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Activation Tagging in Aspen Using A Glucocorticoid-Inducible Two Component *Ac*/Ds-Enhancer Element System

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ABSTRACT

Based on the *Ac*/Ds two element transposition system from maize an activation tagging approach was suggested for the hybrid aspen (*Populus tremula x tremuloides*) line 'Esch5'. A glucocorticoid-inducible two element *Ac*/ATDs element system was used to induce activation tagged variants following two independent transformation steps. In combination with a 35S enhancer tetramer and outward facing two CaMV 35S promoter located near both ends of the ATDs element, expression of genes can be elevated located adjacent to the new integration site of the element. As selective marker for ATDs transposition, knocking-out the expression of a phenotypic marker (*rolC* gene) was considered.

Keywords: Activation tagging, glucocorticoid-inducible, *rolC* gene, *Ac/*Ds, transposon, ATDs element.

INTRODUCTION

In many plant species insertional mutagenesis has been used to generate knockout mutations (Parinov *et al.,* 1999). Insertional mutagenesis techniques are key resources for studying the gene functions in plant species (Pan *et al*., 2005) using forward and reverse genetics strategies (Greco *et al*., 2001). The forward and reverse genetics approaches have been used for the identification of gene function and gene cloning (Takahashi *et al*., 1994). These techniques either use transposable elements (Federoff, 2002) or *Agrobacterium tumefaciens* T-DNA as mutagens (Koncz *et al*., 1992; Azpiroz and Feldmann, 1997). The primary tool for dissecting a genetic pathway is the screen for loss-of-function mutation in which an organism is engineered to lack one or more genes. However, a limitation of loss-of-function screens is that they rarely identify genes that act redundantly. A second class of genes whose entire function is difficult to identify with conventional mutagens, which primarily induce loss-of-function mutagenesis, are those that are required during multiple stages of the life cycle and whose knock-out results in early embryonic or in gametophytic lethality (Goover *et al*., 2004). Genes those are not absolutely required for a certain pathway can still be identified through mutant alleles, if such genes are sufficient to activate that pathway. Similarly, genes that are essential for early survival might be identified through mutant alleles if ectopic activation of the pathways they regulate is compatible with survival of the organism.

The key in either case is the availability of gain-of-function mutations (Goover *et al*., 2004). Gain-of–function phenotypes can either be caused by activation of the resulting protein (Chang *et al*., 1993) or by mutations that alter levels or patterns of gene expression (Schneuwly *et al*., 1987). The traditional way to induce the latter type of mutation has been through chromosomal rearrangements or the use of T-DNA and transposons that bring genes under the control of new promoters or enhancers (Chadwick *et al.,* 1990; Smith *et al*., 1992; Miller *et al.*, 1993; Kluppel *et al.*, 1997 and Brunner *et al.*, 1999). For example, a T-DNA vector was constructed carrying four copies of an enhancer element from the constitutively active 35S promoter for a more directed way of inducing gain-of-function mutation (Hayashi *et al,* 1992). These enhancers can cause transcriptional activation of nearby genes (Suzuki *et al*., 2001).

In this study we describe the establishment of an activation tagging system in poplar based upon the maize *Ac/*Ds transposable element system. A modified Ds element (ATDs; Suzuki *et al*. 2001) containing two CaMV 35S promoters and four tandem repeats of enhancer fragments (En) of the 35S promoter, carrying *rolC* as phenotypic selectable marker was introduced into poplar.

MATERIALS AND METHODS

Gene constructs carrying the transposase gene

The plasmid pindex3-Transposase (15208 bps) was used to design the construct carrying transposase gene under control of the glucocorticoid-inducible-promoter (Fig. 1A). As plant selectable marker the construct carries the hygromycin resistance gene with the CaMV35S promoters.

Gene constructs carrying the ATDs

The activation Ds system (ATDs) has kindly been provided by Y. Suzuki, University of Tokyo, Tokyo, Japan (Suzuki *et al*., 2001). The ATDs contains two cauliflower mosaic virus (CaMV) 35S promoters (Odell *et al*., 1985) and four tandem repeats of enhancer

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fragments (En) of the cauliflower mosaic virus (CaMV) 35S promoters that work for promoter-type and enhancer-type gene activation, respectively are flanked by terminal inverted repeats. For the activation tagging approaches the construct employed contains *rolC* from *Agrobacterium rhizogenes* as phenotypic selectable marker (Fladung, 1999; Kumar and Fladung, 2001a) (Fig. 1B), in the construct the marker genes were located outside of the ATDs element (Fig. 1B). Following transposase induction the genes become promoterless upon excision of ATDs rendering them inactive. For selection of transgenic plants the construct carries the *nptII* gene (Fig. 2) as plant selectable marker.

Plant material, culture and genetic transformation

In vitro culture of hybrid aspen clones (*Populus tremula x tremuloides*) was used for generation of the transgenic lines (Fladung *et al*., 1997). Plants were grown on solid McCown Woody plant medium (WPM, Duchefa M0220) (Lloyd and Mccown, 1980) containing 2% Sucrose, 0.6% Agar (Agar Agar, Serva, 11396). Genetic transformations were carried out using the *Agrobacterium*-mediated approach (Fladung *et al*., 1997). WPM medium for regeneration of transgenic plants was supplemented with 0.01% Pluronic F-68 (Sigma P-7061), thidiazuron (0.01 ?M/L) (601 medium) and antibiotic cefotaxime 500 mg/L(601C medium) for Agrobacteria elimination and kanamycin (50 mg/L) (601K medium) or hygromycin. (10 mg/L) (601H medium) for the selection of transgenic shoots. The cultures were kept under growth room conditions with 24 hour light and 22 ° C (light intensity: 5-8 x 10^3 lux, lamps: Phillips TLM 140W/33RS).

Extraction of DNA, RNA and molecular analysis

The DNA extraction procedure was adapted from the method of Doyle and Doyle (1987). The RNA extraction procedure was adapted from the method of Logemann (1987). Standard PCR techniques were used to detect the transgenes as described by Fladung *et al*. (1997).

In the case of GIP-*transposase* construct PCR was performed by using primers 5'AAA GCC TGA ACT CAC CGC GA3' and 5'TAC TGT CTT CTA TCG AAC3'and 5'TGC GAG GAT CAC TTG TTT TAA3' for amplification of *hph* gene, *GVG* gene, and *Ac Tpase* gene respectively. In the case of the double transgenic plants which had GIP-*transposase/*Ds-AT-*rolC* presence of the second gene construct ATDs non checked using a primer pair amplifying a fragment of 5'GGC TGA AGA CGA CCT GTG TTC TCT3' and 5'ATC GTT GAA GAT GCC TCT GC3' and5'AAA GCC TGA ACT CAC CGC GA3' and 5'ATG GAT TGC ACG CAG GTT CTC3' for amplification of *rolC* gene, *35S* - *rolC* gene, hph gene and *nptII*, genes, respectively.

To check presence of the second gene construct ATDs the primer pair amplifying a fragment of (5'ATC GTT GAA GAT GCC TCT GC3'and 5'GGC TGA AGA CGA CCT GTG TTC TCT3') and (5'ATG GAT TGC ACG CAG GTT CTC3') were used for amplification of 35S-*rolC* gene and *nptII* gene, respectively**,** and the primer pair amplifying a fragment of (5'ATG GAT TGC ACG CAG GTT CTC3' and 5'GGC TGA AGA CGA CCT GTG TTC TCT3') to check for ATDs excision following after the DEX treatment. Amplification was carried out under the following conditions in a PCR reaction. The initial denaturation for 2 min at 94 °C was followed by 40 cycles of denaturation (2 min at 94°C), annealing (2 min at 55 or 52 °C), extension (2 min at 72°C), and a final extension step of 5 min at 72 °C followed by 4°C**.**

RT-PCR Method

RT-PCR reaction was carried out using One-Step Access RT-PCR system (Promega, USA). RT-PCR analyses were performed with RNA isolated from DEX-induced and non-induced leaves and stems of four

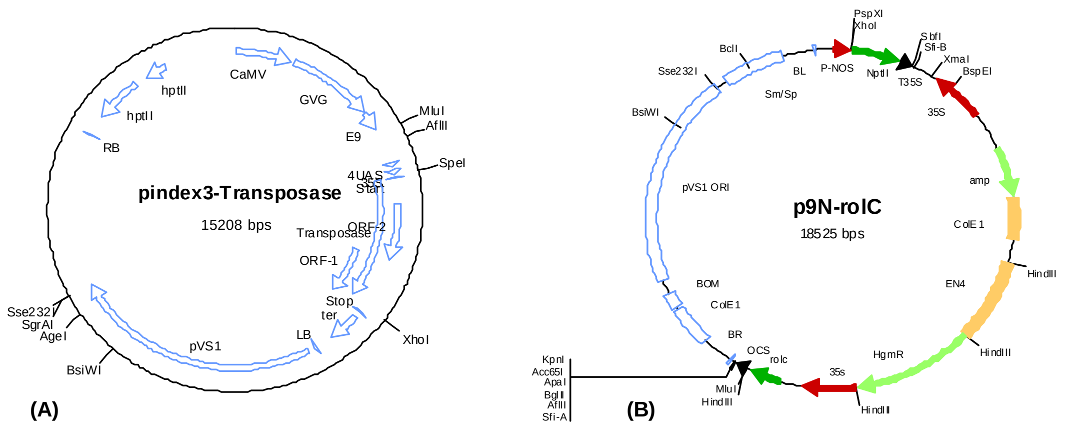

Figure (1): (A) Structure of the plasmid containing the *Ac* transposase and a glucocorticoid-inducible promoter system (GIP = pINDEX3, Ouwerkerk *et al.* 2001), **(B)** Structure of the activation construct (ATDs).

different single transgenic aspen lines containing the GIP-*transposase* gene. The primers 5'TGC GAG GAT CAC TTG TTT TAA3' was used for amplification of the *Ac Tpase* gene.

Induction of transposase

GIP-*transposase* transgenic lines were induced by treating the callus with the corticoid dexamethasone (DEX). The DEX was supplied to *in vitro* grown plants by mixing the corticoid into the media (601CH DEX or 601CHK DEX). The concentration of DEX was 100 µM for five weeks followed by 10 µM for three weeks, every week the callus pieces were transferred to magenta vessels containing 601 HC DEX fresh medium.

RESULTS AND DISCUSSION

Production GIP-*Ac* transposase and GIP-*transposase* / Ds-AT-*rolC* transgenic aspen

Leaf discs and stem segments from aspen *(Populus tremula* x *Populus tremuloides*, Esch5) plants were transformed by *in vitro* co cultivation with *Agrobacterium tumefaciens* strain carrying the binary vector pINDEX3-*transposase*. This vector contains the *hph* gene as selectable marker gene, to obtain transgenic aspen plants containing a single-copy of the transposase element.

The transformed calli appeared from the edge of leaves and stem after 3-4 week of selection on hygromycin containing media, while no callus induction was observed from the control uninoculated sections on selective callus induction medium. Multiplication was carried out by transfering these calli on the same transgenic selective medium. Table (1) shows the mean number of putative transgenic plants produced per construct. About 1.75 independent transgenic plant lines were produced in four independent experiments via *Agrobacterium*-mediated transformation under hygromycin selection in the case of GIP-*transposase*. To confirm the presence of the T-DNA in the regenerated plants, DNA was isolated from transgenic plants and subjected to PCR analysis (Table 1). Results of the PCR analyses are summarized in Table (1) (Fig. 2). The PCR analyses reveal (Table 1, Fig. 3) that seven putative independent transgenic lines contained the *Ac Tpase*, *hph* and *GVG* genes in the case of GIP-*transposase* construct (Fig. 2). A functional *Ac* transposon from maize was successfully transferred in

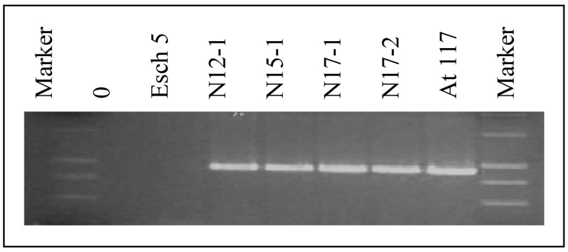

Figure (2): PCR analyses of GIP-*transposase* transgenic lines. Size of the amplified fragment is about 900 bp. N55-1 is a GIP-*transposase* / Ds-AT-*rolC* double transgenic line.

Table (1): Transformation and PCR positive efficiency of single transgenic aspen lines obtained using the three different constructs established in this study.

Construct	Means no. of transformed plants	Transformation efficiency (%)	Transgenic lines tested PCR	Transgenic lines tested PCR positive	PCR efficiency (%)
GIP-*transposase*	1.75	0.5	7	7	100

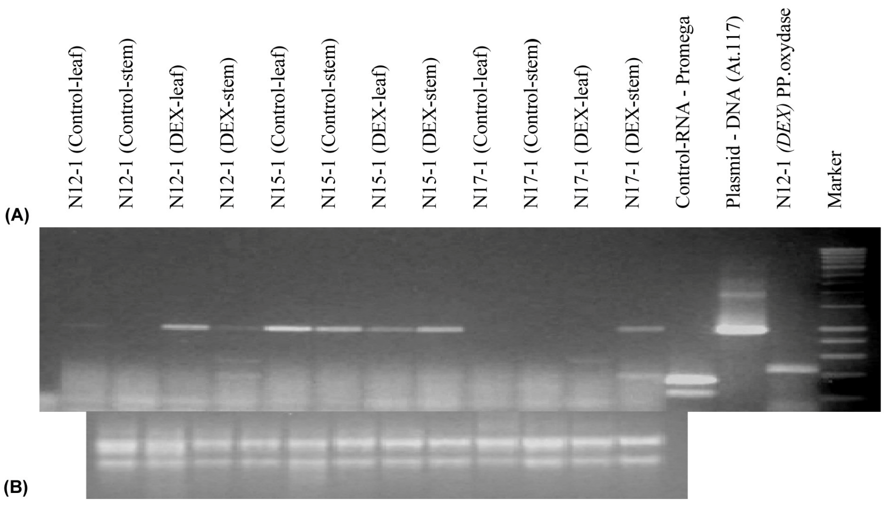

Figure (3): **(A)** RT-PCR of DEX-induced and non-induced leaves and stems of three different single transgenic poplar lines containing the GIP-*transposase* gene construct. **(B)** RNA quality check.

a number of plant species, including poplar (Fladung *et al.*, 1997; Fladung and Ahuja, 1997; Fladung *et al.*, 1997, Kumar and Fladung, 2003a), in tobacco (Charng *et al.*, 2004; Scofield *et al.*, 1992) and in Petunia (Feldman and Kunze, 1991).

Induction of transposase in GIP-*transposase* transgenic lines

Induction of transposase in GIP-*transposase* transgenic lines was achieved by *in vitro* culture by mixing the corticoid (DEX) into the medium. The treatments were sufficient to induce the transposase at high levels but without stressing the plants. RT-PCR of DEX-induced and non-induced leaves of three different single transgenic GIP-*transposase* aspen lines was performed to check for transposase transcription before and after DEX treatments, RNA was isolated from leaves and stems of GIP-*transposase* transgenic lines. RNA quality and amount of the DNase digested RNA was sufficient for RT-PCR (Fig. 3B). RT-PCR results in Figure (3A) showed that out of three GIP-*transposase* transgenic lines two lines (N12-1 and N15-1) showed transposase induction. In N15-1 lines transposase is active also under non-induced conditions. For second transformation with the ATDs construct the lines N12-1 and N15-1 were chosen for super transformation with the ATDs construct.

Establishment of double transgenic aspen

For super transformation with the Ds-AT-*rolC* gene construct the leaves and stem segments of two independent transformed lines (N12-1 and N15-1) containing GIP-*transposase*, were transformed with Ds-AT-*rolC* gene construct. Two months were sufficient to produce plants with well adapted roots that could be used for second transformation. The transformed calli appeared from the edge of the leaves and stems after 3-4 weeks on kanamycin and hygromycin selectable media. The resistant calli were isolated and cultured on the same selective medium. Double transgenic lines were analysed in PCR experiments for presence and complete integration of the second gene construct by using primer pairs amplifying a fragment of either 35S-promoter/*rolC* gene, 35S-promoter/*nptII* gene (Fig. 6 A, B), *hygromycin* gene (Fig. 4), *nptII* gene (Fig. 5).

Activation tagging experiments

Using seven GIP-*transposase*/Ds-AT-*rolC* double transgenic lines an activation tagging experiment was established. The *in vitro* grown regenerating callus pieces were sub-cultivated on DEX-containing media (Fig 8A). The calli were sub-cultivated each week on fresh DEX medium containing regeneration media. On 100 µM 601DEX medium most of the calli were still green but only little regeneration of shoots was observed (Fig. 8B). Therefore, following the period of five weeks on 100 µM DEX medium, the calli were further cultivated for three weeks on media containing 10 µM DEX to initiate regeneration. After three weeks on 10 µM DEX medium shoots were regenerating (Fig. 8C). To check for ATDs transposition, DNA was isolated from the shoots before and after the DEX treatment.

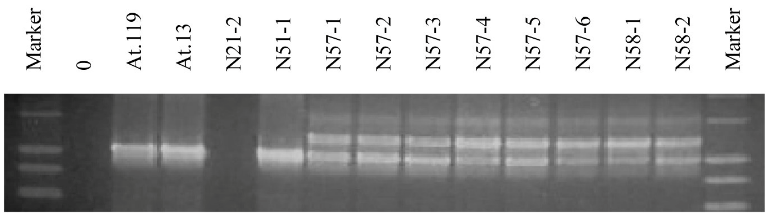

Figure (4): PCR analysis to check presence of the second gene construct ATDs using a primer pair amplifying a fragment of the *hygromycin* resistance gene. In double transgenic lines containing GIP-*transposase* / Ds-AT-*rolC* (N57 and N58) or GIP-*transposase* / Ds-AT-*tms* (N51), two amplification products were detected with exception of the Ds-AT-*tms* containing one.

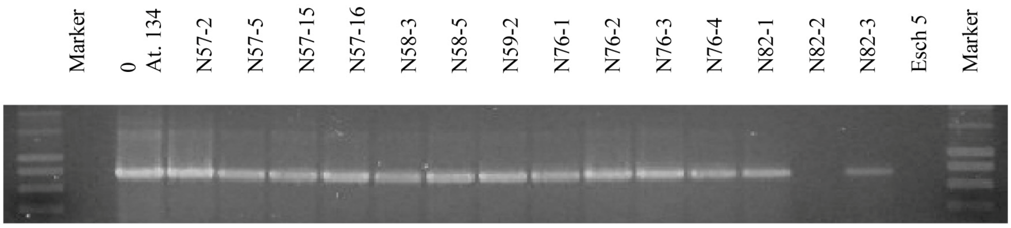

Figure (5): PCR analysis to check presence of the second gene construct ATDs using a primer pair amplifying a fragment of the *nptII* gene. GIP-*transposase* / Ds-AT-*rolC* (N57, N58 and 76) and HSP *transposase* / Ds-AT-*rolC* (N82).

Table (2): Mean number of independent transgenic aspen lines obtained using the different constructs established in this work and tested positive in PCR analyses.

Construct	Transgenic line	Transformation with 2[nd] construct	Means no. of double transgenic aspen lines	Transformation efficiency (%)	Double transgenic lines PCR tested	Double transgenic lines PCR positive	PCR efficiency (%)
GIP-*transposase*	N12-1	Ds-AT-*tms*	1	0.3	1	1	100
		Ds-AT-*rolC*	49. 5	14	27	12	44
GIP-*transposase*	N15-1	Ds-AT-*tms*	1	0.3	1	-	0
		Ds-AT-*rolC*	59	17	18	11	61

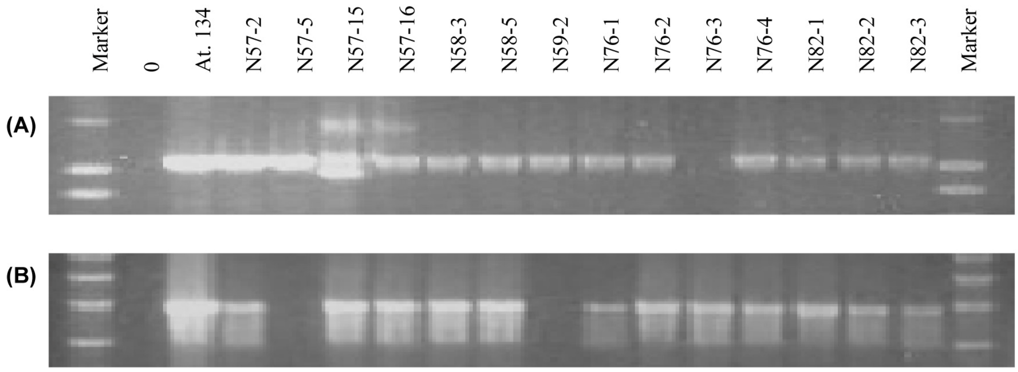

Figure (6): PCR analysis to check complete integration of the second gene construct ATDs at right (A) and left (b) border of T-DNA. (A) Primer pair 35S-promoter / *rolC* gene, (B) primer pair 35S-promoter / *nptII* gene. GIP-*transposase /* Ds-AT-*rolC*.

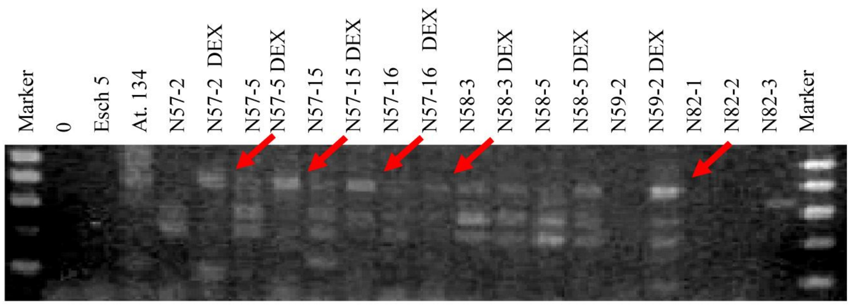

Figure (7): PCR analyses to check for ATDs excision following DEX treatment and transposase induction. The *Agrobacterium* strain 134 reveals a single amplification product of about 9 kb that only can be amplified using Long-Template PCR system (not shown here).

Excision of the ATDs element following DEX treatment was confirmed in PCR experiments using primer located in the *rolC* and *nptII* genes (Fig. 7). For the GIP-*transposase* / Ds-AT-*rolC* double transgenic lines a PCR fragment of about 800 bp is expected following ATDs excision. For all lines tested a specific PCR amplification signal of 800 bp were obtained. In the lines N57-2, N57-5, N57-15, N57-16 and N59-2, no or a very weak signal could be detected in the non-treated lines, however, the other two lines reveal also in non-treated tissue an amplification product. These results are consistent with the RT-PCR results indicating a steady-state level of transposase transcript also in non-induced tissues of N15-1 (used for production of N58-3, N58-5) but not in the N12-1 transgenic line (used for production of N57-2, N57-5, N57-15, N57-16 and N59-2). After eight weeks on DEX treatments the calli were divided in about 12,000 regenerating callus pieces as small as possible and distributed on 200 Petri dishes (Fig. 8D). The number of regeneration shoots which formed from the callus varies between lines. Out of the 10,352 regeneration shoots three putative chlorophyll-defective variants could be detected (Fig. 9).

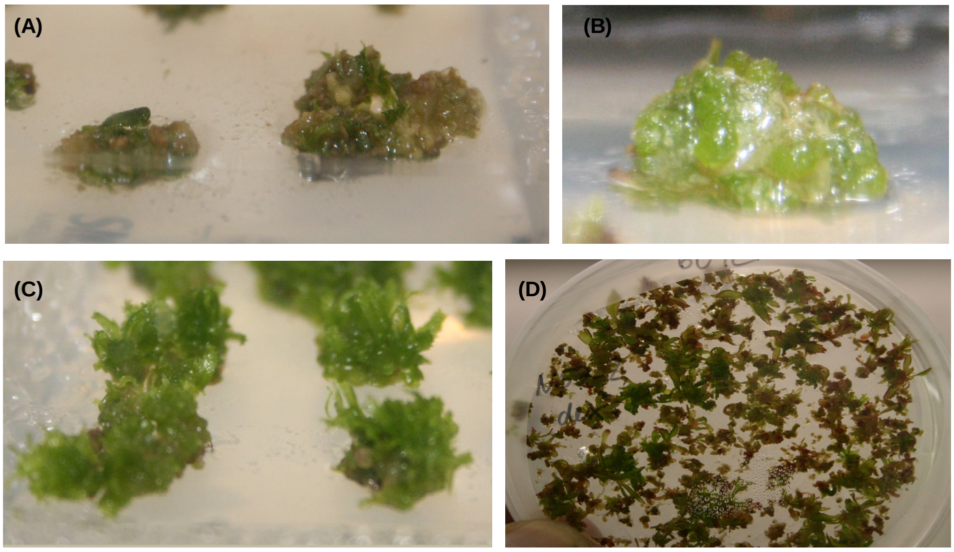

Figure (8): Activation tagging experiment 1: (A) Regenerating callus with removed shoots before starting the experiment. (B) Green callus after five weeks cultivation on 100 µM DEX. (C) Callus with regenerated shoots after three weeks cultivation on 10 µM DEX. (D) Small callus pieces distributed on Petri dishes for regeneration of shoots.

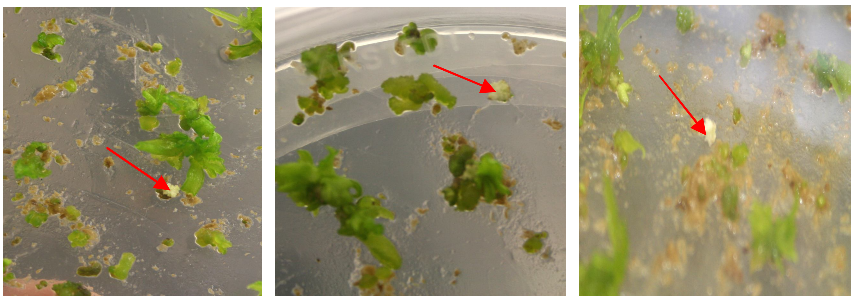

Figure (9): Three putative chlorophyll-defective variant calli (arrows), following DEX treatment and transposase induction.

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الملخص العربـــى

تم الحصول على نباتات الحور المحوره وراثيا والتى تحتوى على الجين GIP-*transposase* الذى يعتمد تنشيط تخليقة على اضافة الهرمون الدهنى glucocorticoid حيث تم تنشيط التخليق الحيوى لهذا الانزيم فى ثلاث نباتات حور المحوره وراثيا كما تم عمل نقل وراثى للجين Ds-AT-*rolC* بواسطة الأجروبكتريم *Agrobacterium tumefaciens* وتم بذلك الحصول على نباتات حور معدلة وراثيا تحتوى على كل من الجينات الاتية GIP-*transposase/* Ds-AT-*rolC* ثم عرضت هذة النباتات للهرمون الدهنى glucocorticoid بغرض تنشيط تكوين انزيم الترانسبوزم حيث تم تعرض كالس النباتات ل glucocorticoid تم اخذ عينات ال (DNA) من النباتات لعمل PCR لمعرفة انتقال ال(ATDs) من مكانه داخل جينوم النباتات وحيث ان ال (ATDs) يوجد عند طرفية الجينين *rolC* and *nptII* genes فامكن استخدام البادئات المخلقة primer الجينين *rolC* and *nptII* genes حيث تم الحصول على ثلاث نباتات بيضاء لم تكمل نموها وكان نسبة انتقال ال (ATDs) باستخدام نظام الGIP ضعيفة.