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Agrobacterium- mediated transformation of Turkish upland rice (*Oryza sativa* L.) for Dalapon herbicide tolerance

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Agrobacterium-mediated transformation of upland rice is established in few numbers of cultivars due to the high cultivar-specificity of regeneration from transformed explants. Further, dehalogenase E (*dehE*) gene had been characterized in *Pseudomonas putida* and it produces an enzyme that degrades dalapon. This study aimed to transform Turkish upland rice with the *dehE* herbicide resistant gene and addresses the challenges of transgenic rice recovery by identifying explant and transformation method. Constructed vector pCAMdehE carrying *dehE* gene was transferred into the rice shoot apex by *Agrobacterium*-mediated transformation. The transformed rice was analyzed for expression of the transgenes by polymerase chain reaction (PCR). Herbicide resistance leaf painting assay was carried out at different dalapon herbicide concentrations to the transgenic rice leaves. Transformation efficiency percentage (putative) was highest (32.66%) in 5 days old explants. PCR analysis resulted in the amplification of the *dehE*, T-DNA border endonuclease (*virD2*) and hygromycin phosphotransferase (*hpt*) genes from the transgenic rice. In addition, dehalogenase activity was proved with higher dalapon tolerance in the rice. Dalapon effects started to appear in the transformed rice at 180 mg/l, while in non-transformed ones at 60 mg/l concentration. The results showed that transformed plants have more tolerance to the herbicide than the non-transformed ones.

Keywords: *Agrobacterium tumefaciens*, dehalogenase E gene, dalapon, transgenics

Introduction

Cultivated rice is one of the world's most important field crops and stable diets for Asia continent[1-2]. The global amount of upland rice produced derived to about 4% of total rice production in the world[3]. Upland rice, waterless agricultural system of cultivated rice is usually directly seeded and grown on limited irrigation conditions[4-5] whereas, lowland rice is not as drought-resistant as upland rice. Farming of upland rice is suitable for rainy mountainous fields, waterlogged, rugged rural and low-lying areas[6]. One of the special varieties of such rice equally grown in limited irrigation habitat in Turkey and has the potential value for further improvement via genetic engineering[7].

Moreover, weeds compete with rice for water and nutrients which serves as origin of pests and infections[8]. Currently, herbicides are commonly used for weed management[9-10] which is the primary xenobiotic compounds in agricultural soil. Over the last 20 years, agricultural soils contamination with xenobiotic remains the most serious problem for global health[11]. Dalapon is a selective systemic herbicide which controls perennial and annual grasses[12] as well kills only certain organisms while sparing non-target varieties of plants. Dalapon has

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been confirmed to be used-up in sugarcane, sugar beets, corn, potatoes, asparagus, grapes, rice, citrus, nut trees and non-crop lands[13-14]. It was also recorded for usage in many non-crops applications including lawns, drainage ditches along railroad tracks and in industrial areas[15]. According to Hilton *et al*[16] dalapon breaks down the pantothenate-synthesis enzyme in the biosynthesis pathway of plants and inhibits synthesis of pantothenate by competing with pantoate, a precursor of pantothenate. Herbicide tolerance is one of the most important traits that have been applied to manage unwanted plants efficiently for rice agriculture[17]. Some bacterial group capable of using the herbicide dalapon has been identified by continuous flow enrichment culture[18-19]. Genes from the bacteria are being engineered to develop herbicide tolerant plants, including the dehalogenase E (*dehE*) gene[20] and bromoxynil (*bxn*) gene[21]. As well, bialaphos resistance (*bar*) gene exists broadly in all *Streptomyces hygroscopius*, which plays an important role in detoxifying herbicide phosphinothricin (PPT) in crop plant[21].

Dehalogenase genes have been incorporated into plants as reported by Buchanon-Wallostan *et al*[22]. They introduced *dehE* gene into the tobacco genome that resulted in the production of transgenic tobacco *Nicotiana plumbaginifolia.* Different types of dehalogenase genes were genetically transformed viz., *Agrobacterium*-mediated transformation[22], electroporation[23] and particle bombardment[24] into several plants including rice, *Arapidopsis thaliana* and tobacco. All these studies focused on the type of explants used like embryogenic callus, immature embryo and shoot apex[25]. So far no report is available on genetic transformation of Turkish upland rice with *dehE* gene using shoot apex as an explant. Therefore, the current research is the first to report on the *dehE* gene transformation into Turkish upland rice toward improving herbicide resistance.

Materials and Methods

Rice Cultivar and Shoot Apex Regeneration

Turkish upland rice variety, namely Kırçeltiği was used throughout the research which obtained from Ondokuz Mayis University, Samsun, Turkey. The variety mature seeds were surface sterilized as described by Karakütük[26]. The sterilized seed were inoculated onto Murashige and Skoog (MS) media and kept at 25°C. Three, four and five days old shoot apices (3 - 4 mm) were excise from the seedling and used for genetic transformation.

Gene Construct and *Agrobacterium* Transformation

In this research, *Agrobacterium tumefaciens* LBA 4404 and pCAMBIA1301 binary expression vector was used. The vector is carrying hygromycin phosphotransferase (*hpt*) and kanamycin (*KAN*) resistant genes, β-glucuronidase (*GUS*) reporter gene from *E. coli* with an intron, driven by cauliflower mosaic virus (CaMV) 35S promoter and *nos* poly-A terminator sequences[27]. Earlier, *dehE* gene isolated from *Pseudomonas putida* strain TF4 (GenBank accession No. MG518568.1) were amplified by PCR using gene specific primers (Table 1). The specific primers were designed by incorporating *Bgl*II and *Bste*II restriction sites (Table 1) for easy ligation with pCAMBIA1301 vector. Gene double digestion with restriction endonuclease (*Bgl*II and *Bste*II) enzymes was performed according to the instructions of manufacturer for pCAMBIA1301 vector. The amplified *dehE* gene flanked with *Bgl*II and *Bste*II was constructed into pCAMBIA1301. The band equivalent to 900 bp is *dehE* gene was successfully inserted into pCAMBIA1301

The *Agrobacterium* transformation was planned as described by Sambrook *et al*[28] with slight modification as described by Kaya *et al* (2013). Competent *A. tumefaciens* strain LBA 4404 was transformed with constructed pCAMBIA1301 vector through freeze-thaw transformation method[29]. Recombinant *A. tumefaciens* were grown for 2 days on *Agrobacterium* (AB) medium with containing 50 mg/l kanamycin[30] at 28°C by shaker at 250 rpm. The overnight culture (1 ml) was transferred to 100 ml of fresh AB medium containing 50 mg/l kanamycin. The cells were pelleted by centrifugation after reaching OD_{600} 0.3 and re-suspended in 20 ml infection MS media containing B5 vitamin, 15 g/L maltose, 10 g/L glucose and 100 µM acetosyringone at pH 5.6. The inoculated strain LBA 4404 were pelleted at 2500 rpm for 15 min, then, re-suspended in equal volume of pre-induction medium[31] supplemented with 20 g/l sucrose, 1 mg/l 2, 4-D, acotesyringone 50 µM, pH 5.6). The culture was shaken for an hour's under same condition for infection of the shoot apex explant.

Table 1 — The dehalogenase gene specific primers.

Primer name	Primer sequence
dehE1 F	5'GGAGC**AGATCT**TATGTTAAACGCTGCG3'
dehE2 R	5'AGAA**GGTAACC**TGGTATTCATAAGTAGTCC3'

Bold letters show *Bgl*II and *Bste*II restriction site

Co-cultivation, *Agrobacterium*-Mediated Transformation and Transgenic Rice Regeneration

To study the effect of hygromycin on shoot apices explant, the *in vitro* regenerated plantlets were initially inoculated into the culture media supplemented with 0.5 mg/l BAP and 0.1 mg/l NAA and various concentrations of hygromycin (0, 25, 50, 75, 100 mg/l). A total randomized design experiment was performed and repeated each three times. Three replicate each with a total of 50 explants were used as treatments and control.

Shoot apices (3, 4 and 5 days old) were introduced into the *A. tumefaciens* suspension for 12 min with shaking. The transformed explants were blot dried on sterile filter paper for few minutes to remove excess bacterium before inoculating onto co-cultivation medium (MS, 30 g/l sucrose, 7 g/l agar, 500 mg/l L-proline, pH 5.8, 800 µM acetosyringone). After co-cultivation for 3 days at 26°C in the dark, the explants were rinsed 4 - 5 times with sterile water containing 500 mg/l cefotaxine, blot dried on sterile paper to remove excess *A. tumefaciens*. The transformed shoot apices were transferred to regeneration medium A (MS, 30 mg/l sucrose, 1 mg/l BAP, 0.1 mg/l NAA, 8 g/l agar, pH 5.8 with 500 mg/l cefotaxime and 50 mg/l hygromycin) and incubated in the light condition under 16 h light photo period for 15 days. The shoot apices subsequently were washed with 500 mg/l cefotaxime and transferred to regeneration medium B (MS, 30 mg/l sucrose, 1 mg/l BAP, 0.1 mg/l NAA, 4 g/l pyhtagel, 8 g/l agar, pH 5.8 with 250 mg/l cefotaxime and 100 mg/l dalapon) and kept in plant growth chamber for further regeneration and selection. Afterwards, shoots of about 2 - 3 cm length were moved to root induction medium (MS, 30 g/l sucrose, 0.1 mg/l NAA, 4, 0 g/l phytagel, pH 5.8 with 30 mg/l hygromycin and 100 mg/l cefotaxime) for root formation then roots of about 4 - 5 cm length were moved to plastic cups for hardening.

Analysis of Putative Transgenic Turkish Upland Rice

Total genomic DNA extraction was done as described by Supari *et al*[32] from leaves of T_o and control plants. PCR amplification of *dehE* gene (primer; Table 1), *virD2* and *hpt* gene (primer; Table 2) were carried out. For *dehE* gene, the PCR conditions were; initial denaturation at 98°C for 30 s, followed by 30 cycles of denaturation at 98°C for 10 s, annealing 50°C for 20 s and extension at 72°C for 20 s. The reaction was finalized by additional extension at 72°C for 5 min. The PCR reaction condition for *hpt* was as previously described by Supari *et al* and *VirD2* gene as described by Arockiasamy and Ignacimuthu[33] from leaves of T_o and control plants. The amplicons were subjected to gel electrophoresis on 1% agarose gel in 1X TAE buffer at 70 V for 40 min. The gel bands at the expected sizes were cut and purified using Zymogen TM Gel Recovery Kit (Zymo Research D4001) for future use.

Herbicide Bioassay

Herbicide dalapon was obtained from Merck (Germany). Transgenic plants and control were analyzed for herbicide resistance (herbicide assay) as modified by Kaya *et al*. Dalapon serial dilutions was made at 20, 40, 60, 120, 180 and 240 mg/ml. Solutions of dalapon were applied to the leaf section of the plants for one week period at one day interval in the plant growth chamber by rubbing the middle part of the rice leaves with a swab. The transformed and non-transformed leaf was exposed to dalapon by a sponge brush over one week period with daily exposure.

Results and Discussion

Dehalogenase E (*dehE*) Gene Isolation and *pCAMdehE* Expression Construct

PCR analysis resulted in the amplification of *dehE* gene from *Pseudomonas putida* strain TF5 genomic DNA using its specific primers as designated by Kaya *et al*. The gel band is around 900 bp expected

Table 2 — The *hpt* gene and *VirD2* gene specific primers.

Primer name	Primer sequence
hpt F	5'GAT GTA GGA GGG CGT GGA TA3'
hpt R	5'ATA GGT CAG GCT CTC GCT GA3'
Vir D2 F	5'ATG CCC GAT CGA GCT CAA GT3'
Vir D2 R	5'CCT GAC CCA AAC ATC TCG GCT GCC CA3'

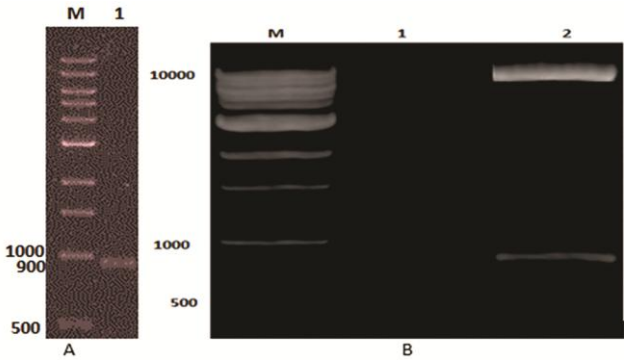

Fig. 1 — (A) Isolated *dehE* gene from *Pseudomonas putida* strain TF5 for ligation with pCAMBIA1301: Lane M; Gene ruler 1 kb plus DNA ladder, Lane 1; amplified *dehE* (900 bp). (B) pCAMddehE: Lane M; Gene ruler 1 kb plus DNA ladder, Lane 1; Negative control; Lane 2; Digested pCAMdehE with *bgl*II and *Bste*II.

size (Fig. 1A). Dehalogenase E enzyme has been proven to have some activity on 2, 2-dichloropropionic acid. Mesri *et al*[34] showed that most wild habitats with diverse population of bacteria survive in close proximity to each other can use similar chemicals in the natural habitats and play a significant function in the microbial bioremediation of polluted area with dalapon herbicide.

Restriction enzyme *Bgl*II and *Bste*II sites were used to ensure simple cloning of *dehE* gene into the plant expression vector. The *dehE* gene was incorporated into pCAMBIA1301 to give pCAMdehE. Now, the T-DNA of the pCAMBIA1301 contains *dehE* from *P. putida* and *hpt* gene with intron driven by cauliflower mosaic virus (CaMV 35 S) and *nos* poly-A terminator sequences (Fig. 2). To ensure that pCAMdehE carries the *dehE* gene, double digestion analysis using *Bgl*II and *Bste*II was conducted and analyzed on agarose gel (Fig. 1b). The size of the pCAMdehE is 9375 bp (Lane 2) after the double digestion analysis.

Chen *et al*[35], Baesi *et al*[36], Kaya *et al*[20] and Malik *et al*[37] also reported on the cloning of genes in pCAMBIA vector using same restriction sites. The cloning position is exactly within the GUS gene which was excised by the respective enzymes to allow for incorporation of the *dehE* gene. The GUS gene serves as an important tool for confirmation of plant transformation studies but it is not desired in this current research. As reported by Kaya *et al* and Mohamed *et al*[38], GUS gene was removed to allow for proper incorporation of the other gene of interest but not *dehE*.

Agrobacterium-Mediated Transformation of Turkish Upland Rice Shoot Apex with pCAMdehE

Selection of transformation vector and its selectable marker gene for plant selection as well as strain of *Agrobacterium* is of paramount importance in genetic transformation of rice. pCAMBIA1301 has been recognized to be efficient in genetic modification *Oryza sativa* using *A. tumefaciens* LBA 4404[39-41]. Therefore, effect of hygromycin on shoot apices prior to transformation was evaluated at different concentrations. At 50 µg/L hygromycin one of the explant was germinated which considered suitable concentration for the analysis of transformants. After the transformation the proliferating shoot apex continues to grow in the co-cultivation medium. In the regeneration medium A, several shoot apices that were co-cultivated indicate transient expression of *dehE* gene and remained green after 15 days of treatment with hygromycin as a selective marker. The shoot explants that were not infected with transformed *A. tumefaciens* did not proliferate in the regeneration medium A, but only in regeneration medium without hygromycin. Similar finding was reported by Arockiasamy and Ignacimuthu[33] and Sarangi *et al*[42].

Our study indicates the hygromycin and dalapon resistance, PCR analysis of transient expression of transgenes and transformation efficiency in 3, 4 and 5 days old shoot explants (Table 3). The result showed that hygromycin expression efficiency was higher in 5 days old explants (42.66%) compared to 4 days (25.33%) and 3 days (18.66%) old. The antibiotic showed clear difference between the transformed and non-transformed plantlets. After 30 days in *in vitro* condition, some of the putative transgenic plant shoots were analyzed for the presence of the transgenes and they were indicated positive in selective medium. This indicated that the putative shoots were successfully transformed with the pCAMdehE T-DNA containing *hpt* and *dehE* gene.

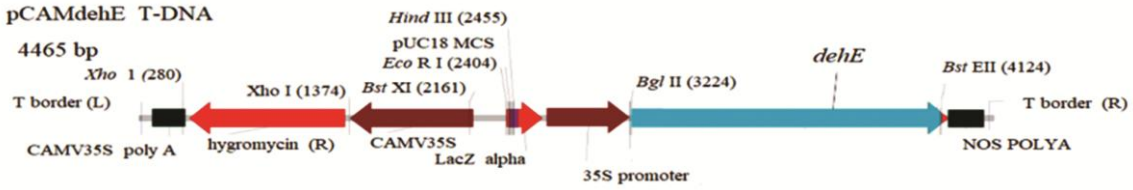

Fig. 2 — T-DNA portion of binary vector pCAMdehE showing restriction sites

Table 3 — Transformation efficiency of Turkish upland rice as calculated on the basis of dalapon resistance.

Explant age (day old)	Number of shoot apices during co-cultivation	Explants grow on selective medium (hygromycin)	Explants grown on selective medium (dalapon)	Number of plants positive for PCR	Bioassay results	Transformation efficiency (%)
3	150	18.66	10.66	12.00	10.66	12.00
4	150	25.33	14.66	14.66	13.66	14.66
5	150	42.66	32.66	32.66	32.66	32.66

Further, more the T_o putative transgenic lines were examined for tolerance to dalapon considering the successful integration of the *dehE* gene.

The result shows that the T_o line has the inhibitory effect of dalapon and continue to grow. The toxic effect of the different concentration of dalapon varied depending on the line and age of the explant. In dalapon selective medium, 5 days old explants was the best (32.66%). For rice genetic transformation, efficient selection involves a substantial level of the marker genes[43-44]. This is in connection with integration and expression of the selectable marker and transgenes in the transformants. The dehalogenase genes has been used as an herbicide marker for selection of transformed plant tissues. *dehE* selectable marker gene has been demonstrated in *N. tabacum*[20,38].

Putative and non-transformed shoots were excised from each culture for genomic DNA isolation. Dehalogenase gene fragments was amplified at expected size of 900 bp from 2 putative lines (Fig. 3a), while no amplification from non-transformed ones. Those lines demonstrated the stable expression of *dehE* gene. Likewise, the genomic DNA was used as template for *hpt* gene presence. This resulted in the amplification of the *hpt* gene (700 bp) only from putative plants (Fig. 3b). Since *VirD2* is existing outside the pCAMdehE T-DNA, it was used to confirm for existence of any contaminating *Agrobacterium*s in the culture (data is not shown). The result of VirD2 PCR showed that the tissues were totally free of bacterial contamination. Equally, higher transformation efficiency was obtained in 5 days old explants (32.66%) compared to 3 and 4 days old.

Many antibiotics were used in plant transformation methods the most frequently applied is hgyromycin[45]. The hygromycin (*hpt*) gene remain the plant selectable marker[46-48]. The results indicated that 50 mg/L hygromycin influenced the growth and development of rice at any stage of development[49]. The outcomes were similar to the findings of Arockiasamy and Ignacimuthu who proved the *Agrobacterium* transformation efficiency using shoot apex explant.

Transgene Stable Expression

The T transgenic rice plants were verified by dalapon leaf paint assay for their resistance to the herbicide. Different concentrations of the herbicide were applied on the leaves of both non-transgenic and transgenic upland rice and kept for seven days at 24°C in green house (16 hrs light, 8 hrs dark). Dalapon herbicide effects begin at 60 mg/L concentration in non-transformed leafs, while the transformed Turkish upland rice leaves resist up to 180 mg/L (Fig. 4). This indicated that the *dehE* transgene has been successfully transformed, integrated and expressed by the Turkish upland rice. The gene provides tolerance to the new variety of Turkish upland rice against dalapon.

The benefit of shoot apex as explant over other regeneration structures such as callus, protoplast culture includes genotype independence[50]. Yuzbasi *et al*[51] reported that retrotransposons led to mutations which are induced by cell culture and the copy numbers due to longer time of incubation. They further described that transgenic plants produced through use of callus and protoplast display somaclonal difference. Interestingly, using shoot meristem was possible and goal for direct and indirect genetic transformation was achieved[52]. Using shoot apex explants optimal regeneration was obtained in rice transformation with limited number of subculture.

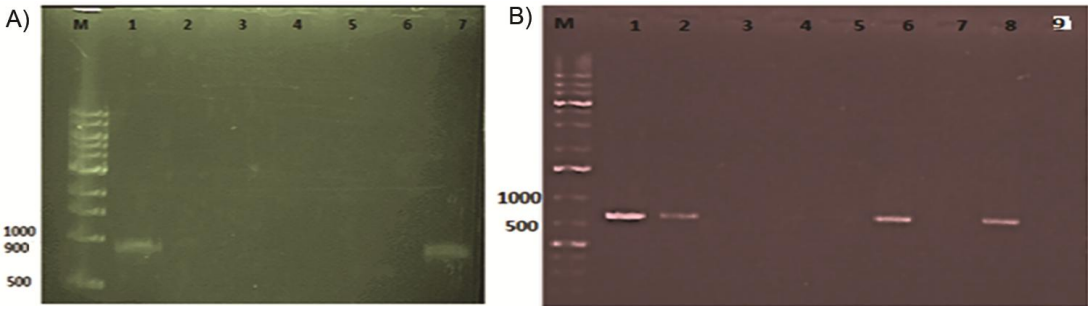

Fig. 3 — (A) PCR amplification of *dehE* gene from gDNA isolated from putative transgenic upland rice. Lane M; Marker 1 kb ladder (promega), Lane; 1, 2, 3, 4, 5 DNA of putative transformed rice, Lane 6; Negative control, Lane 7; Positive control. (B) Amplification of hygromycin gene from putative transgenic upland rice. Lane M; Marker 1 plus kb (promega), Line 1-7; Putative transformed rice, Lane 8; Positive control, Lane 9; Negative control.

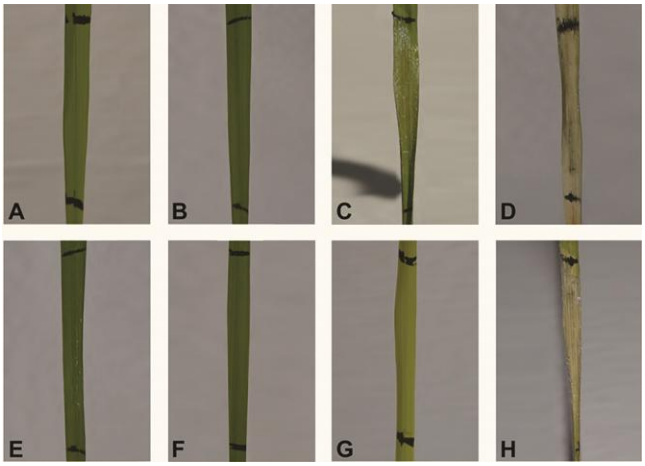

Fig. 4 — Dalapon leaf painting resistance at different concentration by transgenic Turkish upland rice. A: Control, B-D; non-transformed rice leaf in 20, 40, 60 mg/l dalapon treatment respectively, E-H; transformed rice leaf in 60, 120, 180, 240 mg/l dalapon treatment.

Conclusion

Optimum *in vitro* shoot apex induction was achieved from the Turkish upland rice variety. PCR analysis resulted in the amplification of full length *dehE* gene from *Pseudomonas putida* strain TF5 and successfully cloned into the binary vector (pCAMdehE). Subsequent to transformation, the Turkish upland rice genotype determination indicated the transient expression of the transgenes (*dehE*, *hpt* and *VirD* gene). The transgenic rice remained green after 15 days of treatment with hygromycin. The hygromycin resistance was optimal from 5 days old explants and also shows resistance to dalapon herbicide at 180 mg/L. This finding was the first ever from Turkish upland rice variety which might serve as an indication for successful future genetic transformation of other upland rice varieties towards improving resistance to herbicide and reproductive state. The present research technique for the production of genetically modified upland rice was improved and can be applied to other recalcitrant rice cultivars that displayed low regeneration capacity after transformation.

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