0.260	139
3.	25 cm	51	4	51±0.358	5
4.	29 cm	45	26	58±1.098	139
5.	Cont. 129 cm	52	0	00±0.000	0
6.	Cont. 2 cm	50	0	00±0.000	0

- Control 1 is bombarded with tungsten particle -Control 2 is without bombardment

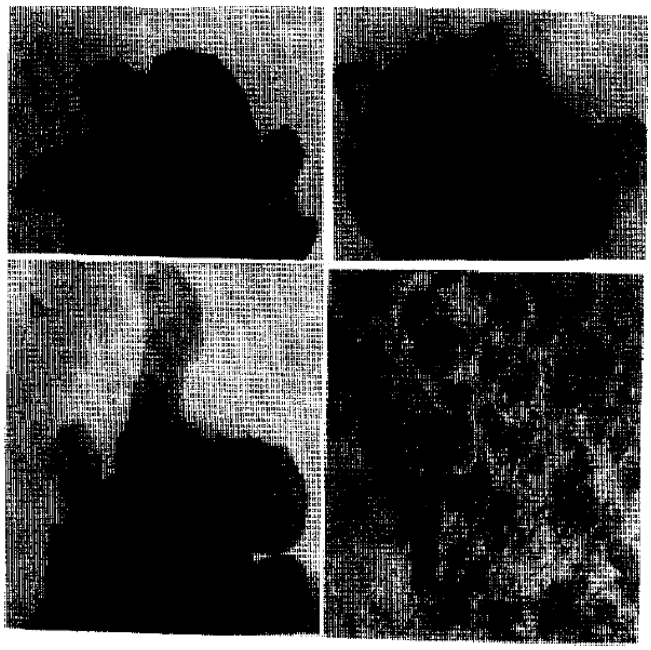

Fig. 1(a-d): Embryogensis of Cicer arietinum L. Variety 6153 from hypocotyl derived callus and transient expression of Beta-glucuronidase gene. a) Glubular shaped embryos. b) Horn shaped embryos c) Germinating embryos d) Transient expression of Beta-glucuronidase in hypocotyl tissue of chickpea.

summary of which is presented in Table 2. Although no morphogensis was observed in BAP 10 μM alone but in combination with NAA and IAA (Indole acetic acid) the calli became embryogenic. The colour of these calli were changed from brownish green to dark green. The horn shaped embryos were germinated in MS medium containing BAP (5 μM) and IAA (0.5 μM). A multiple shoot formation was observed in the same combination with addition to adenine sulphate (0.5mM) and produced 10-15 shoots.

Root Induction: The embryos which germinated on MS medium containing BAP (5uM) IAA (0.5 μM) and adenine sulphate (0.5mM) were devoid of roots.

Experiments were carried out using microcutting to find out appropriate combination for root induction (Table 3). Auxins NAA or IBA at concentration of (0.5-2 μM) were added in MS medium. Microcutting of *in vitro* grown plants were cultured and the number of root regenerated from these microcutting were observed after 10 days. The maximum number of root formation was observed in MS containing IBA (0.5 μM).

The optimum combination of maximum root formation MS containing IBA (0.5 mg/ml) was used for root induction in subsequent experiments.

Plant Production: Embryogenesis observed from hypocotyl explant can be divided into categories (Fig. 1). The horn shaped embryos (Fig. 1b) as well as globular-shaped embryos (Fig. 1a). The cotyledonary stage of embryos was not observed from hypocotyl explant when these various shaped embryos were followed for germination, the globular shaped embryos showed better germination (Fig. 1c). The first leaves which emerged from embryos have hairy surface which latter resulted into a normal shoots. The shoots were transferred on MS containing IBA (0.5) for development of roots.

Table 4 presents the embryogenesis of two local varieties, 6153 and C-44. Chickpea variety 6153 produced 12 percent and 3

percent embryos from hypocotyl explants of 5 and 7 days old seedlings while variety C-44 produced 16 percent and 20 percent embryos. The low percentage of embryo formation in C-44 was observed as compared to variety 6153 when calli were obtained from 5 or 7 days old seedlings. In both cultivars seedlings at the age of 7 resulted in higher embryo formation.

Expression of GUS gene was observed in a group of cells giving blue colour is separate foci (Fig. 1d). Depending upon the distance travelled by DNA coated tungsten particle to the target tissue, 58 percent of the explant showed blue colour and no blue foci was observed in tungsten bombarded control explants (Table 5).

Discussion

In the present investigation it was noticed that hypocotyl explant undergoes regeneration via callus formation. For the induction of callus, the addition of auxin NAA or 2, 4-D with the same concentration of BAP does not make any difference.

However for further development of calli into shoots, the replacement of auxin NAA with IAA has tremendous effect. Two phases of regeneration via callus formation were observed induction phase where either auxin can be used and differentiation phase where only IAA helps in the regeneration of shoot-buds into shoots. Similar two steps regeneration system has been reported in other legumes like *Medicago sativa* (Meijer and Brown, 1987). The typical embryogenesis was observed in the local variety 6153. Both glabular-shaped and horn-shaped embryos were observed as reported in other varieties of chickpea (Sagare *et al*., 1993; Barna and Wakhlu, 1993) and upto 12 percent regeneration of embryos was observed when calli of hypocotyl explants were used. In other studies the regeneration of 50 percent was reported that may be due to the nature of explants used for studies. Generally it appears that in chickpea as the seed germinate and grows into various organs like shoot, root and leaves, its regeneration capacity declines.

The pattern of shoot morphogenesis from hypocotyl explants via callus formation shows that regeneration of shoot buds required the presence of organized meristematic region in the intact callus. This type of shoot morphogenesis has also been reported by Bhojwani and Mukhopadhyay (1984) in another legume *Lathyrus sativus*. However this type of shoot morphogensis can not be used for transformation studies because *Agrobacterium tumefaciens* transform few and not all the cells present in an explant (Draper *et al*., 1988). On the other hand unorganized cells do not regenerate shoots in *Cicer arietinum* (Altaf and Ahmed, 1986). Therefore alternate methods of transformation known as biolistic has been used to transformed hypocotyl tissue of Pakistani variety 6153 and CM 72. Transient expression of GUS gene is observed in more than 50 percent of explant bombarded but the regeneration efficiency and plant recovery percentage is not adequate to use this explant for transformation. The somatic embryogenic via callus is a preferable system but further experimentation is required to insert useful genes in this legume crop.

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