ASSESSMENT THE RESPONSE OF CHICKPEA GENOTYPES TO AGROBACTERIUM -MEDIATED TRANSFORMATION SYSTEM

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(Accepted for publication: May 24, 2016)

Abstract:

Transformation protocol based on the inoculation of chickpea mature embryos with *Agrobacterium* suspension was carried out. Four chickpea lines and one Iraqi local variety were used as recipient to the foreign gene of *Agrobacterium tumefaciens* strain (AgL1). Three plasmids were already inserted in the bacteria cells. The first plasmid carries the bar gene coding for phosphinothricin acetyle transferase (PAT), which confers resistance to the herbicide phosphinothricin or glofosinate ammonium(PPT) and uidA (gusA) gene coding for β-glucuronidase (GUS). The other two plasmids carried the LeEREBP gene which confers drought resistance and bar gene coding for phosphinothricin acetyle transferase (PAT).

Successfully regenerated explants were subjected to selection pressure on 10 mg /l of phosphinothricin PPT and the putative transgenic explants were rooted on root induction medium consisting of MS basal medium with B5 medium vitamins supplemented with 2.5 ml of 1mg /ml IBA in addition to grafting on 7 days old non- transformed rootstock. PCR approved transgenic chickpea. 600 mg/l of PPT was used by painting the leaves of surviving plants to detect the expression of bar gene which encodes for phosphinothricin acetyle transferase and confirmed herbicide resistance in transgenic plants.

Key words: *Chickpea transformation, PPT, PCR, Herbicide resistance*

Introduction

Agrobacterium-mediated transformation has been used successfully in grain legumes for over a decade. Chickpea (*Cicer arietinum* L). It is also known as gram, or Bengal gram, garbanzo or garbanzo bean and sometimes known as Egyption pea.

Chickpea is an important source of protein for millions of people in the developing countries, particularly in South Asia of the tropics and subtropics and is one of the most important grain legumes and is a rich source of dietary proteins as available source for both human and animal nutrition. . In addition to having high protein content (20-22%), chickpea is rich in fi ber, minerals (phosphorus, calcium, magnesium, iron and zinc) and β-carotene. Its lipid fraction is high in unsaturated fatty acids (Pooran *et al*., 2010).

Advances in biotechnology of grain legumes may lead to introduction of novel traits through genetic transformation into chickpea which its grain productivity reduced its yields via both biotic and a biotic factors due to lack resistance to many environmental factors and this problem remains the major cause of significant loss of the product (Singh *et al*., 1994).

Agrobacterium tumefaciens harboring a large plasmid known as tumer- inducing (Ti) plasmid, a fragment of Ti plasmid is transfer DNA (T-DNA) which carries several genes conferring special properties(Gelvin , 2003) . Therefore, the introduction of specific genes into chickpea could be achieved by genetic engineering.

Up to date many of transformation and *In vitro* plant regeneration methods of chickpea had been reported (Fontana, *et al*., 1993; Tewari – Singh *et al*., 2004) and many *Agrobacterium* mediated transformation also had been reported (Krishnamurthy *et al*., 2000; Senthil *et al*., 2004; Sharmin *et al*., 2012).

The aim of this investigation was to enhance a reliable method for mature embryos of chickpea and development of an efficient transformation protocol for different genotype in addition to its response by using *Agrobacterium tumefaciens* . And this investigation was carryout under the authorization of International Centre for Agricultural Research in the Dray Areas (ICARDA)

Materials and methods

Plant material

Chickpea seeds (*Cicer arietinum* L) from seed bank of ICARDA were provided include four Kabuli genotype Flip 86-5, Flip 88-85, Flip 97-706 and Iraqi local one (Duhoki) were used. The seeds were surface sterilized with 75% ethanol (v/v) for one minute followed by 5% sodium hypochlorite for 15 minutes with slow agitation , rinsed three times in sterilized distilled water and soak for about 16 hours supplemented with 10 ml /l (v/v) calcium chloride. Embryos were isolated by splitting cotyledons and keeping them in sterilized distilled water to avoid embryo dehydration at room conditions (ICARDA, 2003).

Agrobacterium strains and plasmid

For inoculation of decapitate embryos, Transgenic AgL1 strain of *Agrobacterium tumefaciens* were used carrying pGREEN plasmid obtained from DMSZ (Germany) and CLIMA (Australia). The first plasmid carries the bar gene coding for phosphinothricin acetyle transferase (PAT), which confers resistance to the herbicide phosphinothricin or glofosinate ammonium and uidA (gusA) gene coding for β-glucuronidase (GUS) used as reporter gene both of which are driven by 35S promoter . The second plasmid carries the LeEREBP gene which confers drought resistance and bar gene coding for phosphinothricin acetyle transferase (PAT). And another one contains same genes which they are driven by 35S promoter. All constructs was available in Biotechnology Lab. ICARDA.

To prepare bacterial inoculums, single colony of each *Agrobacterium* strain was maintained in plastic Petri dish on Luria broth agar. The medium (LB) containing 100 mg/l kanamycin and incubated for one day at 28˚C. A swab of bacterial cells was transferred to 25 ml of liquid LB medium containing 25µl kanamycin and grown in rotary incubator overnight at 28˚C and 150 rpm to an O.D 600 at 1.2 – 1.4. The bacterial culture were centrifuged at 4000 rpm at 4 ˚C for 15 minutes to collect bacterial cells, then the pellet was suspended in 20 ml of Agro suspension containing MS macro – and – micro basal salts (Murashige and Skoog , 1962) supplemented with 12 mg Fe-Na- EDTA, 5 mg manganese sulphate, 2.6 mg zinc sulphate, 2 mg boric acid, 0.075 mg sodium molybdate, 0.0075 copper sulphate , 0.0075 mg cobalt chloride, 0.25 mg potassium iodide , 1 mg nicotinic acid , 10 mg thiamine, 1 mg pyrodoxin , 100 µM acetosyrengone , 0.25 µM thidazuron (TDZ), 15 g sucrose and 15 gm glucose for one liter). Acidity was adjusted to pH 5.7 before autoclaving at 121˚C for 20 minutes.

Transformation procedures

Inoculation and co-cultivation

A scalpel wetted with *Agrobacterium* suspension was used to cut 1-2 mm of both embryo tips (Fig.1A) under sterile conditions. Decapitate embryos were stepped three times with wetted micro needle of *Agrobacterium*. Thereafter, the explants were incubated in 10 ml of *Agrobacterium* suspension for 2 hours under room temperature, blotted dry on sterile filter paper and co-cultivated on filter paper placed on solidified (0.2%) DKW (Driver and Kuniyuki, 1984) supplemented with the same extra amount used in agro suspension solution (micro salts, vitamins, 0.05 µM TDZ, sucrose and glucose) for six days at 22 ˚C in dark (Fig.1B). To remove the eliminate *Agrobacterium*, the explants were rinsed three times with sterile distilled water after removing incurved roots, followed by immersing them into 150 mg/l ticarcillin three times 3,5,5 minutes subsequently.

Regeneration and elongation

The medium (DKW) containing (0.005 µM TDZ) was used for explants regeneration for one week, then the regenerated ones were transferred to elongation medium {(DKW supplemented with 1 g/l ethanosulforic acid (MES), 2 mg 6-Benzyleamino purine (BAP) and 0.01 mg Indol 3- butaric acid (IBA) per one liter} for three weeks at a light intensity of 75 µmol.photon.m1s-1 (ICARDA, 2003). (Fig.1C).

Selection

For selection, DKW medium supplemented with 5 mg/l PPT were used, eight explants per plate were cultured after subdividing them into three parts (original shoot derivate from the apex and two auxiliary buds (Fig. 1D). Each selective stage remained two weeks and the selective pressure was increased to 10mg/l PPT and 10g/l polyvinyle pyrolidone (PVP) by adding them to the media at the last three subcultures. The survival green shoots were subjected by repeated excision of the branches to fresh selection medium for seven rounds (ICARDA, 2003).

Rooting

Two methods were used for root formation:(i)putative transgenic shoots were isolated and cultured on root induction medium consist of MS basal medium with B5 medium vitamins supplemented with 8g/l agar, 1.5% sucrose and 2.5 ml of 1mg /ml IBA (Fig.1E). (ii) half strength MS medium were used to germinate chickpea seeds for 7 days followed by grafting small putative transgenic explants (Fig. 1F), and incubated in the dark for 3 weeks. the successful plantlets were transferred to plastic pots containing soil mixture consist of (1:1:1) (clay, sand: peat moss) in controlled growth room conditions; 22 C˚; 16/8 h photoperiod, and light intensity of 75 µmol photon m1s-1., the plants were covered with polyethylene bags for one week, then they were punctured to reduce the humidity followed by removing them after 2 weeks (Fig.1G,H) for acclimatization(ICARDA, 2003).

GUS assay

For GUS assay, 4 ml x-gluc (1mg/ml) was mixed with 6 ml GUS buffer [100 mM phoshphate buffer , 1 mM Na2 EDTA , 0.5 mM K3[Fe (CN)6]. 3H2O].Co-cultivated samples (decapitate embryos) were taken and immersed in 200 µl solution , incubated for 16 h at 37˚C. Green tissues were cleared overnight with 100% absolute ethanol (Jefferson, 1987).

Genomic DNA extraction

Modified CTAB method (Doyle and Doyle, 1990) was used for genomic DNA isolation from 0.5 g young leaves, grinded with liquid nitrogen followed by maceration in 800 µl of CTAB buffer (3% CTAB, 1.4M NaCl, 0.2% mercapto ethanol, 29 mM EDTA, 100 mM Tris-Hcl (pH, 8.0), 0.5% PVP) mixed and incubated for 30 minutes at 60˚C , 800 ml of chloroform-iso amylealchohol (24:1) was added with shaking , then were centrifuged for 10 minutes at 14,000 rpm, followed by transferring the liquid phase to new micro centrifuge tube. 2/3 volume of pre-cold isopropanol was added to the liquid phase and mixed gently , thereafter , DNA were pelleted using same condition of centrifugation .Supernatant was discarded and the pellet washed in 20 µl washing buffer (76% ethanol, 10 mM ammonium acetate), the pellet was air-dried after removing the buffer and re suspended in 200 µl TE buffer (10mM Tris- HCl (pH,8.0), 1mM EDTA)supplemented with 10 mg/ ml RNAse A; and incubated for 30 min. at 37 C˚ followed by adding 100 µl of 75mM ammonium acetate and 750 µl ethanol with mixing . The supernatant was discarded and the pellet was dried an re suspended in 20 µl of sterile distilled water (ICARDA, 2003).

Polymerase chain reaction (PCR)

The primers used for the amplification of a specific bar sequence (264 bp) were 5'-GCAGGAACCGCAGGAGTGGA-3' and 5'-AGCCCGATGACAGCGACCAC-3'.PCR reaction was carried out in 20 µl total volume containing 2.0 µg genomic DNA, 0.4 µM of each primer, 10 x PCR buffer (100 mM Tris-HCl, 500 mMn KCl, 15mM MgC2), 200 µM each dNTPs and 1.0 unit of Taq DNA polymerase. The PCR conditions were 4 min initial denaturation at 94ºCº, followed by 30 cycles for 90 sec. denaturation (94º Cº), 90 sec. annealing (62º Cº), 30 sec. extension (72º Cº), and finally a 5 min extension step at 72 Cº. Also , the primers sequence 5'-CACAATCCCACTATCGTTCGC-3'and 5'-TCCGTCCACTCCTGCGGT-3' were used to amplify 294 bp of the 35S promoter sequence with the following PCR condition: : 94º Cº (4min), followed by (30) cycles for (1 min) at 94º Cº , (1 min) at 60 Cº, (2 min) at 72º Cº and finally (7 min) at 72º Cº. Also the Sequences primer was used to amplify 473 bp of the LeEREBP : 5'-TTC TGA TGA TGA TGA TGA TG -3' (20 bp) and 5'-TAA AAG ACA CAT TCT CGA AG -3' with the following PCR condition : 94 Cº (5 min), followed by (35) cycles for (30) sec at 94º Cº , (40) sec. at 56º Cº, (1) min at 72º Cº and finally (7) min at 72Cº. Also the primer sequence 5'- AGA TTT CCA TTT GAC TAG TG-3' and 5'- AAA GTC ATT TTG CTC TCT AC-3' were used to amplify 477 bp of the rd29A primer with the following PCR condition : 94º Cº (5 min), followed by (35) cycles for (40 sec.) at 94º Cº , (40sec.) at 55Cº, (40 sec.) at 72ºCº and finally (5) min at 72ºCº.

Electrophoresis

PCR products were separated by electrophoresis at 80V for 1.30 h in 1.2% agraose gel. Thereafter, the gel was stained in ethidium bromide solution (0.5 µg /ml) with slow agitation for 20 min, and visualized under UV light in the gel documentation device. A digital photo was taken for further analysis.

Results and Discussion

Tissue culture and plant transformation

Decapitate mature embryos of four Chickpea lines were tested for Agrobacterium – mediated transformation by using AgL1 strain of Agrobacterium . In this study a total 4844 mature embryos were subjected to Agrobacterium (table 1), the explants were regenerated to produce 4-5 shoots (Fig.1B). The putative transgenic shoots were derived from the apical of the embryos followed by subjecting them to selection pressure (Fig.1D) which inhibit the shoot formation by elimination of untransformed cells (Kar *et al.,* 1997), and the chosen protocol leads to directly shoot formation without an intermediate callus phase.

There was different response for transformation as a result of using different lines and constructs, which display a different transformation efficiency ranged between 0-6.6. The results also showed that the efficiency of transformation in Iraqi local and Flip 88-85 was 0 by using the constructs LeEREBP/35S, and LeEREBP/ rd29A. While Flip 88-85 line reports the highest transformation efficiency with Gus / rd29A construct (6.6) followed by Flip 97-706 by using LeEREBP/35S (5.8).

Table1: Transformation efficiency in different Chickpea lines

Line	Construct	No. of co-cultivated mature embryo	No. of putative transgenic explants	Efficiency %*
Flip 88-85	rd29A/ LeEREBP	400	3	0.75
Flip 88-85	35S /LeEREBP	327	0	0
Flip 97-706	rd29A /LeEREBP	202	4	1.98
Flip 97-706	35S /LeEREBP	205	12	5.8
Iraqi Var.	rd29A /LeEREBP	422	0	0
Iraqi Var.	35S /LeEREBP	688	0	0
Flip 86-5	rd29A /LeEREBP	1090	5	0.45
Flip 86-5	35S /LeEREBP	1181	0	0
Flip 86-5	rd29A /Gus	119	2	1.6
Flip 88-85	rd29A /Gus	210	14	6.6

*The transformation efficiency was obtained by dividing the number of independed clones ×100 with the total number of co cultivated embryos.

Rooting

Putative transgenic regenerates showed direct rooting in the medium consisting of MS basal medium with B5 medium vitamins supplemented with 8g/l agar, 1.5% sucrose and 2.5 ml of 1mg /ml IBA during 6 weeks by forming of 2-5 roots /plantlet with high range between 0.5-8cm (Fig.1E), while the others which fail in direct rooting were successfully grafted by using an alternative micrografting technique (Krishnamurthy *et al*., 2000) on germinated seeds for 7 days on half strength MS medium (Fig1D). After 5 weeks the new rooted plantlets were transferred to soil mixture (1:1:1) (clay , sand and peat moos) for acclimatization .15% of them were fail to remain healthy .As a result or consequence of this , thirty four of putative transgenic shoots were successfully drafted derived from 20 clones which has been sub cultured continuously (table 2) and only (16) of them (47%) were gained as a transgenic T0 plant and the other (18) (53%) were died and not developed to transgenic plants during the acclimatization period.

Table 2: in vitro clones and recovery of T0 plants

Line	Construct	No. of clones	No. of successfully grafts	No. of T0 plants	PCR	Seed no.
Flip 88-85	rd29A/ LeEREBP	1	4	2	+	3
Flip 88-85	35S /LeEREBP	0	0	0	-	0
Flip 97-706	rd29A /LeEREBP	1	4	2	+	4
Flip 97-706	35S /LeEREBP	6	4	2	+	5
Iraqi Local	rd29A /LeEREBP	0	0	2	-	0
Iraqi Local	35S /LeEREBP	0	0	0	-	0
Flip 86-5	rd29A /LeEREBP	2	1	0	-	0
Flip 86-5	35S /LeEREBP	0	0	0	-	0
Flip 86-5	rd29A /Gus	1	6	2	+	7
Flip 88-85	rd29A /Gus	9	16	8	+	22

As a result of above data or facts we can conclude that the line Flip 88-85 cocultivated with the construct rd29A / GUS was advanced when we compare it with the other line followed by Flip 86-5 line with the same construct, while the Iraqi local line didn't show any response for genetic transformation by using these two strains of A*grobacterium tumefaciens* and that may be is due to lack of purity for this line. Moreover, the results we obtained showed that the construct with the promoter 35S was less response compared with the same genes with the promoter rd29A which display more transformation potential.

CUS Expression

Most of used embryos stained with blue color after treatment with X-gluc. at the apices which is very important for giving the evidence of establishment of transformation protocols although, didn't mean that all samples will form transgenic plants.

Polymerase Chain Reaction (PCR)

The results of table 2 display that all tested transgenic T0 plants developed in growth room and T1 plant that derived from transgenic T0 seeds showed positive PCR using specific primers produced the fragments of used genes (264bp for bar gene, 294 bp for 35S and 437 bp for LeEREB gene. (Fig.2 A,B,C,D).

Although the protocol which has been used in our study is not far away from Krishnamurthy (2000) and Kiesecker (2000) protocol, getting higher transformation efficiency to transform our local lines is conceder a promising results to Enhance the resistance of new crops for both biotic and abiotic factors, moreover, new studies are needed to increase Chickpea genetic transformation, in addition to study the segregation of the genes and physiological features of the plant derived from T0 seeds.

600 mg/l of PPT was used by painting the leaves of surviving plants to detect the expression of bar gene which encodes for phosphinothricin acetyle transferase and confirmed herbicide resistance in T0 and T1 plants.

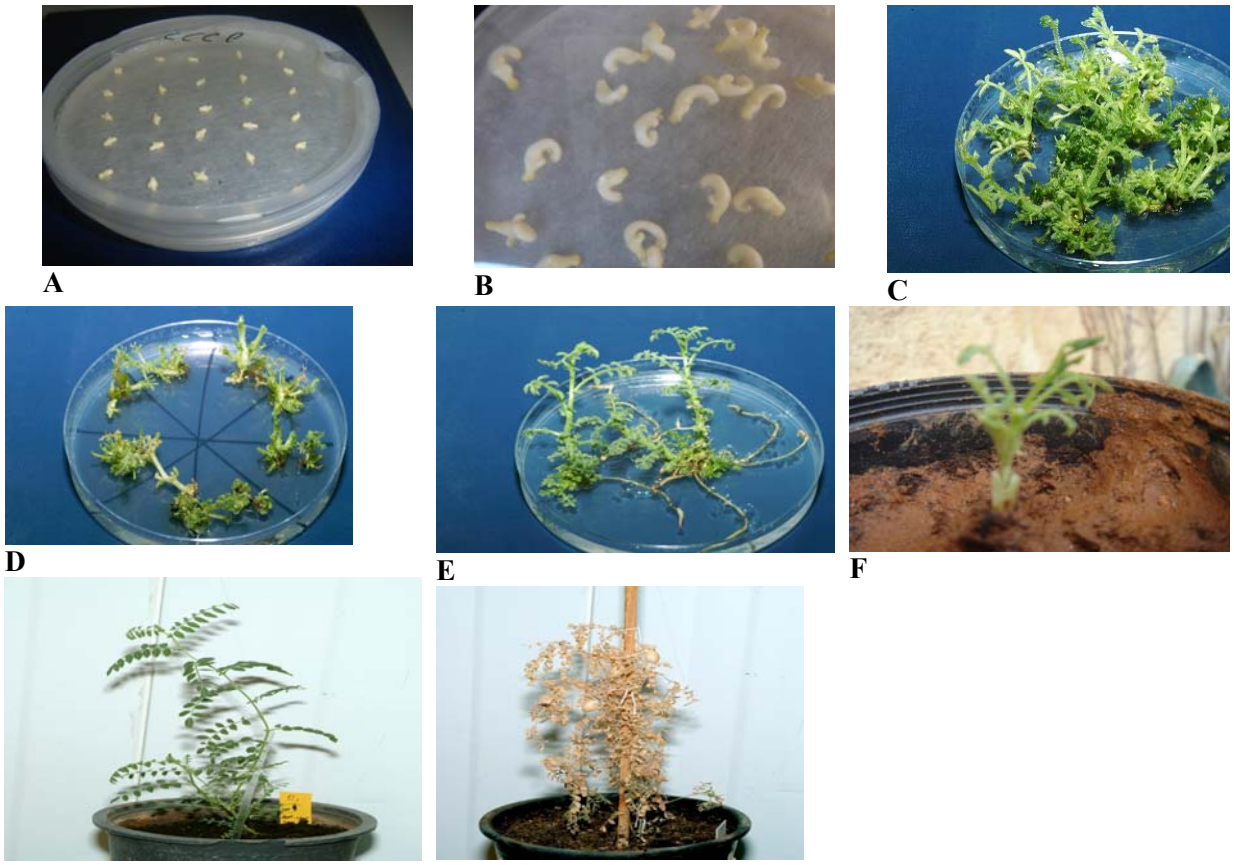
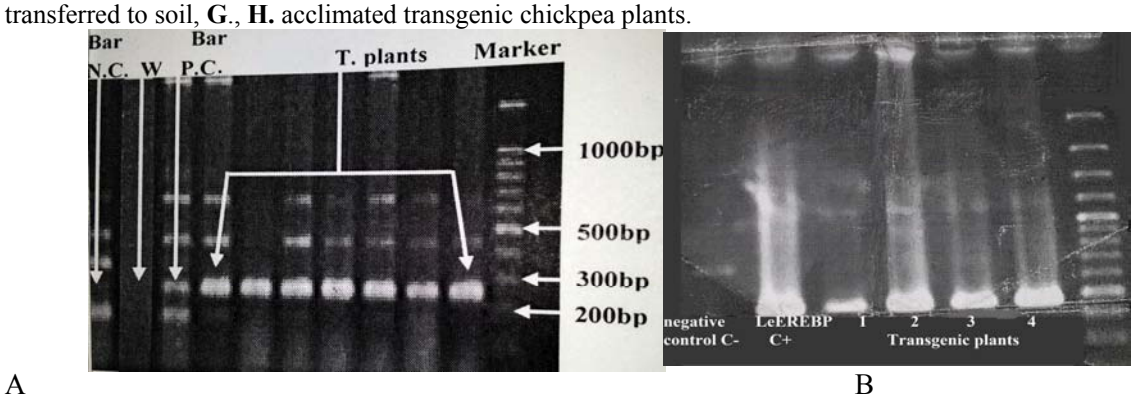


Fig.1 A cocultivated embryos, **B**. cocultivated embryos after 6 days, **C**. elongation embryos , **D**. selection on medium supplemented with phosphinothricin, **E**. rooting of putative transgenic explants. **F**. Grafted shoots transferred to soil, **G**., **H.** acclimated transgenic chickpea plants.



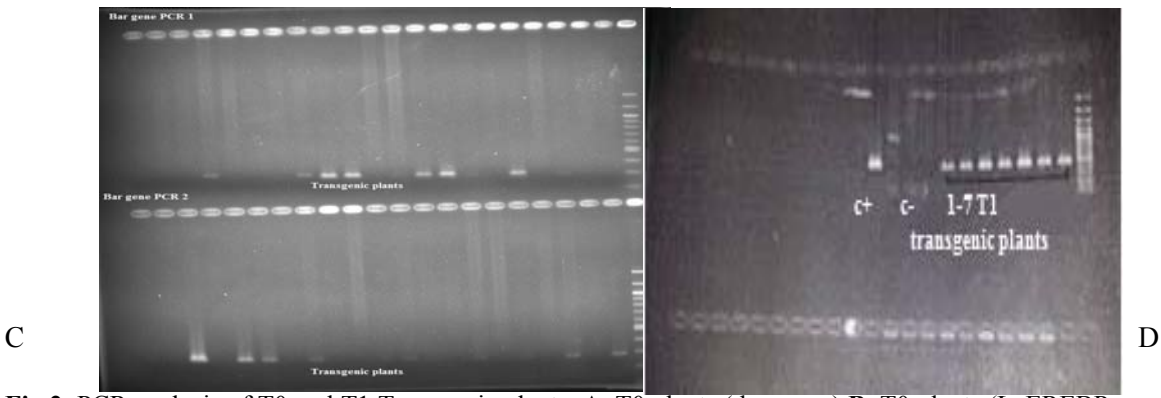


Fig 2. PCR analysis of T0 and T1 Transgenic plants. **A.** T0 plants (bar gene) **B.** T0 plants (LeEREBP gene), **C.** T1 plants (bar gene), **D.** T1 plants (LeEREBP gene)

Acknowledgments: We would like to thank Ms. Nahid Al Sokhny , Reem Dabbagh and all ICARDA staff that supported this investigation.

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پوختە:

پروتوکولەکا ڤەگوهاستنا ژینیتیکی ل سەر بناغێ ڤاکسینکرنا ئاوەلەمێن گەهشتی یێن نوێکا دگەل تێکهەلێ ئەگروبەکتیریەم هاتە بدەستڤەئینان دئەڤێ ڤەکولینێ دا. چوار توخمێن نوێکا دگەل جورەکێ نوێکێن عیراقی هاتنە تاقیکرن ژبو پێشوازیکرنا جینێ بیانی یێ Agrobacterium tumefaciens ژ .(AgL1). سێ پلازمید هاتنە ژوورێخستن دناف خانێن بەکتریایێ. پلازمیدێ ئێکێ کودێ جینێ phosphinothricin acetyle transferase (PAT) بخوڤە دگرت، ئەوێ جینێ بەرگیریێ بو ژناڤبەرێ ئادەی phosphinothricin of glofosinate ammonium and uidA (gusA) زێدەکری بو β-glucuronidase (GUS). هەردوو پلازمیدێن دیتر جینێ LeEREBP بخوڤەدگرت ئەوێ جینێ بەرگیریا هشکاتیێ زیدەکری دگەل کودێ جینی بو phosphinothricin acetyle transferase (PAT).

پارچێن رووەکی ئەوێن هاتینە بدەستڤەئینان ب سەرکەفتیانە هاتنە سەرەدەریکرن دگەل فشارێ ژێگرتی ل سەر 10 ملگم/ لتر ژ PPT وریهدان هاتە بدەستڤەئینان ل سەر ئەفان پارچێن رووەکی دناف بیاڤێ چاندنێ MS دگەل ڤیتامینێن بیاڤێ B5 یێ زێدەکری ب 2,5 ملگم/ لتر ژ IBA دیسان دگەل پیککرناوان ل سەر شتلێن دژیێ 7 روژان دا. PCR هاتە بکارئینان ژبو چەسپاندنا نوێکێن هاتینە تاقیکرن. 600 ملگم/ لتر ژ PPT هاتە بکارئینان ژبو بویاغکرنا بەلگێن رووەکێن ساخ ژبو شلوڤەکرنا بوچوونا ارجینێ یێ phosphinothricin acetyle transferase دیسان ژبو چەسپاندنا بەرگیریا ژناڤبەرێ ئادەی دناف رووەکێن .

تقييم استجابة سلالات نبات الحمص للتحول الوراثي بوساطة بكتيريا *Agrobacterium tumifaciens*

الملخص :

تم تطبيق نظام او بروتوكول للتحول الوراثي باعتماد تلقيح الاجنة الناضجة مع معلق الاكروبكتيريوم باستخدام اربعة سلالات لنبات الحمص فضلا عن سلالة اخرى تزرع محليا في كوردستان العراق كمستقبلات لمورثات سلالة AgL1 لبكتيريا *Agrobacterium tumefaciens* المهندسة وراثيا والتي كانت تحتوي على ثلاث بلازميدات . الاول يحوي على المورثة bar التي تشفر للانزيم phosphinothricin acetyle transferase (PAT) والذي يمنح المقاومة للمبيد العشبي phosphinothricin or glofosinate ammonium (PPT) والمورثة uidA (gusA) التي تشفر للانزيم β-glucuronidase (GUS). أما البلازميدين الاخرين فيحملان المورثة LeEREBP التي تمنح المقاومة للجفاف فضلا عن وجود المورثة bar المذكورة اعلاه على نفس البلازميد.

عرضت النباتات المتمايزة والنامية بعد مرحلة التلقيح بالبكتيريا الى عملية انتخاب باستعمال 100 ملغم / لتر من المبيد العشبي PPT ثم تم تجذير النبيتات التي نجحت في عبور الانتخاب باستعمال الوسط MS كاساس والمدعم بفيتامينات الوسط الغذائي B5 و 2.5 مل من 1 ملغم/ مل من الاوكسين IBA . اما تلك التي فشلت في تجذيرها بتلك الطريقة فقد اتبعت طريقة التطعيم الدقيق على اصول نباتات الحمص المجذرة بعمر 7 ايام وغير الملقحة بالبكتيريا .

أثبتت تفاعلات التسلسلي المتعدد PCR باستخدام جهاز التدوير الحراري في تضخيم اعداد المورثات المدروسة والمأخوذة من النباتات المفترض تحولها وراثيا والتي اجتازت مراحل الانتخاب تحولها وراثيا ، اعقب ذلك اختبار تلك النباتات الحاملة للمورثة bar من خلال مسح اوراقها ب 600 ملغم / لتر PPT للكشف عن مقاومتها لهذا المبيد والتي اثبتت نجاحها في مقاومته.