

CHAPTER THREE

DESIGNING *CYCLIN* GENE CONSTRUCTS AND TRANSFORMATION OF BANANA

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3.1 Introduction

One of the limitations in plant genetic engineering is the successful delivery of transgenes and regeneration of plants thereafter. This has been attributed to factors that are species and genotype-specific requirements for an ideal explant as well as need for a suitable method of T-DNA delivery and *in vitro* procedures (Cheng *et al.*, 2004). Among the available plant transformation techniques, *Agrobacterium* has been preferred due to its simplicity, delivering of single copy numbers and intact T-DNA (Dai *et al.*, 2001; Hu *et al.*, 2003; Veluthambi *et al.*, 2003).

Transgenes for expression are cloned into expression cassettes comprising of the transgene, selectable marker gene, promoter to drive the transcription of these genes and their respective transcription terminator. The Cauliflower mosaic virus CaMV35S promoter has been widely used as a universal constitutive promoter (Yoshida and Shinmyo, 2000). However, studies have demonstrated low efficiency of CaMV35S in some monocot plants, including banana (Sagi *et al.*, 1995; Chowdhury *et al.*, 1997). This prompted searches for alternative promoters, such as the polyubiquitin-1 promoter from maize (Wilmink and Dons, 1993; Atkinson *et al.*, 2004). Although strong promoters are intended to deliver high expression of transgenes, excessive transcripts may lead to gene silencing (Stam *et al.*, 1997). Irrespective of the promoter and transformation systems used, only a small proportion of cells exposed to T-DNA are ultimately transformed (Wilmink and Dons, 1993; Sreeramanan *et al.*, 2006). Thus, selectable marker genes are included in the constructs to facilitate identification of the few transformants.

Conventionally, assembly of gene constructs uses a number of shuttle plasmids in which PCR amplified DNA fragments are cloned and combined through restriction and ligation reactions (Sambrook *et al.*, 1998). This strategy also requires confirmation of orientations and integrity of the inserts which can be laborious. However, using recombinase enzymes that recognize specific DNA sequences, Gateway cloning and binary expression vectors were developed that are easy to use, facilitate combination of DNA fragments in defined

orientations with high fidelity (Hartly, *et al.*, 2000; Karimi *et al.*, 2005; Magnani *et al.*, 2006).

The objectives of this part of the study were (i) to construct expression vectors for ectopic overexpression of a *CyclinD*-type gene from *Arabidopsis* and banana, (ii) to transform banana with these gene constructs and (iii) to regenerate transgenic banana plants. Both Gateway and the conventional system were applied to make the vectors with maize polyubiquitin-1 and 35S promoters. The T-DNA was delivered into banana cells by using the *Agrobacterium* system (*Agrobacterium tumefaciens*; strain AGL1). PCR and Southern blotting was performed on the regenerants to confirm the integration of the *cyclin* gene.

3.2 Materials and Methods

3.2.1 Construction of transformation vector

3.2.1.1 Isolation of ubiquitin promoter

The maize poly-ubiquitin promoter was amplified by PCR from genomic DNA isolated from maize plants (line B73) using specific primers ATTB4Fw (5'-GGGGACAACCTTGT ATAGAAAAGTTGTGCAGCGTGACCCGGTTCGT-3') and ATTB1Rv (5'-GGGGACTG CTTTTTGTACAACTTGAGAGGGTGTGGAGGGGGT-3'). Primers were designed based on consensus sequences of maize polyubiquitin-1 promoters (GenBank accessions AY452736.2, DQ141598.1 and S94464.1). These primers were designed to amplify the promoter region, excluding the 5' untranslated region and the introns to achieve a lower expression of the gene. *AttB* sequences were added at the primer ends to facilitate cloning of the PCR product into the Gateway® pDonor vector (pDONR-P4-PIR; Invitrogen Life Technologies). *AttB* sites are recognized by recombinase that catalyzes reciprocal double DNA exchange between two specific DNA sites (Magnani *et al.*, 2006). A 30 µl PCR reaction was performed containing 1.5 mM MgCl₂, 0.3 mM dNTPs, 0.3 µM each of primer and 1 unit of Platinum®Taq DNA Polymerase High Fidelity (Invitrogen). The cycling program consisted of an initial DNA denaturing of 2 min at 94°C, followed by 35 cycles of

30 sec at 94°C, 30 sec at 55°C, 1 min at 68°C and a final extension of DNA strands for 7 min at 72°C. The PCR products were run on a 1% agarose gel from which the desired DNA fragment was purified using Qiaquick^R gel extraction kit (QIAGEN GmbH; Cat # 28706). The purified DNA fragment was inserted into the pDONR-P4-PIR plasmid by mixing 50 ng of the PCR product with 1 µl of plasmid and 1 µl of BP ClonaseII enzyme, and then incubating the mixture for 6 hrs at 25°C. The cloned fragment was then transferred to *E. coli* cells following the method outlined in section 2.3.8 and transformants were selected on a Luria Bertani (LB) medium containing 10 g/l tryptone, 5 g/l yeast extract, 10 g/l NaCl₂, and 15 g/l agar and 50 µg/ml kanamycin. Plasmid DNA was isolated from cells of three kanamycin-resistant colonies using a High Pure Plasmid isolation Kit (Roche Applied Science Cat. No. 11754777001) and DNAs were then sequenced. The correctness of sequence was verified by blasting it against the NCBI databank and by analysis with TSSP software (<http://www.Softberry.com>).

3.2.1.2 Construction of *Arath;CYCD2;1* transformation vector

Plasmid pDONR221 carrying the *Arath;CyclinD2:1* gene (Accession number At2g22490) was purified from an *E. coli* (strain JM 109). A multi-site Gateway® binary vector pK7m24GW that carries a neomycin phosphotransfase II (*nptII*) selectable gene, under the control of cauliflower mosaic virus (CaMV) 35S, was used as destination vector. The final gene cassette was prepared by mixing *Arath;CyclinD2:1* and ubiquitin promoter entry plasmids with the binary vector DNA in a ratio of 2:1:1 together with 2 µl of LR clonase enzyme and 2 µl clonase buffer. The mixture was incubated for 16 hrs at 25°C. The reaction was stopped by addition of 1 µl of proteinase K and incubating the mixture for 10 min at 37°C. An aliquot of 150ng DNA from the LR reaction was transferred into *E. coli* cells and cells were selected on LB medium supplemented with 100 µg/ml streptomycin. The plasmid was extracted and the success of the cloning was checked by digesting 500ng of the plasmid DNA with 10 units of XbaI restriction enzyme for 1 hr at 37°C to generate a 2.4 and 8.0 kbp fragments. Fig. 3.2 illustrates the procedure that was followed for cloning of the ubiquitin promoter and construction of *Arath;CYCD2;1* transformation vector.

3.2.1.3 Construction of a *Musac;CYCD2;1* transformation vector

To create an expression vector for *Musac;CyclinD2;1* the *cyclin* coding sequence was cloned as an EcoRI fragment, after cutting the cyclin DNA sequence from the TOPO cloning vector, between a double CaMV35S promoter sequence and a CaMV terminator sequence of the vector pLBR19. For that, both vectors (TOPO and pLBR19) were individually digested with 1U of EcoRI for 2 hrs at 37°C in a 20 µl reaction mixture. The digested TOPO plasmid was run on a 1% agarose gel to isolate the *cyclin* coding sequence. The digested pLBR19 plasmid was immediately purified to remove the enzyme. Precipitation was performed adding water to a total volume of 30 µl. An equal volume of cold (4°C) phenol : chloroform : isoamyl alcohol 25:24:1 was added and mixed gently. The mixture was centrifuged for 3 min at 13000 rpm. The supernatant was transferred to a new Eppendorf tube to which 1/10 of sodium acetate (pH 5.2) was added. Two and half volume of 96% ethanol was added and the mixture kept for 1 hr at -80°C. This was followed by centrifugation for 15 min at 4°C and 13000 rpm. The supernatant was removed and the pellet was washed with 200 µl of 70% ethanol and centrifuged for 5 min at 4°C and 13000 rpm. The pellet was dried and re-suspended in 20µl water.

For dephosphorylation of pLBR19, phosphatase was diluted five-times. Dephosphorylation of plasmid was performed by mixing 6µl of purified plasmid with 2 µl 10x phosphatase buffer, 2 µl of a 1 to 10 diluted phosphatase and incubating the mixture for 30 min at 37°C. The reaction mixture was placed on ice and purified again to remove the phosphatase. The *cyclin* coding sequence was ligated into the digested plasmid pLBR19 by mixing 6 µl of purified DNA fragment with 2 µl of the de-phosphorylated plasmid (ratio of 3:1), 1 µl T4-ligase buffer and 1 µl ligase. Ligation was conducted for 16 hrs at 16°C after which the reaction was terminated by heating the reaction mixture for 10 min at 65°C. The ligate (6 µl) was transferred into *E. coli* competent cells following the procedure outlined in section 2.2.1.4 and transformed cells were selected on LB medium containing 100 ug/ml kanamycin. Five bacterial colonies were selected from which plasmid DNA was extracted. To confirm the presence of cyclin containing plasmids, the cyclin coding sequence was

amplified from plasmid DNA by PCR using the cyclin gene specific forward primer (5'-ATGAGTCCAAGTTGTGACTGCG-3') and reverse primer (5'-TCATCTGGTTGTTTTCCTCCTCT-3'). Because EcoRI sites were located at both ends of the *cyclin* gene insert, the ATG position was confirmed by digesting the plasmid with EcoRV to generate a 898 and a 1587 bp fragment.

To obtain the final construct for transforming *Agrobacterium*, the cyclin coding sequence with the promoter and terminator sequences was cloned into the de-phosphorylated plasmid pBIN19 following the procedure described above. The final expression vector was designated *pBin-35S-Musac;CycD2;1* and the procedure used in assembling is shown in Figure 3.3.

3.2.1.4 *E. coli* transformation

Plasmid DNA was transferred into *E. coli* (strain JM 109) using the heat shock method. The procedure involved placing 100 µl of the competent bacteria cells from -80°C storage and thawing them on ice for 30 min. One microgram of plasmid DNA was added to the bacteria cells, gently mixed and left to stand on ice for another 30 min. Heat shock was performed by immersing the Eppendorf tube containing the bacteria-plasmid mixture for 45 sec in a 42°C water bath and immediately cooling the tube on ice for 2 min. SOC medium (250 µl) was immediately added to the bacterium and the mixture incubated for 1 hr at 37°C and shaking at 150 rpm. Excess medium was removed by spinning the cell suspension for 2 min at 8000 rpm. The supernatant was reduced to 100µl in which the bacterium pellet was re-suspended before spreading onto 25 ml LBA plates supplemented with appropriate antibiotics specific for the plasmid. Cultures were incubated for 16 hrs at 37°C. Single colonies were inoculated into 5 ml of liquid LB medium and cultured for 16 hrs at 37°C under 150 rpm shaking. Plasmids were isolated using the GeneJET™ plasmid miniprep Kit (Fermentas Life Sciences, cat K0503).

3.2.2 Transformation of Agrobacterium

Transformation of *Agrobacterium tumefaciens* (strain AGL1) was carried out using the heat shock technique (Sambrook, *et al.*, 1989). An empty expression vector pBin19 was used as a control for the over-expression of the banana cyclin. To transform *Agrobacterium*, 100 μ l competent cells from a -80°C storage were thawed on ice for 30 min. About 600 ng plasmid DNA containing the cyclin construct was added to the competent cells that were gently mixed and left on ice for another 30 min. The cells were heat-shocked by flashing the tube into liquid nitrogen for 2 min followed by immediate thawing on ice for 2 min. Pre-warmed (37°C) SOC medium (250 μ l) was added and the mixture incubated for 4 hrs at 28°C and shaking at 200 rpm. At the end of incubation, the tube was centrifuged for 2 min at 8000 rpm. The supernatant was discarded and the bacterium pellet was re-suspended and spread on a yeast/mannitol medium (YM) containing 0.4 g/l yeast extract, 10 g/l mannitol, 0.1 g/l K_2HPO_4 , 0.4 g/l KH_2PO_4 , 0.1 g/l NaCl_2 and 0.1 g/l MgSO_4 . Selection media were supplemented with 25 $\mu\text{g/ml}$ rifampicin and 250 $\mu\text{g/ml}$ carbenicillin to select for transformed *Agrobacterium* cells. In addition, 100 $\mu\text{g/ml}$ streptomycin and 300 $\mu\text{g/ml}$ spectinomycin was used for the *Arabidopsis CYCD2;1* expression vector, and 100 $\mu\text{g/ml}$ kanamycin for the *Arabidopsis CYCD2;1* expression vector. Cultures were incubated for 3 days at 28°C .

To confirm the presence of the gene constructs in the bacteria, single colonies were inoculated into 5 ml of LB medium containing the respective antibiotics and incubated for 3 days at 28°C and shaking at 200 rpm. Back ups of the selected colonies were maintained as streaks on plates containing YM medium. Isolated plasmids were digested with XbaI for *Arabidopsis;CYCD2;1* and KpnI and XbaI for *Musac;CYCD2;1* constructs. The corresponding back up colonies of the positive plasmids were maintained at -80°C as 20% glycerol stocks.

3.2.3 Transformation of banana cells

3.2.3.1 Embryogenic cell suspension of banana

An embryogenic cell suspension used in the study was generated from immature male flowers of banana cultivar ‘Sukalindiizi’ (AAB). The flowers were aseptically isolated from male buds and cultured into 100 cm petri dishes containing full strength MS macro- and micro-nutrients (Murashige and Skoog, 1962) supplemented with 4.09 μM biotin, 5.7 μM IAA, 5.4 μM NAA, 18.1 μM 2,4-D, 72 mM sucrose and solidified with 2.3 g/l phytigel. The pH was adjusted to 5.8 before autoclaving the medium. Cultures were sealed with parafilm and incubated in the dark at 28°C without sub-culturing until callus formation, which occurred after 5 months. Embryogenic callus, characterized by friable callus and embryos, was transferred to 5 ml of liquid MS medium and incubated in the dark with agitation on a rotary shaker at 90 rpm. Culture medium was changed every 10 days and when the cell suspension had established it was bulked by transferring 2 ml packed cell volume into 250 ml Erlenmeyer flask containing 50 ml MA2 medium (Appendix I). The culture medium comprised of 1x MS macro- and micro-nutrients enriched with 4.09 μM biotin, 680 μM glutamine, 100 mg/l malt extract, 4.5 μM 2,4-D, 130 mM sucrose at pH 5.3. Cells were transferred into fresh medium six days prior to use for transformation.

3.2.3.2 Preparation of *Agrobacterium* cells

Agrobacterium cells carrying the *cyclin* constructs were streaked onto YM agar medium containing the appropriate selection antibiotics and incubated for 3 days at 28°C. A single colony was inoculated into 25 ml of liquid YM medium and incubated at 28°C under shaking at 200 rpm for 3 days. The culture (5 ml) was transferred into 20 ml LB medium with the selection antibiotics and grown overnight at 28°C and shaking at 200 rpm. The bacterial cells were harvested by centrifugation for 10 min at 5000 rpm. Virulence of the bacterium was induced by suspending the pellet into 25 ml of BRM medium (Appendix II) supplemented with 200 μM acetosyringone (3'-5'-dimethoxy-4-hydroxyacetophenone) and

incubating for 2 hr at 25°C with agitation at 70 rpm. At the end of incubation, the bacterial suspension was adjusted to an OD of 0.6 using BRM.

3.2.3.3 Transformation of banana cells

Banana cells were harvested by transferring the suspension into a 50 ml Falcon tube and left to settle. The medium was decanted and the cells were heat shocked by addition of warmed (42°C) MA2 medium and the mixture incubated for 5 min at 42°C. The medium was removed and 2 ml of settled cell volume was transferred into new Falcon tubes to which 10 ml of induced *Agrobacterium* cell suspension was added. MA2 medium was added to the non-transformed cells to act as a control. A surfactant, Pluronic F68, was added to each Falcon tube to a 0.02% final volume to reduce the surface tension of the medium and facilitate contact of the bacterium and plant cells. Cell interaction was further enhanced by a gentle agitation of the banana-bacterium cell suspension followed by centrifugation for 3 min at 900 rpm. This was repeated with a resting interval of 30 min between the centrifugation.

To enable *Agrobacterium* to integrate the transgene, banana and bacterial cells were co-cultivated by aspirating the cell mixture onto sterile nylon mesh placed over a Whatman filter paper that absorbed the excess liquid. The mesh with the embedded cells was plated onto petri dishes containing MA2 medium supplemented with 300 µM acetosyringone. Petri dishes were sealed with cling film to prevent dehydration and incubated in the dark for 5 days at 22°C. At the end of the co-cultivation period, the plant cells were transferred into Falcon tubes and washed four times with MA2 medium containing 200 µg/ml timentin (ticarcillin disodium + potassium clavulanate) to suppress *Agrobacterium* growth.

3.2.3.4 Selection and regeneration of transformed banana shoots

To select and regenerate transformed cells, aliquots of the washed cells were drawn and a thin layer of cells was aspirated onto sterile nylon mesh placed over a Whatman filter paper. The cell-loaded meshes were placed onto 50 cm plates containing embryo initiation

medium (M3, Appendix III). The medium was supplemented with 50 µg/ml geneticin (G-418 disulfate) as a selectable agent to use the neomycin phosphotransferase II (*nptII*) selectable marker gene. Timentin was also incorporated into the medium to eradicate the bacterium. Cultures were incubated in the dark at 26-28°C. Five rounds of transfer of the mesh onto fresh medium were carried out at 2 wks intervals. To reduce the incidences of escapes, cell clusters that had formed were further cultured on selection medium for 1 month. Surviving embryos were induced to develop by plating them on RD1 medium comprising of half-strength MS salts, full-strength MS vitamins, 20 mg/l ascorbic acid, 88 mM sucrose and solidified with 2.3 g/l phytagel. Embryos that developed were germinated on M4 medium (Cote *et al.*, 1996) comprising of MS salts (Murashige and Skoog, 1962), Morel vitamins (Morel and Wetmore, 1951), 0.22 µM BAP, 1.1 µM IAA, 88 mM sucrose, pH of 5.8. Non-transformed cells that were used as a control were cultured on similar media devoid of geneticin.

3.2.3.5 Proliferation and establishment of banana regenerants

Germinated embryos were proliferated on MS medium supplemented with 22 µM BAP. Plants were rooted on growth regulator free MS medium containing 30 g/l sucrose for 4 wks. Weaning, potting and growth evaluation of the plants was done in the containment glasshouse (Level 3). Weaning involved removing the shoots from the culture jars, cutting back the roots to two centimeters, washing off the nutrient medium and potting in 200 ml plastic cups containing moist pasteurized forest top soil and farm yard manure mixed at a ratio of 12:1. Plants were hardened by maintaining them under a low transparent plastic tent for 3 wks after which the humidity was reduced by gradual opening of the sides of the tent during the fourth week. Subsequently, the plants were transferred, with their intact soil, into 3 L pots containing 2 kg of the same potting substrate. Watering was done daily and the temperature was maintained at 27-32°C and humidity at 30-60% through intermittent misting.

3.2.4 Molecular analysis of regenerated banana

3.2.4.1 DNA isolation

To conveniently handle several samples while using little amount of tissue, genomic DNA for PCR was isolated using the miniprep method of Dellaporta *et al.* (1983), with slight modifications. From *in vitro* plants, about 20 mg of leaves were aseptically dissected and placed in 1.5 ml Eppendorf tubes. Leaf material from potted plants was harvested by placing the leaf lamina between an opened Eppendorf tube and by closing the cover, a leaf disc of 12-15 mg was extracted. The tubes containing the samples were flashed in liquid nitrogen and the frozen leaf material was ground with a micro-pestle. Extraction buffer (500 µl) containing 100 mM Tris pH 8, 50 mM EDTA, 500 mM NaCl, 2% PVC (MW 10000 or 20000), and 1% Na₂SO₃ as an antioxidant were added followed by 33 µl of 20% SDS. The mixture was vortexed and incubated for 10 min in a water bath at 55°C. Potassium acetate (5 M; 160 µl) was added, the mixture was vortexed briefly and centrifuged for 10 min at 13000 rpm. Supernatant (450 µl) was transferred to a new Eppendorf tube and an equal volume of cold isopropanol was added. The mixture was vortexed briefly and centrifuged for 10 min at 13000 rpm. The supernatant was discarded and 200 µl of 70% ethanol was added to the pellet and the contents were centrifuged for 5 min at 13000 rpm. The pellet was suspended in 20 µl of 50 µg/ml RNase solution and incubated for 30 min at 37°C. To precipitate the DNA, 1/10 volume of sodium acetate and 2 volumes of absolute ethanol were added to the pellet and the mixture was centrifuged for 10 min at 13000 rpm. The pellet was washed with 70% ethanol followed by centrifugation for 10 min at 13000 rpm. Ethanol was removed and the pellet was dried in a concentrator for 20 min at 30°C. The pellet was finally re-suspended in 100 µl of sterile double distilled water.

Genomic DNA for Southern blot analysis was isolated using hexadecyltrimethylammonium bromide (CTAB) maxi prep method described by Ude *et al.* (2002). Leaf samples (5 g) from fully open leaf of potted regenerated plantlets were homogenized in liquid nitrogen using a mortar and pestle. The powder was transferred to a 50 ml Falcon tube to which 20

ml of 100 mM Tris pH 8, 20 mM EDTA, 1.4 mM NaCl, 4% CTAB and 1% Na₂SO₃ as an antioxidant pre-warmed to 65°C was added and the mixture was thoroughly inverted ten-times. The suspension was incubated at 65°C for 30 min with occasional mixing. The mixture was allowed to cool for 5 min after which 10 ml of chloroform : isomyl alcohol (24:1, v/v) was added and mixed by continuous inverting of the tube for 15 min. The mixture was centrifuged for 10 min at 6000 rpm after which the supernatant was transferred into a new Falcon tube. DNA was precipitated by adding 2/3 volume of ice-cold isopropanol to the supernatant followed by a gentle inverting of the tube. The content was centrifuged for 10 min at 6000 rpm and the supernatant was discarded. The pellet was rinsed with 70% ethanol and left to dry at room temperature after which it was suspended in 600 µl sterile double distilled water. RNA was digested by addition of 10 µl of 10 µg/ml RNase solution and incubation for 30 min at 37°C. DNA was precipitated by adding 1/10 volume of sodium acetate and 2 volumes of absolute ethanol followed by centrifugation for 5 min at 6000 rpm. The pellet was washed with 70% ethanol and allowed to dry at room temperature after which it was re-suspended in 200 µl of sterile double distilled water. The integrity of the DNA was checked by running 2 µl of the DNA on a 1% Agarose gel. DNA was visualized by immersing the gel briefly in 1µg/ml ethidium bromide solution and viewing it under UV light.

3.2.4.2 Polymerase chain (PCR) reaction

Polymerase chain (PCR) reaction was performed on the regenerants to confirm the presence of the transferred genes. PCR was performed in a 20 µl reaction volume using 40 ng of genomic DNA, 1.5 mM MgCl₂, 0.2 mM dNTPs, 0.3 µM each of the forward primer and reverse primer and 0.5 unit of Taq DNA polymerase. Amplification of a 326 bp fragment within the *Arabidopsis* *cycD2;1* coding sequence was performed with primers (5'-GCAAGCTCTAACTCCATTCTCC-3') and (5'-CCTGCTCCTGCGATAAACTA-3'). The PCR cycling program consisted of 3 min at 95°C; 35 cycles of 30 sec at 94°C, 30 sec at 60°C, 30 sec at 72°C and a final extension of DNA strands with 7 min at 72°C. A 553 bp band from neomycin phosphotransferase II (*nptII*) selectable marker gene was amplified using primers (5'-GAGGCTATTCGGCTATGACTG-3') and (5'-GGCCATTTTCCACCA

TGATA-3') using the same cycling program. PCR to confirm the regenerants containing the banana *cyclin* coding sequence was performed with forward primer (5'- GAGAGAGA CTGGTGATTTTCAGC-3') which had been designed within the 35S promoter region and a reverse primer MgwFF2 (5'-GCTCTCTCTCGACCAACAAGCTCAAC-3') located within the transgene to generate a 500 bp DNA fragment. A similar PCR program was used as described above with the exception that an annealing temperature of 64°C was used. PCR products were run on a 1% agarose gel in 1x TAE buffer. To visualize the PCR products, the agarose gels were stained after electrophoresis by immersing them briefly in 1 µg/ml ethidium bromide solution. Gels were visualization under UV light and photos were captured with a gel documentation system.

3.2.4.3 Southern blot analysis

Southern blot analysis was performed on PCR positive plants containing the *cyclin* sequence to confirm the stable integration of the transgenes into the banana plant genome. Genomic DNA (12 µg) was digested for 17 hrs with *BamHI* and the digested products were run on a 0.8% agarose gel containing 0.1µg/ml ethidium bromide in 1x TAE buffer. Gels were then blotted onto a positively charged nylon membrane (Roche, Cat # 1 417 240) by capillary transfer using 20x SSC (saline sodium citrate) solution and cross-linked by UV light exposure. Probes were labeled with digoxigenin 11-dUTP (DIG) (Roche) using PCR and primers mentioned in section 3.2.3.6.4. Membrane hybridization was performed at 42.7°C that was calculated from the equation: $T_m = 49.82 + 0.41 (\% G+C) - (600/l)$ [where l = length of hybrid in base pairs] and $T_{opt} = T_m - (20 \text{ to } 25^\circ\text{C})$. Hybridization signals were detected with the CPD-Star system (chemiluminescence) following the manufacturer's instruction (Roche manual version 4 October 2004, Cat # 12 041 677 001). Images were captured on a Kodak X-ray film (Sigma Chemical company Cat # P7167-1GA; Eastman Kodak Company N.Y) by exposure of blots to the film for 5 min. Developing and fixing of films was performed for 10 min using Kodak reagents, with a 10 sec rinsing interval between, and final rinsing with water.

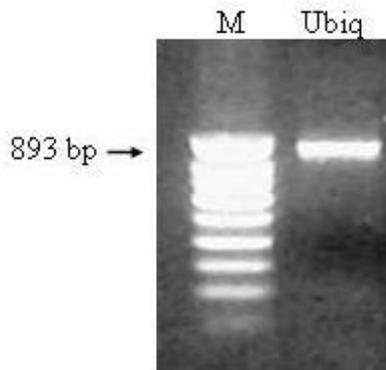
3.3 Results

3.3.1 Gene construct

Polymerase chain reaction (PCR) with ATTB4Fw and ATTB1Rv primers amplified a 893 bp maize polyubiquitin promoter sequence (Fig. 3.1A). Examination of the sequence using the promoter prediction program (TSSP) confirmed the presence of the TATA box, a promoter region and an enhancer element (Fig. 3.1B). The promoter and *Arabidopsis;CyclinD2;1* coding sequences were combined in the Gateway® destination binary vector pK7m24GW (Fig. 3.2). The resultant 10,496 base pair expression vector was designated pExpression B4-UBiB1-D2-B3 (Fig. 3.2D) and the T-DNA cassette is shown in Figure 3.4A. The cassette has XbaI restriction sites that can be used to confirm its presence based on restriction bands with the size of 2.4 and 8.0 kbp.

Using a conventional cloning approach, a 14,344 bp expression vector for the *Musac;CyclinD2;1* coding sequence under the control of the CaMV35S promoter was also constructed (Fig. 3.3). The gene cassette (Fig. 3.4B) is detectable by double digestion with KpnI and XbaI to generate a 2.4 and 8.0 kbp fragment and both gene constructs carry a neomycin phosphotransferase (*nptII*) coding sequence to be used as a selectable marker for transformed plant selection.

A



B

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TGCAGCGTGACCCGGCTGTGCCCTCTCTAGAGATAATGAGCATTGCATGTCTAAGT
TATAAAAAATTACCACATATTTTTTTGTCCACACTTGTTTGAAAGTGCAGTTTATCTA
TCTTTATACATATATTTAACTTTACTCTACGAATAATATAATCTATAGTACTACAA
TAA TATCAGTGT TTTAGAGAATCATATAAA TGAACAGTTAGACATGGTC TAAAGGAC
AATTGAGTATTTTGACAACAGGACTCTACAGTTTATCTTTT TAGTGTGCATGTGTT
CTCCTTTTTTTTGGCAAATAGCTTCACCTA TATAA TAC TTCATCCATTTTA TTAGTA
CATCCATTTAGGGTTTAGGGTTAATGGTTTTTATAGACTAA TTTTTTTAGTACATCT
ATTTTATTC TATTTTAGCC TCTAAA TTAAGAAAAC TAAAC TC TATTTTAGTTTTTT
TATTTAATAATTTAGATATAAAA TAGAATAAAA TAAAGTGACTAAAAAT TAAACAAA
TACCCTTTAAGAAAT TAAAAAACTAAGGAAACATTTTCTTGTTTCGAGTAGATAA
TGCAGCCTGT TAAACGCCGTCGACGAGTC TAA CCGGACCAACCAGCGAACCGCA
GCGTCGCGTCGGGCCAAGC GAA GCA GACGGCACGGCATCTCTGTGCTGCCTCTGGA
CCCCTCTCGAGAGTTCCGCTCCACC GTTGGACTTGCTCCGCTGTCGGCATCCAGAAA
TTGCGTGGCGGAGCGGCAGACGTGAGCCGGCACGGCAGGCGGCCCTCCTCCTCCTC
ACGGCACGGCAGCTACGGGGGATTCCTTCCACC GCTCCTTCGCTTTCCTCCTCCTC
GCCCGCGGTAAATAATA GACACCCCTCCACACCTCT
  
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Fig. 3.1 Isolated maize ubiquitin-1 promoter. (A) PCR amplification product from maize (line B73). (B) 893 bp nucleotide sequence of the promoter. M, Smart Ladder 100-1000 bp (Eurogentec®); Ubiq, lane with ubiquitin-1 PCR band; TATA region is boxed; CAAT sequences are underscored; bent arrows indicate first nucleotide position of promoter (double underline) and enhancer (underlined) regions, respectively.

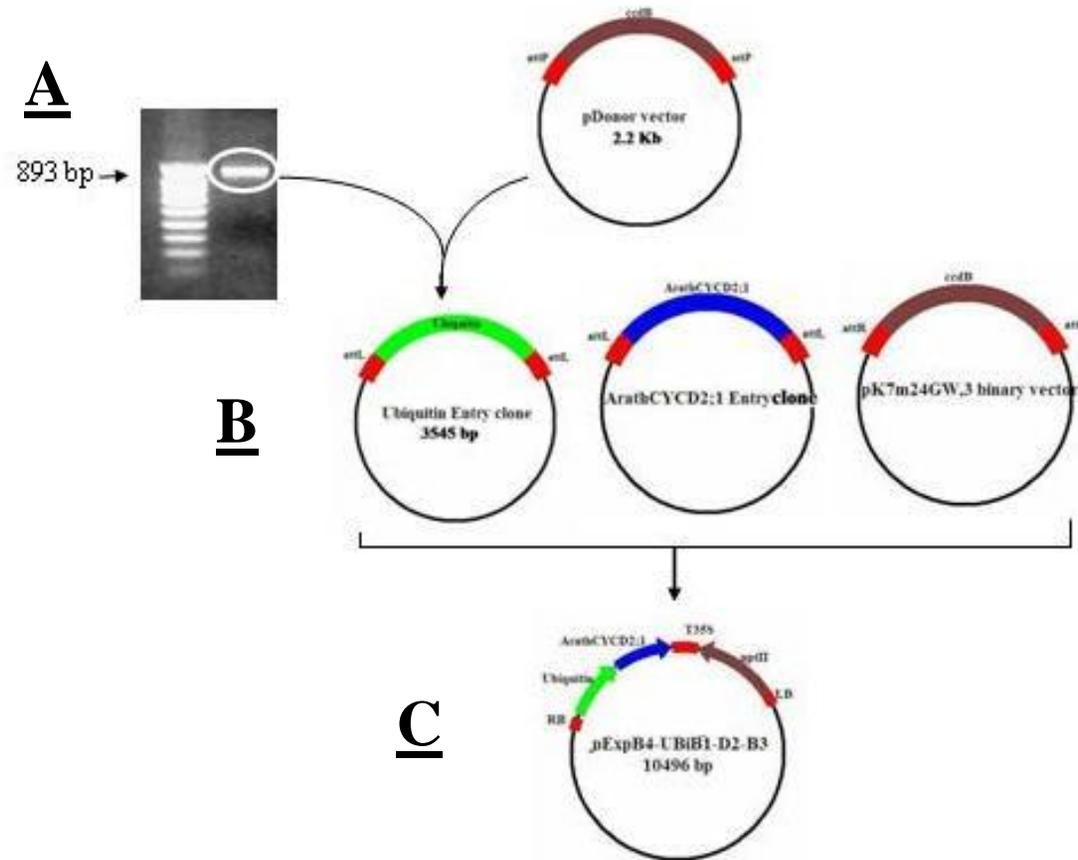


Fig. 3.2 Diagrammatical representation of the construction of the *Arabidopsis;CYCD2;1* expression vector using the Gateway™ cloning system. (A) ubiquitin promoter isolated from maize by PCR with primers containing *attB* site and ligated into pDONR vector by PB recombination reaction; (B) combination of the gene and promoter from their respective entry vectors into destination vector by LR recombination reaction; (C) Final destination vector pExpression B4-UBiB1-D2-B3.

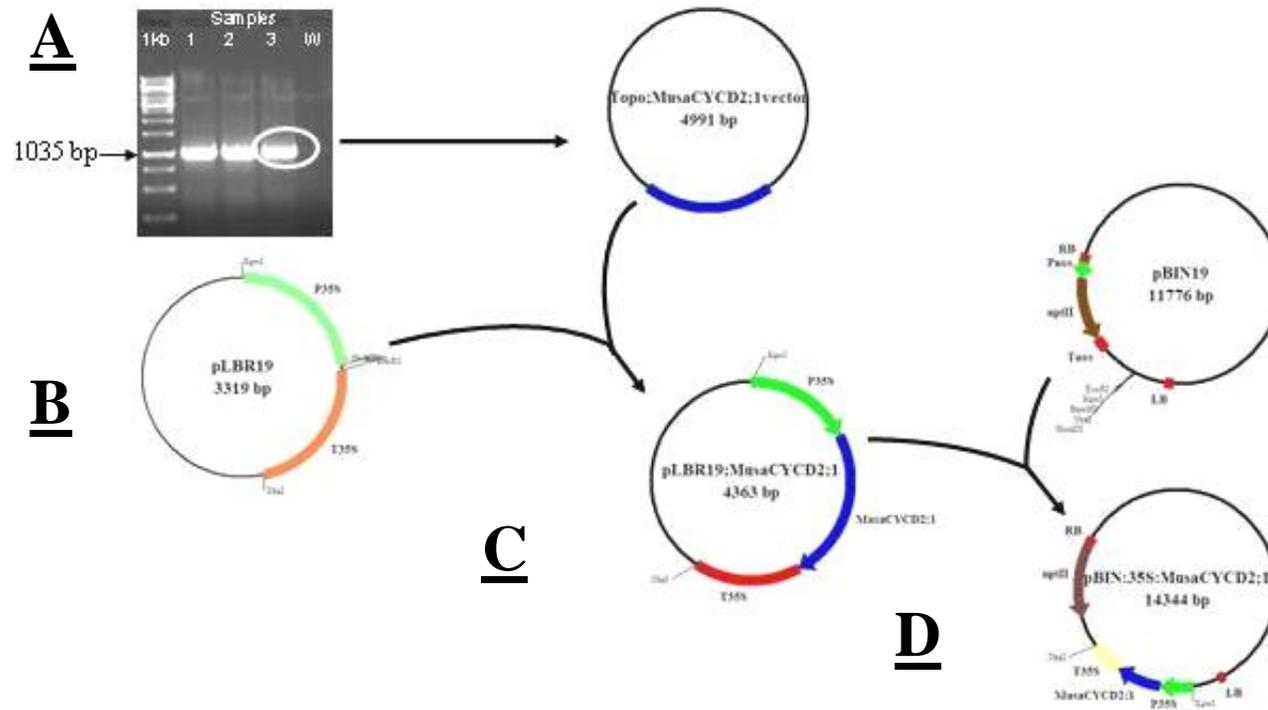


Fig. 3.3 Diagrammatical representation of the construction of the *Musac;CyclinD2;1* expression vector. (A) *Musac;CyclinD2;1* cDNA cloned into TOPO vector; (B) cDNA cloned between the 35S promoter and terminator of pLBR19 vector; (C) construct ligated into multiple cloning sites of expression vector pBIN19; (D) Final vector pBIN:35S: *Musac;CYCD2;1*.

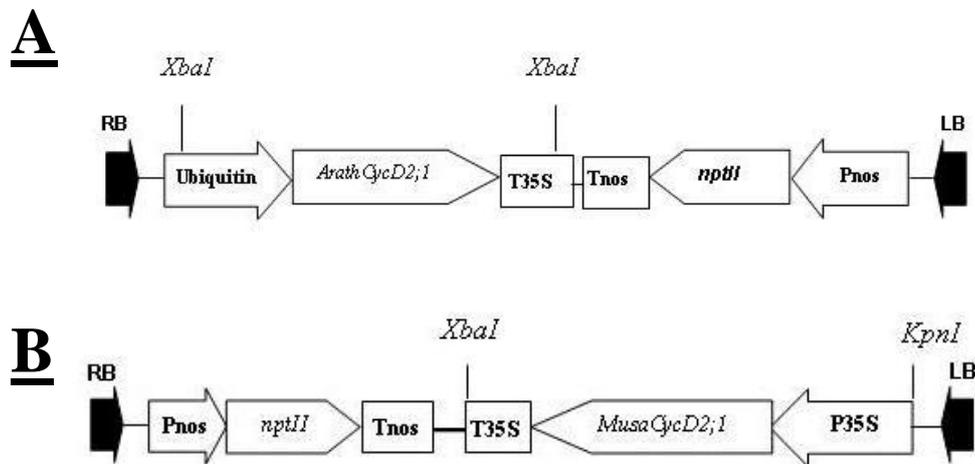


Fig. 3.4 (A) *Arath;CyclinD2;1* gene cassette in expression vector pExpression B4-UBiB1-D2-B3 vector. (B) *Musac;CyclinD2;1* gene cassette in expression vector pBIN:35S:*Musac;CyclinD2;1*. Restriction sites for gene insertion verification are indicated.

3.3.2 Plant transformation, selection and regeneration

The banana cells that were co-cultivated with *Agrobacterium* turned brown when plated onto regeneration (M3) medium fortified with geneticin. However, recovery and proliferation became evident as white spots against a background of black dead cells. This recovery was observed after 5 wks for *Arath;CYCD2;1* and *Musac;CYCD2;1* and 6 wks for pBIN19 on selection medium. The recovered cells formed clusters when transferred onto fresh medium (Figs. 3.5A; 3.6A and 3.6B). Cell clusters were plated in direct contact with M3 medium to increase selection. Embryos formed in the surviving cell clusters were cultured for another month on RD1 medium. These embryos germinated when they were plated on M4 medium (Fig. 3.5C and 3.6C). Comparison of the shoots arising from the plated embryos showed a significantly lower regeneration frequency in cell cultures co-cultivated with *Arabidopsis;CyclinD2;1* compared to the untransformed control (Table 3.1). Cells co-cultured with the banana *cyclin* showed considerably higher regeneration compared with the ones where an empty vector pBIN19 was used (Tables 3.2).

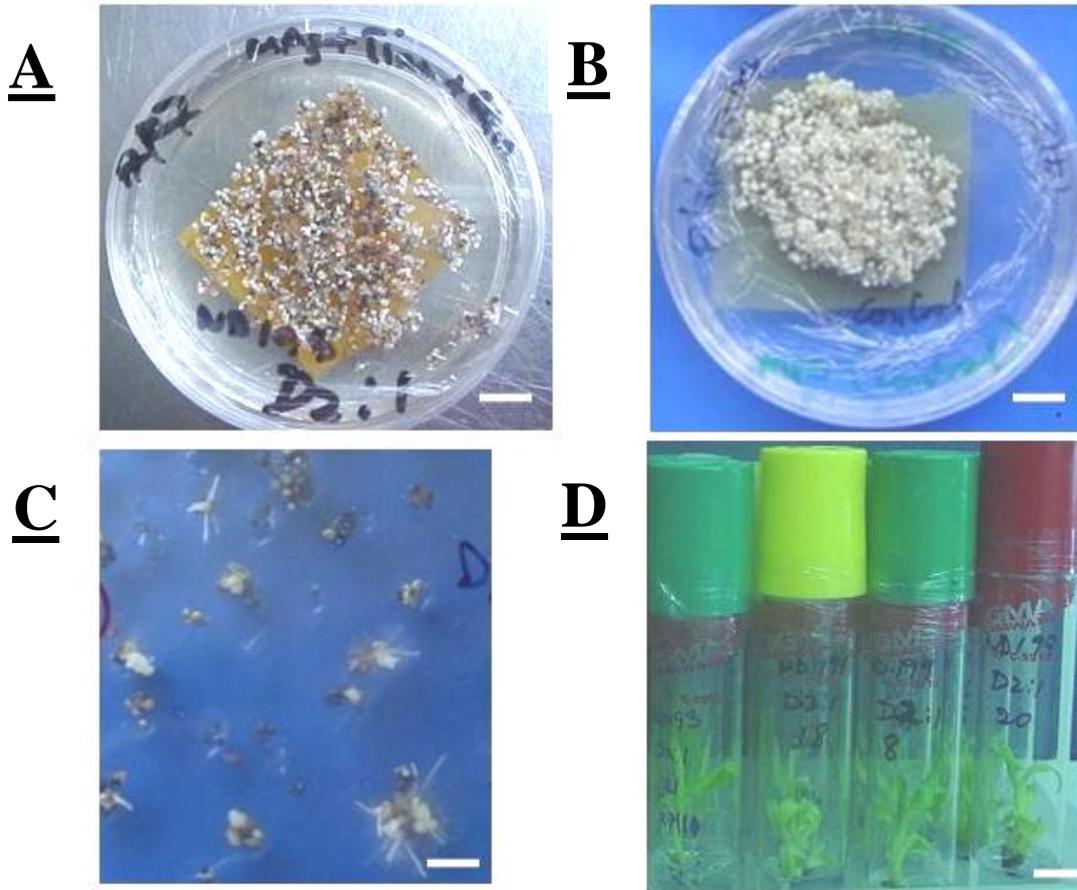


Fig. 3.5 Response of banana cells transformed with *Arath;CyclinD2;1* gene. (A) selection and regeneration of transformed cells, 8 weeks on selection medium, bar = 1.5 cm; (B) regeneration of non transformed cells, bar = 1.3 cm; (C) germinating embryos, bar = 0.8 mm; (D) rooted regenerants = 1.5 cm.

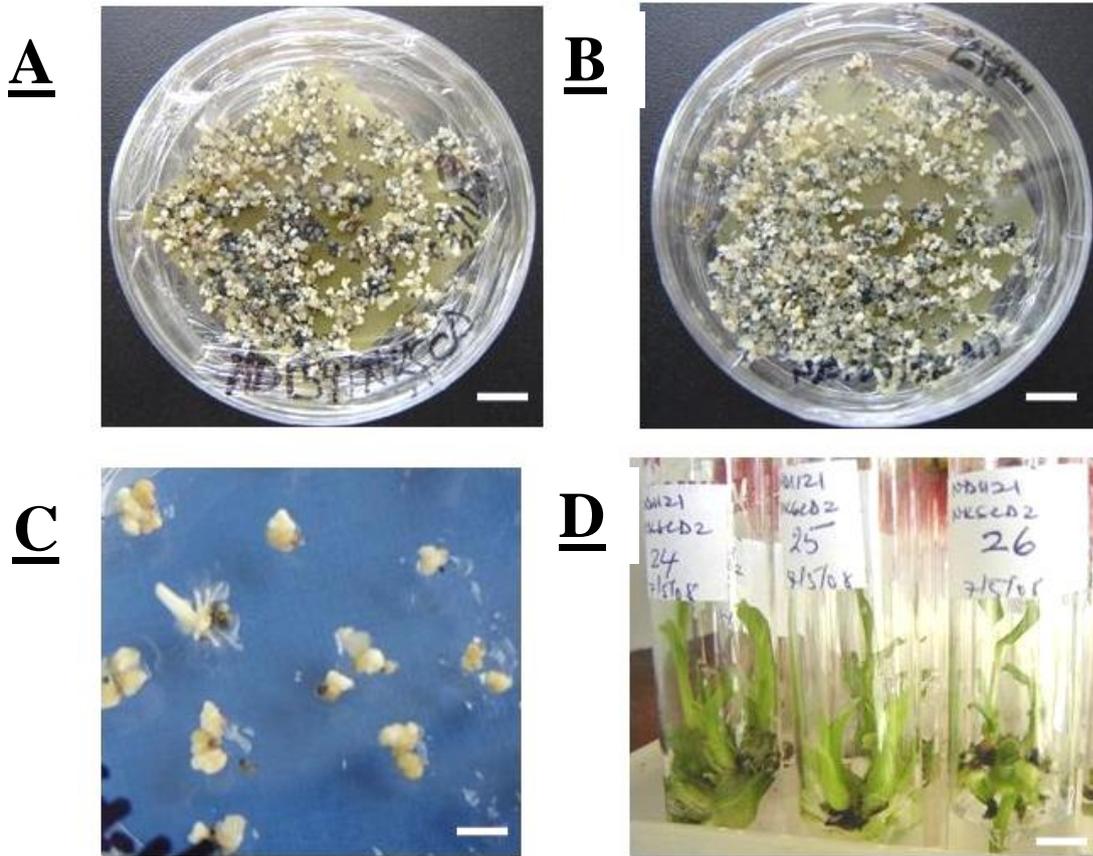


Fig. 3.6 Response of banana cells transformed with *Musac;CyclinD2;1* gene. (A) selection and regeneration of *Musac;CyclinD2;1* transformed cells, 8 wks on selection medium, bar = 1.5 cm; (B) selection of regeneration of cells transformed with empty vector pBIN19, 8 wks on selection medium, bar = 1.5 cm; (C) Germinating embryos, bar = 0.9 mm; (D) proliferating regenerants = 1.0 cm.

Table 3.1 Regeneration frequency of Sukalindiizi cells transformed with *Arath;CyclinD2:1*.

Construct	Clones plated	Shoots	Regeneration frequency (%)
<i>Arath;CYCD2;1</i>	622	93	14.8±1.2
Non transformed (Control)	71	34	47.6±6.2
T-test (<i>P</i> value)			0.031

Table 3.2 Regeneration frequency of Sukalindiizi cells transformed with *Musac;CyclinD2;1*.

Construct	Clones plated	Shoots	Regeneration frequency (%)
<i>Musac;CYCD2;1</i>	250	36	14.1 ± 1.7
pBIN19 (Control)	250	17	6.8 ± 0.8
T-test (<i>P</i> value)			0.001

Table 3.3 Number of positive transformants from randomly selected regenerants.

Cell line	Gene	Number	PCR positive*	Percentage
S. Ndiizi: line 193	<i>Arath;D2:1</i>	47	24	51.1
S. Ndizi: line 195	<i>Musac;D2;1</i>	36	25	69.4
S. Ndizi: line 195	pBin19	14	7	50.0

*PCR with DNA from *Arath;D2:1* transformants was performed with gene and *nptII* specific primers. *nptII* detection was used on the pBin19 regenerants; a forward primer in the 35S promoter and reverse in the gene region was used for the *Musac;CyclinD2;1* regenerants.

3.3.3 Confirmation of transgene insertion

PCR of genomic DNA from regenerated plants from *Arabidopsis;CycD2;1* transformation amplified a 323 bp and a 553 bp DNA fragment that were absent in the non-transformed control plants and in the buffer (Fig. 3.7A). Some of the regenerants tested negative with no DNA fragment amplified from DNA of both *Arath;CycD2;1* and neomycin phosphotransferase II (*nptII*) genes. Only samples that showed bands for both genes were considered as positive transformants giving a transformation frequency of 51.1% (Table 3.3). DNA of regenerants of *Musac;CycD2;1* produced a 500 bp DNA fragment that was absent in the control carrying an empty vector pBIN19 (Fig. 3.9A). Further, PCR with *nptII* primers and DNA from regenerants transformed with plasmid pBIN19 showed that 50% were transformed (Table 3.3). Out of the potted plants, PCR amplified a fragment from DNA of 69.4% and 50% of the *Musac;CycD2;1* and pBIN19 transgenics, respectively (Table 3.3). Primers flanking the *Musac;CycD2;1* coding sequence generated a 2 kbp fragment in the transformed and also non-transformed samples *cyclinD2* but an extra 1kbp fragment in transgenic plants (Fig. 3.9B).

When Southern blot analysis was carried out using genomic DNA from different putative *Arath;CycD2;1* transgenic plants, DNAs from 6 plants out of 9 tested DNAs showed the presence of the cyclin gene. No hybridization product was detected in the non-transformed control (Fig. 3.8). From the Southern blot of the *Musac;CycD2;1* regenerants three DNAs of the putative transformed plants and one DNA from a plant transformed with the empty vector hybridized with a *nptII* probe (Fig. 3.10). No signal was observed in the non-transformed plants.

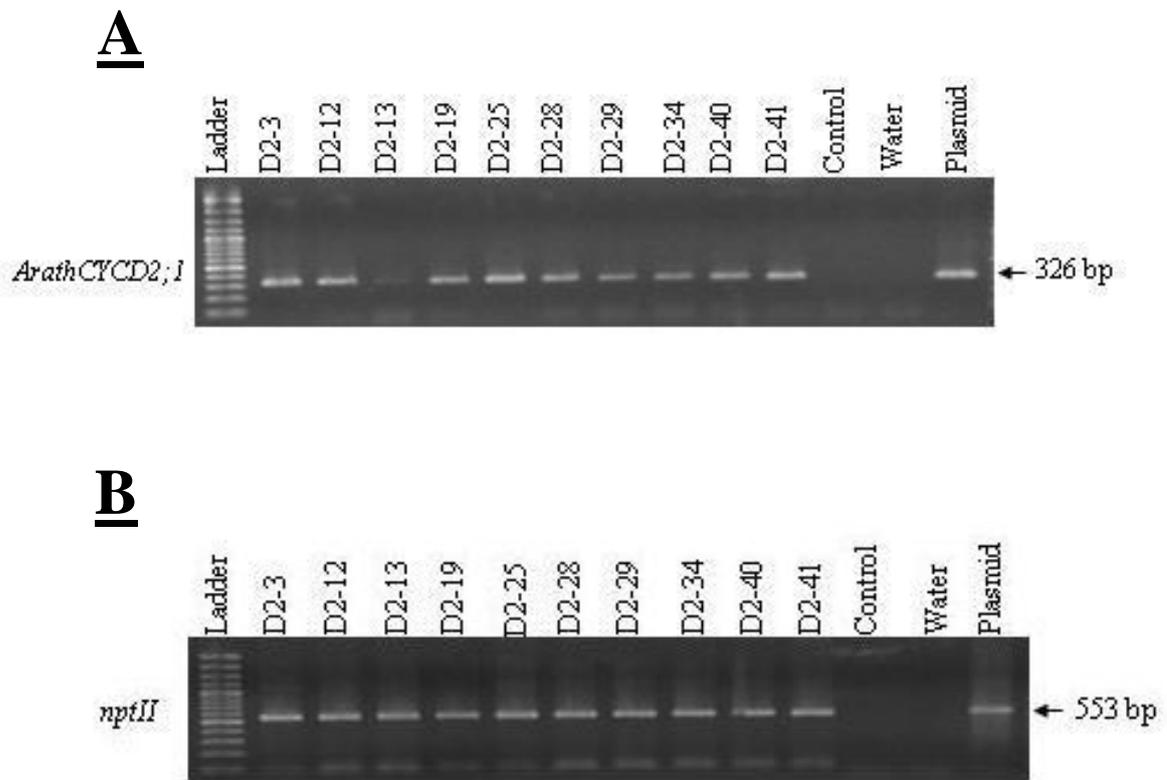


Fig. 3.7 PCR analysis of greenhouse potted *Arabidopsis;CyclinD2;1* regenerants. (A) PCR with *Arabidopsis;CyclinD2;1* specific primers; (B) PCR with neomycin phosphotransferase II (*nptII*) specific primers; L, 100bp DNA ladder; Control is a non-transformed plant, plasmid is vector pExpression B4-UBiB1-D2-B3 DNA carrying the transgene.

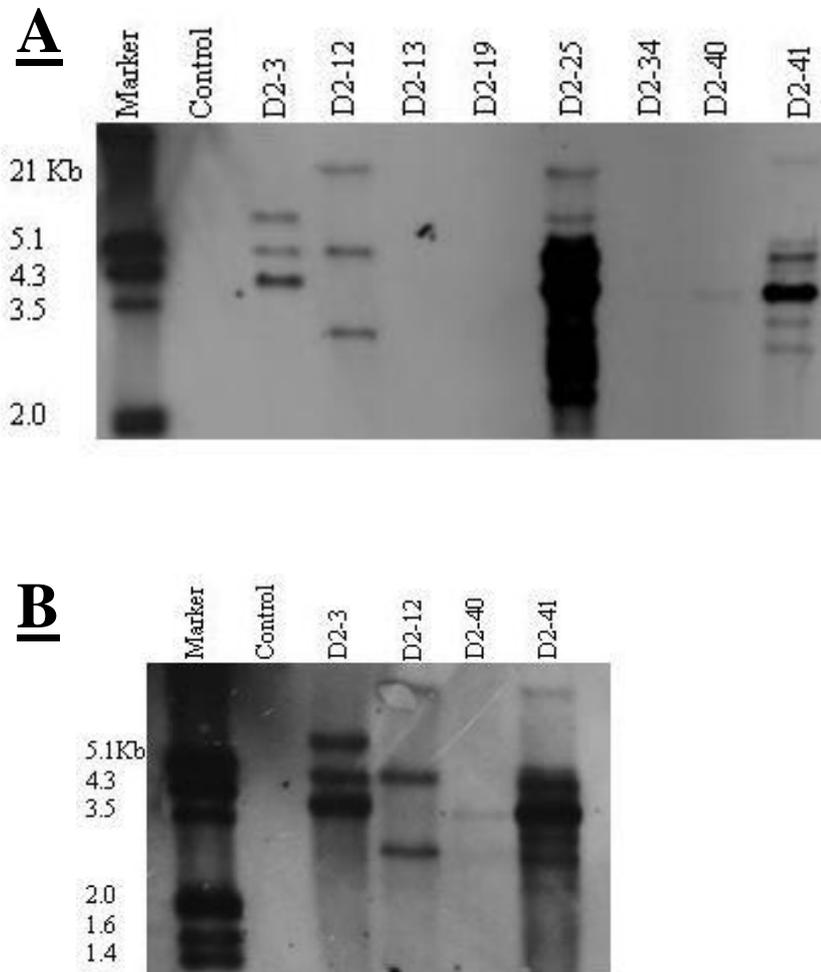


Fig. 3.8 (A and B) Southern blot of *Arath;CyclinD2;1* putative transformed bananas. Genomic DNA from selected transformed plants and a control (non transformed) were digested with *BamHI*, fractionated on 0.8% agarose gel and probed with a DIG-labeled 650 bp *Arath;CyclinD2;1* DNA fragment. Marker is a digoxigenin-labeled DNA molecular weight marker III.

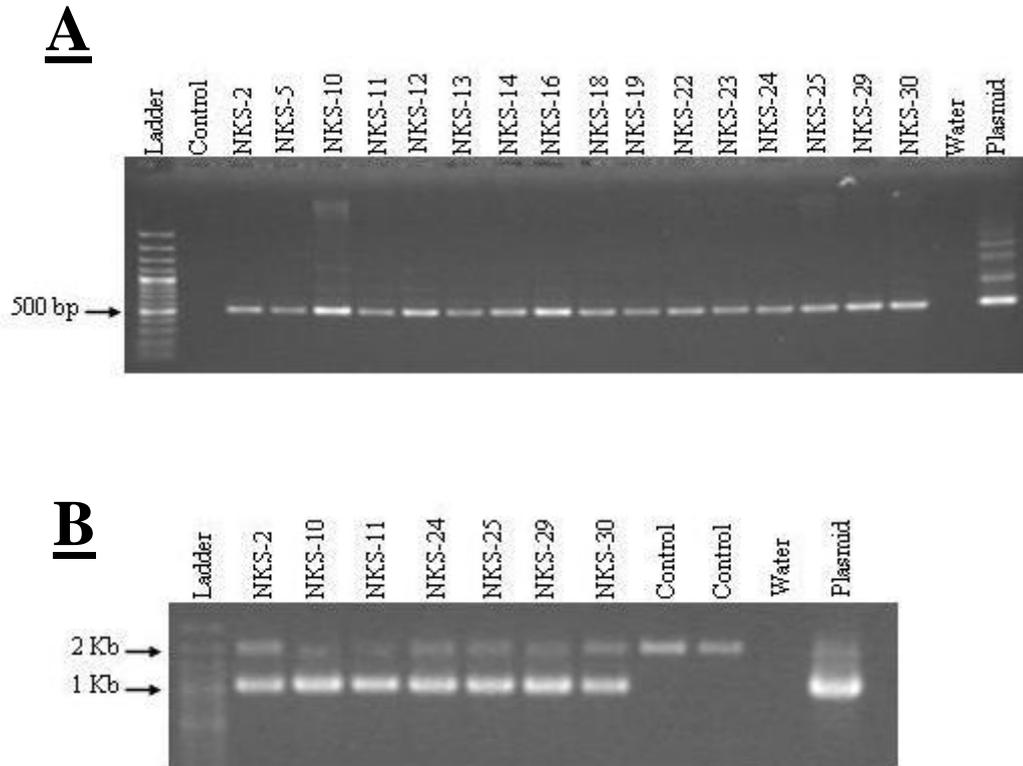


Fig. 3.9 PCR analysis of DNAs from greenhouse-grown *Musac;CyclinD2;1* transgenic plants. (A) PCR with a forward primer designed in the promoter and reverse primer in the cyclin gene coding regions; (B) PCR with primers flanking the gene coding sequences; L=100bp DNA ladder; NKS are lines that were randomly selected; Bands: 1 kbp represents the genomic *Musac;CyclinD2;1* and the 2 K bp is the *Musac;CyclinD2;1* cDNA transgene. Controls are the non transformed plants; Plasmid is pBIN:35S: *Musac;CyclinD2;1* vector DNA used in the transformation.

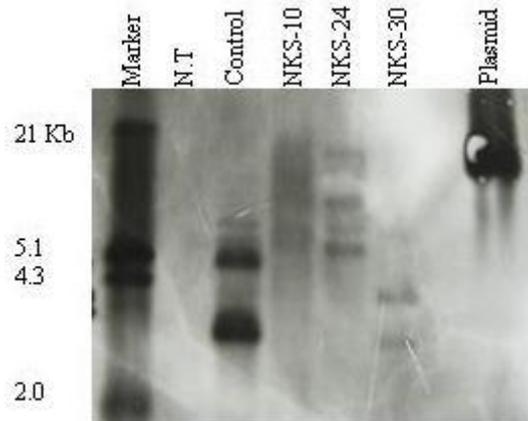


Fig. 3.10 Southern blot of banana plants transformed with *Musac;CyclinD2;1* coding sequence. Genomic DNA from selected transformed lines and non-transformed control were digested with *BamHI*, fractionated on 0.8% agarose gel and probed with DIG 750 bp labeled neomycin phosphotransferase II (*nptII*) gene fragment. Marker is a digoxigenin-labeled DNA molecular weight marker III; N.T is a non transformed plant; control is a transgenic carrying an empty pBIN19 vector.

3.4 Discussion

The results obtained from the *Arabidopsis* and *Musac;CyclinD2;1* gene constructs provide a broad comparison due to difference in cell lines, promoters and *cyclin* genes that were used. Generally, transformation and regeneration of bananas over-expressing *Arath;CYC;D2;1* and *Musac;CYC;D2;1* were achieved. The determined regeneration efficiency of 48% was much higher than previously reported (15%) cultivar ‘Mas’ belonging to the same AAB genotype (Jalil *et al.*, 2003). Shoot regeneration in banana is generally low and the low frequency of conversion of plant embryos into plants is a common problem (Gaj *et al.*, 2004). Even highly proliferative cells may lack the embryogenic competency (Namasivayam, 2007). In addition, the low regeneration might be attributed to a stress condition due to the long *in vitro* duration required to generate callus and a cell suspension and further the constant exposure to growth regulators (Bardini *et al.*, 2003). In this study, a 3-fold lower regeneration frequency

was obtained in transformed than in non-transformed cells. This could be due to failure of some cell clusters that had survived the long selection process to finally regenerate into shoots or to a low expression level of the antibiotic (*nptII*) gene. Also the prolonged selection regime used could have been stressful enough to reduce regeneration (Bardini *et al.*, 2003). Further, *Musac;CyclinD2;1* over-expression resulted in a higher regeneration rate when compared to cells co-cultured with an empty vector and it might be interesting to study in more detail if over-expression of cyclin affects plants regeneration.

Molecular evaluation of the regenerants identified almost half of the lines to be escapes. Survival of untransformed cells could be attributed to the detoxification of the antibiotic in the selection medium by the surrounding transformed cells. Such cross protection is a common phenomenon in aminoglycoside antibiotics, such as geneticin (Wilmink and Dons, 1993). Furthermore, accumulation of toxic compounds released from dead untransformed cells can also contribute to the low regeneration of transformed cells (Lindsey and Gallois, 1990). These occurrences are likely to be high in banana cells that grow in clusters.

In conclusion, this study has demonstrated the practicality of genetic transformation of banana with *CyclinD2;1* gene homologs. In the next part of the study, expression of the *cyclin* transgenes was investigated.