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ORIGINAL ARTICLE

Agrobacterium Mediated Transformation of Fld and GUS Genes into Canola for Salinity Stress

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Salinity is one of the major abiotic stress which limits wide spread canola cultivation. One way to overcome this problem could be transfection, to produce tolerable species. Cotyledonary and hypocotyls explants obtained from 4 and 7 days old seedling of Elite and RJS003 varieties were utilized in this study. Genetic transformation was implemented through *Agrobacterium tumefaciens* LBA4404 containing PBI121 plasmid and *Agrobacterium tumefaciens* C58, LBA4404, AGL0 and EHA 101 strains which contain P6u- ubi- fvt1 construct. The T-DNA region of P6u- Ubi- Fvt1 plasmid included HPT (Hygromycin phosphotransferase) plant selectable marker and Fld (flavodoxin) gene. PBI121 plasmid had NptII (Neomycin phosphotransferase) plant Selectable marker and β-glucuronidase (GUS) reporter genes. Transfected explants were analyzed by PCR and histochemical assay for Fld and Gus genes, respectively. Our data indicated that the cotyledonary explants of both cultivars were incompetent to be infected with Fld gens. However, the transformation in Elite hypocotyls explants with *Agrobacterium tumefaciens* C58 and LBA 4404 strains were confirmed through PCR product and histochemical evaluation for Fld and GUS genes, respectively. Therefore, the result of this manuscript may to certain degree fulfill the endeavor appointed to this oilseed.

Key words: Salinity, Brassica napus L, Transformation, Agrobacterium tumefaciens, Fld, GUS

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Salty soil and salinization of lands are a growing problem for plant production (Flowers, 2004). According to FAO report, 6% of the world's total land and 20 % of irrigated land is salt affected (FAO., 2005). Since irrigated land contributes to one-third of the world's food production, engineering crops capable of growing in saline environments is one of the essential needs for today's agriculture (Hussain

et al., 2008). Chloroplast ferredoxin has a basic role in transferring a reduced electron from photosynthetic electron transport chain (PETC) to various oxidative pathways (Tognetti *et al*., 2006). In the numerous microorganisms such as Cyanobacteria, after exposure to diverse abiotic stress condition, level of Fd is decreased and flavodoxin rank is significantly increased. The compensatory Fld elevation offers tolerance for the microorganism. Moreover, transferring Cyanobacterial Fld into the plants provides multiple stress tolerance phenotype in which bacterial Fld take place of plant Fd-dependent reactions in vitro (Zurbriggen *et al*., 2007). Cyanobacteria and chloroplast share same prokaryote ancestor. Therefore, gene exchange between these two is rational (Cardoza *et al*., 2003). Canola (*Brassica napus* L.) is an important oil crop and holds the third rank in global oil production after soybean and palm seed (Cardoza *et al*., 2003). Canola oil is widely used as cooking oil. It has lowest saturated fatty acid content and has application for production of margarine (Mashayekhi *et al*., 2008). It seems that improvement of plant through conventional breeding methods is limited (Yamaguchi *et al*., 2005). But using of tissue culture and molecular genetics technique provides a new opportunity to plant breeding (Moravcikova *et al*., 2009). There are several reports about modification in canola for qualitative and quantitative trait such as improvement in oil component (Knutzon *et al*., 1992), herbicide tolerance (De Block *et al*., 1989), insect resistance (Stewart *et al*., 1996) and protein composition (Altenbach *et al*., 1992). Transformation was performed with different kinds of explants such as stem internodes (Fry *et al*., 1987), stem segment (Pua *et al*., 1987), cotyledonary petiol (Moloney *et al*., 1989), hypocotyl segment (Dellaporta *et al*., 1983; Radke *et al*., 1988). Therefor, detecting extension variety and protocol to improveability transformation is needed. In this manuscript, potential regeneration of Elite and RJS003 cultivares and their interaction effect with 4 strains of Agrobacterium to transfer Fld and GUS genes into canola has been evaluated.

MATERIALS AND METHODS

Chemicals, Plant material and transformation vector

All of the chemicals and antibiotics used in this research were taken from Sigma- Aldrich, U.S.A and phytohormones purchased from Duchefa Company, Netherland. Two plasmids were used for transformation. P6u-ubi-fvt1 plasmid carrier Fld (Flavodoxin) gene under the control of Ubi (ubiquitin) promoter and NOS (Nopalin synthase) terminator. Its plant selectable marker was the Hpt (Hygromycin phosphotransferase) gene with Ubi promoter and 35S (CaMV) terminator. Insertion of this gene to the explants, confer resistant to Hygromycin antibiotic.

PBI121 construct encoding UidA (β-glucronidase) gene with 35S promoter and NOS terminator. Its selectable marker was NptII (Neomycin phosphotransferase) gene that controlled with NOS promoter and terminator. Correctly transfer this gene to explants cause resistant to kanamycine.

Seeds of two commercial canola (*Brassica napus L.*) cultivars, RJS003 and Elite, were provided from Seed and Plant Improvement Research Institute, Oilseeds Research. Seeds surface were sterilized for 1 min with 96% (v/v) ethanol and followed by rinsing for 15 min in 3 % (v/v) commercial sodium hypochlorite and 0.1 % (v/v) tween-20 as a surfactant. Seeds washed five times with distilled

water and germinated on half-strong MS medium (pH=5.2) (Murashige, Skoog, 1962) including 30 mg/l sucrose and 8% agar (w/v), without phytohormones, cultures were incubated at 28 C˚ and 16/8 h light/dark (or day/ night) photoperiod. Cotyledon and hypocotyls explants were excised from 4 and 7day-old seedling respectively.

Transformation and plant regeneration

The Agrobacterium of different strains was grown overnight in liquid LB medium containing 75 mg/l rifampcin and 50 mg/l streptomycin, until its OD_{600} reached to 0/8. After centrifugation of bacteria in 4000 rpm for 10 min, the sediment resuspended in half-strong MS medium (pH=5.2) plus 0.05 mM acetocyringone and 30 mg/l sucrose (Murashige, Skoog, 1962). On the other hand, 15 Hypocotyls segments with average 1 cm length placed in precultured medium which contain MS medium supplemented with 1 mg/L 2.4-D (2,4-dichlorophenoxyacetic acid), 30 g/l sucrose, 8 % agar for 3 days. Explants were submerged for 5 min with Agrobacterium suspension with mentioned concentration in petri dish. After drying on the sterile Whatman 3 mm filter paper for 10 min, the explants were co-cultured in the precultured medium for more 2 days. With the intent to identify transfected explants, they were moved to callus induced medium (CIM) containing 1mg/l 2,4-D, 250 mg/l cefotaxim and selective 50 mg/l hygromycin antibiotic for next 2 weeks. Growing callus were sub-cultured on the CIM medium for coming 2 weeks. Then, calluses were transferred on the shoot inducing medium (SIM) containing 4 mg/l BAP (6-Benzylaminopurine), 2 mg/l zeatin, 5 mg/l silver nitrate ($AgNO_3$), 50 mg/l hygromycin and 250 mg/l cefotaxime for 2 weeks (Fig 1). Plantlets were cut and used for PCR profile evaluation. Datas were collected for callus induction and the shoot induction frequency and they were calculated as follows:

CIF= (number calli from one variety with one Agrobacterium strain)/(total inoculated explants) ×100

SIF= (number shoots from one variety with one Agrobacterium strain)/(total inoculated explants) ×100

In order to evaluate canola seed derived cotyledon capacity to be transfected, cotyledon with 1-2 mm long petioles were used for incubation, we were careful not to cut the meristemic tissue. So that only their cut edge were incubated with Agrobacterium strain for 10 seconds. Then, they were taken out and dried on sterile Whatman 3 mm filter paper and placed on the co-culture MS medium supplemented with 4.5 mg/l BAP, 30 mg/l sucrose and 8% (w/v) agar without antibiotic. 2 days after co-culture, explants were transferred to shoot inducing medium (SIM) and stayed for 6 weeks. The basic medium for SIM is MS medium plus 4.5 mg/l BAP, 250 mg/l cefotaxime and 50 mg/l hygromycin. All the cultures maintained at 24±2 ˚C and 16 h photoperiod using cool white daylight fluorescent lights at 50 µmol $m^{-2}sec^{-1}$. Appearance of yellowish explants which start to shrinkage and death in selectable medium which contains antibiotic represent failure of the target sequence transfection into explants. To ensure transfection T-DNA of PBI121 construct, cotyledon and hypocotyls explants were culture on the selection medium containing 50 mg/l kanamycin, this is implemented because of NptII gene in the construct. Histochemical investigation was implemented to confirm transformation in callus

stage.

Confirmation of transformation

Transformation was confirmed using Histochemical GUS assay for UidA gene and PCR with specific primer for fld gene (Jefferson, 1987).

Polymerase chain reaction

Putative transformed plantlets that were able to survive onto selectable medium, squashed and used for PCR. PCR detection kit was purchased fome Cinnagen Company, Iran, and DNA extraction was carried out according to Dellaporta method (Dellaporta *et al*., 1983) from transformed and non-transformed (Control) explants. The reaction mixture (25µl) contained 100 ng DNA, 2 µl dNTPs, 1 µl each primer, 0.3 µl Taq polymerase, 2 µl $MgCl_2$, 2.5 µl PCR Buffer and we add water until the volume reaches 25 µl. The forward and reverse primers pairs used for DNA amplification were 5′ CTACGGTACTCAAACTGG 3′, 5′ GCGATCGTCTGTTAAGTC 3′ for Fld gene and 5′ AGAATCTCGTGCTTTCAGCTTCGA 3′, 5′ TCAAGACCAATGCGGAGCATATAC 3′ for HPT gene. PCR was carried out using the Bio- Rad. After initial denaturation of DNA at 94˚C for 5 min, 35 cycle of amplification implemented in following order: denaturation at 94˚C for 1 min, followed by 1 min at 55˚C as primers annealing and 1 min at 72˚C for extension . For final extension 5 min at 72˚C was carried out. Amplicons were run on electrophoresis system using 1% agarose gel and visualized stained with ethidium bromide. After the test, using the detector, the band was observed and photographed with Syngene GelDoc instrument.

Histochemical GUS assay

The Histochemical assay for detection of β-glucuronidase (GUS) activity was carried out according to the method of Jefferson . The analyzed tissues were immersed in GUS staining solution containing 1 mmol/l of 5-bromo-4-chloro-3-indolyl glucuronide (X-gluc), 50 mmol/l sodium phosphate and 0.1 % of triton X-100, PH=7 for 24 h at 37C˚ in dark place. After discoloring in 70% ethanol for 5 min to improve contrast, GUS activity was visible as blue spots at the areas of enzyme activity.

RESULTS

Hypocotyl explants precultured on MS medium supplement with 1 mg/l 2.4-D for 3 days, during this term was simulated for callus formation. Hypocotyls segments after incubation with different strain of Agrobacterium was cultured on co-culture medium with 1 mg/l 2,4-D for 2 days. Callus was induced from both cut ends of hypocotyls segment onto CIM medium with 1 mg/l 2,4-D plus selectable antibiotics for 2 weeks. We used of hygromycin (50 mg/l) for selection , so only transformed hypocotyls segment with high vigority were able to survive on this medium and all of non- transformed explants were eliminated in the early stages. The remaining callus were transformed onto shoot induction medium supplement with 4 mg/l of BAP, and selectable antibiotics for 2 weeks, many of callus weaked and discarded in this period (table 1).

After incubation with different Agrobacterium strains, cotyledonary explants put onto co-culture medium containing 4.5 mg/l BAP for 2 days. Then they transferred to SIM with 4.5 mg/l BAP plus antibiotics for 2 weeks. All the explants died during this period and did not show symptoms of shooting (table 2).

Our findings showed, transformation ability in hypocotyl segment was better than cotyledon in this varieties. Despite, the highest production of callus by Elit*C58, we saw highest shoot regeneration in Elit* LBA4404 (PBI 121) treatment.

In the Elit variety highest callus formation belonged to Elit*C58 treatment and Elit* LBA4404 (PBI 121) was in the next level. Also, Elit* LBA4404 (PBI 121) had highest shooting ability that fallowed with Elit*C58. Other treatments haven't regeneration potential.

At the RJS003 variety, RJS003*C58 treatment showed highest callus production and RJS003* LBA4404 (PBI 121) being after that. Best shooting response observed in RJS003*C58 treatment that fallowed by RJS003* LBA4404 (PBI 121) and other treatment died during this test.

Conclusion of transformation showed direct dependence to interaction between bacterial strain, plant variety and explants regeneration ability. Accordingly we used from randomized complete block for analysis our data with SAS software in 1 percent level of significant. Analysis of variance (ANOVA) results, showed witch interaction between variety and bacterial strain is significant (Table 3). So their means of comparison do with LSD test (Fig. 2). This investigate showed significant difference between Elit*C58 and Elit* LBA4404 (PBI 121) interactions. The best interaction belongs to C58 bacterial strain with Elit cultivar. Also, there were significant differences in interaction between RJS003 variety with bacterial strains. RJS003 variety with C58 strain had superlative operation and RJS003* LBA4404 (PBI 121) was in the next ranks. Generally in this investigate interaction between Elit cultivar with C58 bacterial strain was better than RJS003 cultivar with C58, that could be found through their meaning of comparison (Fig. 2).

We performed PCR analysis with the Fld gene specific primers for confirming transformation plantlet incubated with T-DNA region relevant to C58, made up band in 495 base pair (bp) verified gene transfer (Fig. 3). In the present study, we used of histochemical assay to confirmed transferred T-DNA region of LBA4404 strain, blue spots at the ends of hypocotyls demonstrated GUS gene active (Fig. 4).

Table 1. Calli and shoot induction frequency from different variety hypocotyls.

Genotype	Agrobacterium strains	Number of explants	Callus inducing explants	Callus induction %	Shoot induction %
Elit	C58	480	449	93	28
	LBA4404 (P6u-Ubi-Fvt1)	480	0	0	0
	AGL0	480	0	0	0
	EHA101	480	0	0	0
	LBA4404 (PBI 121)	480	422	87	32
RJS003	C58	480	379	78	24
	LBA4404 (P6u-Ubi-Fvt1)	480	0	0	0
	AGL0	480	0	0	0
	EHA101	480	0	0	0
	LBA4404 (PBI 121)	480	353	73	23

Table 2. Calli and shoot induction frequency from different variety cotyledon.

Genotype	Agrobacterium strains	Number of explants	Shoots inducing explants	Shoot induction %
Elit	C58	480	0	0
	LBA4404 (P6u-Ubi-Fvt1)	480	0	0
	AGL0	480	0	0
	EHA101	480	0	0
	LBA4404 (PBI 121)	480	0	0
RJS003	C58	480	0	0
	LBA4404 (P6u-Ubi-Fvt1)	480	0	0
	AGL0	480	0	0
	EHA101	480	0	0
	LBA4404 (PBI 121)	480	0	0

Table 3. Analysis of variance (ANOVA) from percent induced explants.

S.O.V	Degree of freedom (df)	M.S
Repeat (r)	5	2.60130 ^{ns}
Variety (v)	1	459.21388 **
Bacterial strains (b)	4	24701.35768 **
Interaction v×b	4	134.48495 **

Figure 1: Organogenesis process in canola. A) Inflation hypocotyls ends onto preculture medium. B) callus generation onto CIM. C) shoot generation onto SIM.

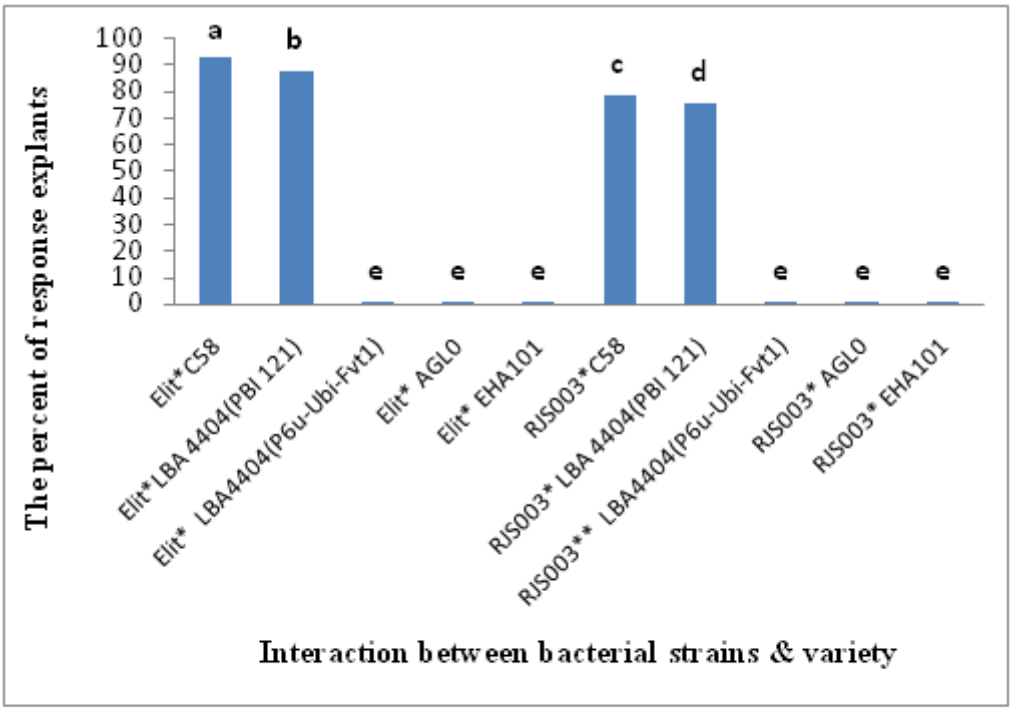

Figure 2: Means of comparison with LSD test for interaction between variety and bacterial strains.

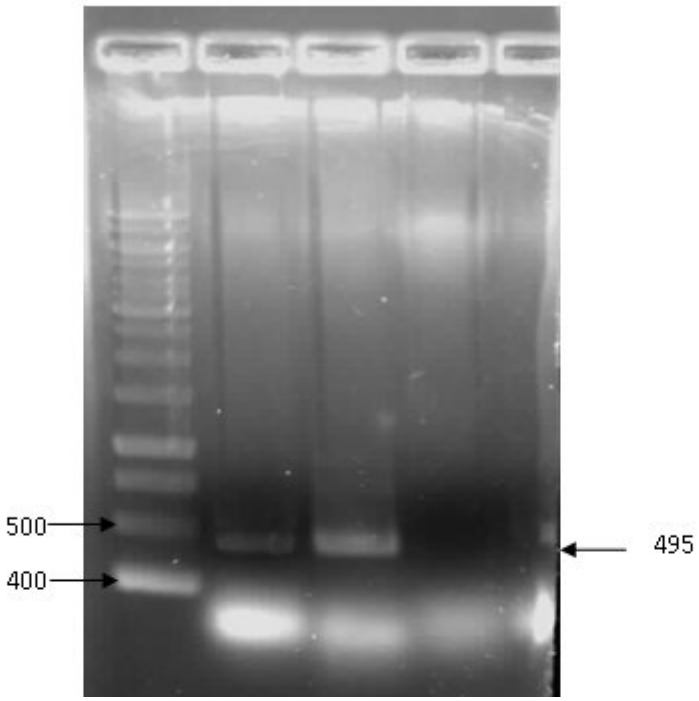

Figure 3: PCR analysis of transgenic canola with P6u-Ubi-Fvt1 (Fld). From left to right respectively: DNA marker, transgenic canola, a positive control from plasmid DNA, non-transgenic control canola DNA, purified water.

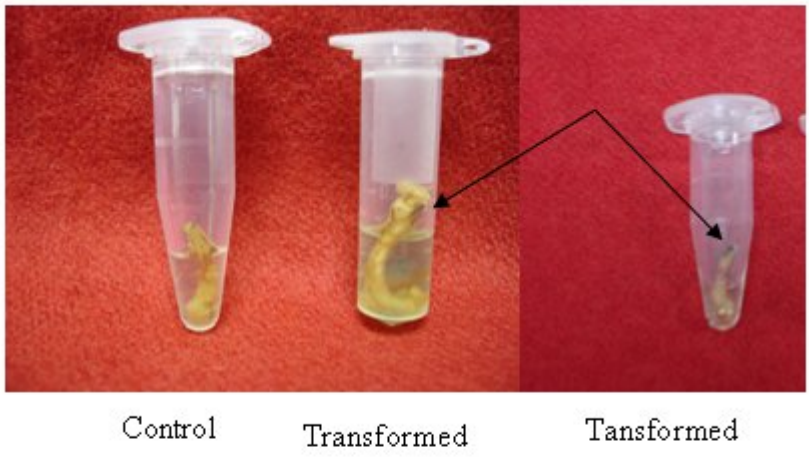

Figure 4: Histochemical detection of GUS activity in callus. The GUS activity (see black arrows) was detected as a blue spot in the ends of hypocotyl explants.

DISCUSSION

Fld gene transfection has recently directed the focus on biotechnology to generate resistant plants (De la Riva *et al*., 1998). This is of far more importance in Middle East countries which suffers from drought and salinity. We used *Agrobacterium tumefaciens* and Fld genes to produce transformed *Brassica napus* which is one of three worldwide used oilseed plants. Using Agrobacterium is most common system for Brassica napus transformation because of reduction in transgene copy number, the stable integration with fewer rearrangements of long molecules of DNA with defined ends and the ability to generate lines free from selectable marker genes (Jones *et al* ., 2005; Travella *et al*., 2005). Our results displayed efficiently Canola hypocotyls transfection which is conformed through PCR profile and histochemical analyses. This piece aligns with studies in which Fld genes were transferred into tobacco and alfalfa plants (Redondo *et al*., 2009).

Elite hypocotyl explants with C58 and LBA44404 agrobacterium strains acquired the better outcome in our study. It has been shown that, efficiency of transformation depends on bacterial strains, preculture, incubation and co-culture time, as well as levels of phytohormones during regeneration. Zhang *et al*., (2006) obtained higher number of transformed plants with cotyledon and hypocotyl segments.

Furthermore, we found more than 90% callus and almost one third shoot generation with Elite hypocotyls in medium containing 2,4-D and BAP. We used from 2,4-D for callus induction and blend of BAP, Zeatin and silver nitrate for shoot induction, our method similar to protocol used by Cardoza *et al*. (2004) and Meloney *et al.* (1989). The result of callus creation and regeneration has been reported differently in the articles. For example, best callus regeneration were observed when 2,4-D and BAP and silver nitrate were added into the medium while variable shoot induction detected with altered concentrating of NAA plus BAP and silver nitrate (Ali *et al*., 2007). Bano and colleagues studied regeneration protocol for *B. juncea* and reported maximum callus production on MS

medium supplement with BAP, NAA and maximum shooting in the same basal media with BAP, NAA, kinetin and IAA (Bano *et al*., 2010). In another study, no difference in percentage of explants that formed callus formation was reported however, a great varies in shooting ability of *B. napus* Australian cultivars were testified (Zhang, Bhalla, 1999). Al-Naggar *et al*., investigate on five cultivars with 4 stages of 2,4-D and they summed up with determined significantly different regeneration capability. In a way, Maximum shoot regeneration showed in Srew4 cotyledon on MS medium containing 4 mg/L BAP (Al-Naggar *et al*., 2010). It has been demonstrated that, this amount or variety could b4 that the genotype-specific action (Moghaieb *et al*., 2006).

In this research, cotyledon explants didn't response to transformation with used 4.5 mg/l BAP in both of variety. We used 4.5 mg/l BAP for shoot induction. However, Khan *et al*. reported 57.14 % regeneration from Tori-7 variety with 2.5 mg/l BAP (Khan *et al*., 2009). Other search obtained shooting from cotyledon explants with 2 mg/l NAA. These results showed variation in shooting ability in different variety (Zhang *et al*., 2006).

GUS is one of the main reporter gene that determined efficiency a method for transformation.

Since GUS reporter gene was transferred with our protocol, it is recommended applying this method as a basic scheme to transfer other genes. Besides, Elit hypocotyls explants with C58 Agrobacterium showed good transformation and regeneration. Considering to importance of Fld gene in stress tolerance, it calls for more examination to find out other varieties interaction.

REFERENCES

Al-Naggar AMM, Shabana R, Rady MR, Ghanem SA, Saker MM, Reda AA, Matter, M. A.; Eid, S. A. M. (2010) In vitro callus initiation and regeneration and in some canola varieties. *International Journal of Academic Research*. **2(6):** 356-361.

Alam khan MM, Hassan L, Ahmad SD, Shad AH, Batool F. (2009) In vitro regeneration potentiality of oil seed Brassica genotypes with differential BAP concentration. *Pak. J. Bot*. **41(3):** 1233-1239.

Ali H, Ali Z, Ali H, Mehmood S, Ali W. (2007) In vitro regeneration of Brassica napus L., cultivars (Star, Cyclone and Westar) from hypocotyles and cotyledonary leaves. *Pak. J. Bot*. **39(4)**: 1251-1256.

Altenbach SB, Kuo CC, Staraci LC, Pearson KW, Wainwright C, Georgescu A, Townsend J. (1992) Accumulation of a Brazil nut albumin in seeds of transgenic canola results in enhanced levels of seed protein methionine. *Plant Mol. Biol*. **18**: 235-245.

Bano R, Khan MH, Khan RS, Hamid Rashid H, Swati ZA. (2010) Development of an efficient regeneration protocol for three Genotypes of Brassica juncea. *Pak. J. Bot*. **42(2)**: 963-969.

Cardoza V, Stewart CN. (2003) Increased Agrobacterium-mediated transformation and rooting efficiencies in canola (Brassica napus L.) from hypocotyl segment explants. *Plant Cell Rep*. **21(6)**: 599-604.

De Block M, De Brower D, Tenning P. (1989) Transformation of Brassica napus and Brassica oleracea using Agrobacterium tumefaciens and the expression of the bar and neo genes in the transgenic plants. *Plant Physiol*. **91**: 694-701.

de la Riva GA, Cabrera JG, Pardo CA, Padron RV.

(1998) Agrobacterium tumefaciens: a natural tool for plant transformation. *Electronic Journal of Biotechnology*. **1(3)**.

Dellaporta SL, Wood J, Hicks JB. (1983) A plant DNA minipreparation: Version II. *Plant Mol Biol*. **1**: 19-21.

FAO. (2005) Management of irrigation-induced salt-affected soils.

Flowers TJ. (2004) Improving crop salt tolerance. *J Exper Botany*. **55**: 307-319.

Fry J, Barnason A, Horsch RB. (1987) Transformation of Brassica napus with Agrobacterium based vectors. *Plant Cell Rep*. **6**: 321-325.

Hussain TM, Chandrasekhar T, Hazara M, Sultan Z, Saleh BK, Gopal GR. (2008) Recent advances in salt stress biology - a review. *Biotechnology and Molecular Biology* **3(1)**: 8-13.

Jefferson RA. (1987) Assaying chimeric genes in plants: The GUS gene fusion system. *Plant Mol Biol Rep*. **5**: 387-405.

Jones HD, Doherty A, Wu H. (2005) Review of methodologies and a protocol for the Agrobacterium-mediated transformation of wheat. *Plant Methods*. **1**: 5.

Knutzon DS, Thompson GA, Radke SE, Johnson WB, Knauf VC, Kridl JC. (1992) Modification of Brassica seed oil by antisense expression of a stearoyl-acyl carrier protein desaturase gene. *Proc Natl Acad Sci USA*. **89**: 2624-2628.

Mashayekhi M, Shakib AM, Ahmad-Raji M, Ghasemi Bezdi K. (2008) Gene transformation potential of commercial canola (*Brassica napus* L.) cultivars using cotyledon and hypocotyl explants. *African Journal of Biotechnology* **7(24)**: 4459-4463.

Moghaieb REA, El-Awady M A, El Mergawy RG, Youssef SS, El-Sharkawy AM. (2006) A reproducible protocol for regeneration and transformation in canola (*Brassica napus* L.). *African Journal of Biotechnology*. **5(2)**: 143-148.

Moravcikova J, Madyagol M, Galova Z, Vaculkova E, Libantova J. (2009) Optimalisation of genetic modification of rapeseed. *Acta fytotechnica et zootechnica Mimoriadne Cislo.* 479-485.

Murashige T, Skoog FA (1962) Revised medium for rapid growth and bioassays with tobacco tissue culture. *Physiol Plant*. **15**: 379-473.

Pua EC, Palta AM, Nagy F, Chua NH. (1987) Transgenic plants of Brassica napus L. *Biotechnology and Molecular Biology*. **5**: 815-817.

Radke SE, Andrews BM, Moloney MM, Crouch ML, Krid JC, Knauf VC. (1988) Transformation of *Brassica napus* L. using Agrobacterium tumefaciens: developmentally regulated expression of a reintroduced napin gene. *Theor Appl Genet*. **75**: 685-694.

Redondo FJ, de la Pena TC, Morcillo CN, Lucas MM, Pueyo JJ. (2009) Overexpression of flavodoxin in bacteroids induces changes in antioxidant metabolism leading to delayed senescence and starch accumulation in alfalfa root nodules. *Plant Physiol*. **149(2)**: 1166-1178.

Stewart CNJ, Adang MJ, All JA, Raymer PL, Ramachandran S, Parrott WA. (1996) Insect control and dosage effects in transgenic canola containing a synthetic Bacillus thuringiensiscryIAC gene. *Plant Physiol*. **112**: 115-120.

Tognetti VB, Palatnik JF, Fillat MF, Melzer M, Hajirezaei MR, Valle EM, Carrillo, N. (2006) Functional replacement of Ferredoxin by a

Cyanobacterial Flavodoxin in Tobacco confers broad-range stress tolerance. *The Plant Cell*. **18**: 2035-2050.

Travella S, Ross SM, Harden J, Everett C, Snape JW, Harwood WA. (2005) A comparison of transgenic barley lines produced by particle bombardment and Agrobacterium-mediated techniques. *Plant Cell Rep*. **23(12)**: 780-789.

Yamaguchi T, Blumwald E. (2005) Developing salt-tolerant crop plants: challenges and opportunities. *TRENDS in Plant Science* **10(12)**: 615-620

Zhang Y, Singh MB, Bhalla PL. (1999) Genetic transformation of Australian cultivars of oilseed rape (*Brassica napus* L.). 10th international Rapeseed congress, Canberra, Australia.

Zhang Y, Xu J, Han L, Wei W, Guan Z, Cong L and Chai T (2006) Efficient shoot regeneration and Agrobacterium-mediated transformation of Brassica juncea. *Plant Mol. Biol. Rep*. **24**: 255a-255i.

Zurbriggen M, Tognetti VB, Valle EM, Carrillo N. (2007) Cyanobacterial Flavodoxin provides multiple Stress tolerance. ISB News Report.