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FUNCTIONAL ANALYSIS OF CYCLIN D2;1-TYPE GENES EXPRESSED IN TRANSGENIC BANANA PLANTS

By

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DECLARATION

This thesis is my original work and has not been presented for a degree in any other University.

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DEDICATION

To my wife Susan Talengera, daughter Norah Nampiima, and sons Andrew Talengera and Simon Mukoka.

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SUMMARY

Early maturity is one of the most important aerial growth traits next to bunch size, in determining banana productivity. However, low seed fertility in banana and the lack of breeding lines limit the application of conventional breeding for this trait. Genetic transformation with a *CyclinD2*-type gene, responsible for the CyclinD2 protein sub unit, which modulates the cell cycle progression at the G1/S phase, enhanced growth of tobacco plants. On this basis, investigations were carried out on the possibility of increasing the growth rate of banana plants through transformation with and expression of a *CyclinD2*-type coding sequence. *Arabidopsis thaliana;CyclinD2;1* (*Arath;CycD2;1*) gene and its banana ortholog were over-expressed in banana to test their growth enhancement potential. The banana *CyclinD2;1* (*Musac;CycD2;1*) was isolated from an East African highland cooking banana (AAA) cultivar 'Nakasabira' and a cDNA was created by PCR using degenerated primers which was followed by genome walking. Characterization of the banana cyclin protein revealed an IWKVHAHY motif that was found to be conserved across the Musaceae family. Phylogenic analysis revealed a higher protein sequence identity of this banana cyclin to *CyclinD2;1* of monocot plants than that of Arabidopsis. This cyclin was also found to be expressed highly in meristematic tissue which linked it to the cell cycle. The coding sequence was submitted to the GeneBank under accession number HQ839770. Arabidopsis and banana *CyclinD2;1* gene coding sequences under the control of a constitutive promoter were used to transform embryogenic cells of the banana cultivar 'Sukalindiizi' (AAB) using the Agrobacterium transformation system. A higher relative expression of *Arath;CyclinD2;1* was found in the shoot than in the root apices and expression reduced transcript amounts of the endogenous banana *CyclinD2;1*. Plants of transformed banana line D2-41 had the highest *Arath;CyclinD2;1* transcript amount and exhibited a significantly faster leaf elongation rate, better root growth, faster first leaf opening and a bigger lamina composed of bigger epidermal cells than non-transformed control plants. Banana plants transformed with *Musac;CyclinD2;1* had a higher transcript amount of the transgene in the root apices when compared to the shoot apices. The higher transcript amount in the roots of plants of transformed line NKS-30 was related to faster

root growth and development of an extensive root system. Overall, this study has provided evidence that expression of cyclin coding sequences in transformed banana is related to growth promotion. Specifically, *Arath;CyclinD2;1* promoted shoot growth while the *Musa* homolog promoted root growth. Shoot and root growth phenotypes obtained in this study might have the potential to improve banana productivity in terms of short plant growth cycle, increased bunch weight, improved plant anchorage and increased plant resistance to root nematode damage. Future work should assess the produced plants in the field to allow transformed plants to exhibit their full potential and to be able to fully evaluate the vegetative and flowering phases.

Thesis composition

Chapter 1 describes the botany of the banana plants and the demand for a fast maturing banana plant and extensive root growth and the possible contribution of these characteristics to productivity. This chapter also contains the limitations of conventional methods to breeding for these traits and the molecular option targeting the *CyclinD2*-type gene to accelerate the cell cycle. *Chapter 2* covers the results obtained for isolation and characterization of the *CyclinD2;1*-type gene ortholog from banana including a phylogenetic analysis of the isolated banana cyclin gene sequence. *Chapter 3* describes the creation of Arabidopsis and banana *CyclinD2;1* gene constructs suitable for banana transformation, their delivery into transformation into banana cells using the Agrobacterium-based transformation system, regeneration of transformed plants and finally the detection of the transferred transgene in transformed banana using molecular biology tools. *Chapter 4* reports about the expression analysis of both the endogenous cyclin banana gene and of transferred cyclin transgenes in selected regenerated banana plants using the technique of quantitative RT-PCR. *Chapter 5* outlines the phenotypic evaluation of transformed banana plants to detect any changed shoot and root growth characteristics and to determine a possible relationship between changed characteristics and transcript amounts of transgenes. Finally, *chapter 6* summarizes the novel results found in this study and evaluates the set initial working hypothesis. Further, recommendations for further research work, based on the results obtained in this study, are outlined.

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CHAPTER ONE

INTRODUCTION

1.1 The banana plant

Banana (*Musa* spp.) belongs to the order Zingiberales, family Musaceae, section Eumusa and genus *Musa* (Simmonds, 1962; Purseglove, 1972; Stover and Simmonds, 1987). A banana plant is a tree-like, giant perennial tropical monocotyledonous herb of two to nine meters tall (Fig. 1.1). The plant has a pseudostem composed of compact overlapping leaf sheaths. The basal stem is condensed (corm) and only elongates through the pseudostem at flowering to eject the inflorescence. Cultivated bananas have descended from two wild seeded forms, *Musa acuminata* Colla (AA; 2n =22) and *M. balbisiana* Colla (BB; 2n = 22) to give rise to AA, AB, AAA, AAB and ABB genomic composition (Simmonds, 1962; Purseglove, 1972; Stover and Simmonds, 1987). Triploids resulted from fertilization of egg cells with unreduced chromosome, caused by chromosome restitution, in one diploid parent by haploid pollen from another parent. With the exception of a few wild and fertile diploids and in conventional breeding work where seed set and germination can occur, banana plants propagate vegetatively through suckers that arise from the corm of the mother plant (Purseglove, 1972; Stover and Simmonds, 1987).

1.2 Banana breeding

1.2.1 Conventional breeding

Along with diseases, such as black Sigatoka and Fusarium wilt resistance and yield, early maturity and root system development have been traits of interest to classical banana breeders for improving banana productivity (Stover and Buddenhagen, 1986; Vuylsteke *et al*., 1995; Gowen, 1996; Tenkouano *et al*., 1998). However, no conventional breeding scheme has been developed to address these traits. Instead, fast maturity has been a secondary selection parameter in hybrid development (Vuylsteke *et al*., 1993; Tenkouano *et al*., 1998; Ortiz and Vuylsteke, 1998a; 1998b; Ortiz *et al*., 1998). Differences in maturity time due to ploidy levels has been reported where diploids have a short fruit filling duration than triploid (Swennen and Vuylsteke, 2001). However, diploids are unattractive for large scale and commercial cultivation due to their low yields and growth vigour (Swennen and

Vuylsteke, 2001). In addition, diploids have been developed through human selection for their pathernocarpic fruits and seed sterility (Simmonds, 1962). Therefore, their biology restricts the introgression of their fast maturity trait into other banana variety through crossing breeding. The majority of the cultivated genotypes that constitute the popular varieties are triploids and highly sterile which limits their improvement through conventional breeding (Crouch *et al*., 1989). In the few cases where seeds have been obtained, the low germinations limit the recovery of hybrids (Vuylsteke *et al*., 1993; Ssebuliba *et al*., 2005). Among the fertile banana cultivars (Ssebuliba *et al*., 2005), the variability in their maturity time is not significant (Makumbi, 1995).

Application of cross breeding in banana is still limited by factors such as the low seed and pollen fertility, triploidy levels of the economically important cultivars, slow propagation, and long generation time from one generation to the next (Vuylsteke *et al*., 1993; Crouch *et al*., 1998; Sweenen and Vuylsteke, 2001; Ssebuliba *et al*., 2005). Where hybrids have been recovered failure of the fruits to retain the desirable quality has been a drawback (Purseglove 1972; Pillay *et al*., 2004).

1.2.2 Genetic engineering of banana

Genetic transformation has been used to introgress important agronomic traits in a number of monocotyledonous crops (Cheng *et al*., 2004). In banana, this strategy has a potential for traits lacking in the *Musa* germplasm and for seed sterile cultivars (Crouch *et al*., 1998) and the potential has been demonstrated for resistance against bacterial wilt (Tripathi *et al*., 2010). However, employing the technique is limited by the complex gene interaction that influences quantitative traits (Crouch *et al*., 1998). Gene transfer approaches and explants that have been used in banana include electroporation of protoplasts (Sagi *et al*., 1994), particle bombardment of embryogenic cells (Sagi *et al*., 1995; Becker *et al*., 2001) and co-cultivation of *Agrobacterium tumefaciens* with embryogenic cells (Ganapathi *et al*., 2001; Khanna *et al*., 2004; Talengera *et al*., 2010; Tripathi *et al*., 2010). *Agrobacterium* has also been used on wounded corm discs (May *et al*., 1995; Tripathi *et al*., 2008). Of the gene delivery systems, *Agrobacterium* is prominent for its simplicity and delivery of intact

transgenes and low copy numbers and low incidences of transgene silencing (Dai *et al*., 2001; Hu *et al*., 2003; Veluthambi *et al*., 2003). In banana, *Agrobacterium* is reported to give higher transformation frequencies and lower number of integration sites compared to particle bombardment (Arinaitwe, 2008). The use of corm discs as transformation explants can give results in a shorter time when compared to embryogenic cells (Tripathi *et al*., 2008), but it carries the risk of generating chimeric transgenics (Khalil *et al*., 2002; Jain, 2011). Strosse *et al*. (2004) noted that plants regenerated from embryogenic cell suspension (ECS) are from single cells. However, generating banana ECS is a relatively long process and is highly genotype and cultivar dependent (Strosse *et al*., 2003 and 2004).

1.2.3 Factors influencing transgene expression

Expression of a transgene can be regulated by the strength and tissue specificity of a promoter used in the transgene construct (Potenza *et al*., 2004; Dietz-Pfeilstetter, 2010) and by inclusion of an intron into the gene construct (Rose and Baliakoff, 2000; Cheng *et al*., 2004). However, an integrated transgene can be silenced following failure at the transcription level caused by DNA-methylation commonly induced by promoter homology (Park *et al*., 1996; Stam *et al*., 1997). Similarly, silencing can be post-transcriptional where transcription occurs at a low level or its mRNA fails to accumulate as a result of high homology of the transgene with a native gene (Matzke and Matzke, 1995; Stam *et al*., 1997; Kanno *et al*., 2000). High homology of the transgene and the endogenous gene can lead to the silencing of both genes (Stam *et al*., 1997). Strong promoters and multiple transgene copies lead to high transcript levels that result into gene silencing when a threshold level is exceeded (Stam *et al*., 1997). A transgene integrated in a head to head and tail to tail can further induce methylation or cause DNA to DNA or RNA to DNA paring that induces their degradation in the cell (Stam *et al*., 1997; Lakshminarayan *et al*., 2000). Due to the random integration of transgenes, the site of integration influences the extent of transcription (Park *et al*., 1996; Stam *et al*., 1997; Muskens *et al*., 2000; Yoshida and Shinmyo, 2000; Gelvin, 2003; Filipecki and Malepszy, 2006; Qi and John, 2007). Transgenes integrated within a methylated chromosome area are more likely to get methylated and silenced. Genes inserted within a CG rich chromosome region or high gene

density regions will be easily transcribed while those in A/T rich areas are prone to silencing. According to Meyer (1995) and Down *et al*. (2001), transgene expression is also influenced by prevailing growth environment of the transgenic plants.

1.3 Banana growth and development

Where soil fertility, disease and pests are not critical constraints, fast maturity has been identified as the most important aerial growth trait, next to bunch size, in determining banana productivity (Stover, 2000). Maturity time determines the duration of the production cycle that comprise the vegetative growth phase and reproductive phases.

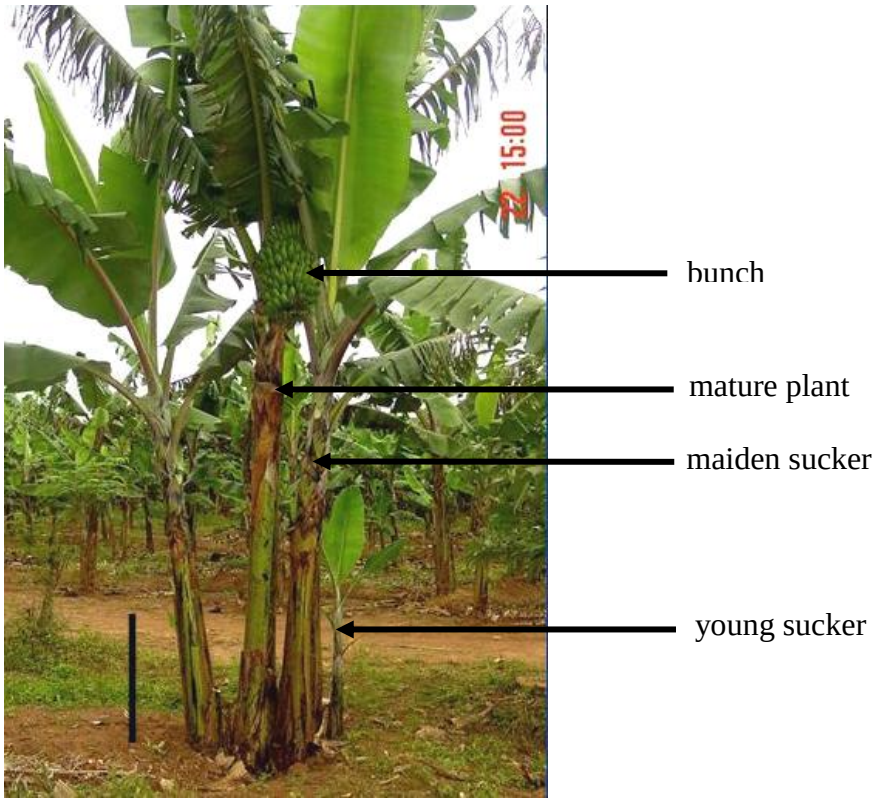


Fig.1.1 Growth cycle of a banana plant. Numbers show the successive vegetative phases before the plant flowers. Bar on the left = 1m

The vegetative phase covers the duration a plant takes to flower and shooting interval between ratoon crops while the reproductive phase covers the fruit filling time (Tenkouano *et al*., 1998). From a field germplasm evaluation study conducted in Uganda (Makumbi, 1995), bananas take 13-17 months from sucker emergence to flowering, and 4-5 months for the fruits to mature. Surveys in East Africa (Karamura *et al*., 2004; Gaidashova *et al*., 2005) and West Africa (Efenden *et al*., 2003) established maturation rates as one of the criteria used by farmers to select cultivars to grow. The relatively long cropping duration of banana was identified as disincentive to farmers' adoption rate of improved production technologies, such as fertilizer, when they consider banana growing as a long-term investment (Van Asten *et al*., 2004). Similarly, long cropping duration of banana has also been a drawback to conventional breeding efforts due to the more than 2 years required generating a crop cycle from a seed (Crouch *et al*., 1998; Pillay *et al*., 2002).

1.3.1 Banana leaf and root growth and productivity

Physiological studies of banana have correlated leaf production with the plant development, architecture and yield. Depending on the cultivar, floral initiation in banana is induced after the plant has emitted a specific number of leaves (Stover and Simmonds, 1987; Swennen and De Langhe, 1985, Swennen and Vuylsteke, 2001). Further, growth studies in banana have positively correlated leaf emergence and growth rates to faster growth and bunch weight (Swennen and De Langhe, 1985). In addition, Swennen and De Langhe (1985) observed a positive correlation between leaf emergence rates, growth rates and early flowering in plantains (AAB). Because the banana pseudostem consists of overlapping leaf sheaths, foliar growth has a direct influence plant height and circumference of the pseudostem (Stover and Simmonds, 1987).

The root system determines the plant's ability to obtain both water and mineral nutrients from the soil (Taiz and Zeiger, 2006). In banana, a positive relationship between root development and aerial growth has been recorded (Blomme *et al*, 2001; Lecompte *et al*., 2002). Because banana plants are characterized by a shallow root system (Purseglove, 1972; Stover and Simmonds 1987), an improved root system is a desirable trait against

toppling and nematode damage (Tenkouano *et al*., 1998). However, breeding for root growth is not feasible with the available conventional approach.

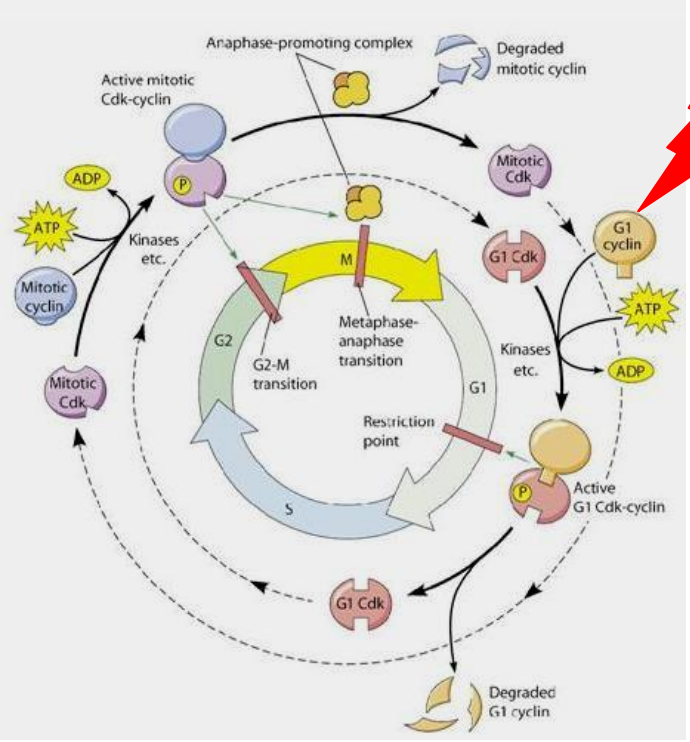
1.3.2 Biotechnology and banana plant architecture

Mutagens, such as gamma rays, can induce genetic variability in plants from which rare traits can be selected for (Van Harten, 1998). This approach has generated a fast maturing Grand Naine banana cultivar "Novaria" (Roux, 2004). However, useful mutation occurs at a very low frequency and can affect other non targeted genes (Van Harten, 1998). In light of these limitations, genetic transformation that is considered to be precise in modifying traits of interest without altering other plant characteristics (Sharma and Ortiz, 2000) remains a desirable option. In particular, over-expression of Arabidopsis *CyclinD2;1* gene in tobacco gave transgenic plants with faster shoot and root growth, faster leaf emission and earlier flowering (Cockroft *et al*., 2000; Boucheron *et al*., 2005) and this cyclin type has also been used in this study to possibly change the banana architecture. *CyclinD*-type is a protein sub unit that plays a major regulatory role of the cell cycle progression at the G1/S transition (Meijer and Murray, 2000; Shen, 2002; Inzé and De Veylder, 2006; Brooker, 2009; Fig. 1.2).

1.4 Plant cell cycle

Cells reproduce themselves through the process of cell division cycle or cell cycle (Taiz and Zeiger, 2006). In plants, growth and development is achieved through regulated cell division, cell expansion and differentiation (De Veylder *et al*., 2003; Taiz and Zeiger, 2006; Brooker, 2009). The phases of the cell cycle and their molecular control mechanism are widely documented (Sorrell *et al*., 1999; Black and Azizkhan-Clifford, 1999; Meijer and Murray, 2000; Shen, 2002; Dewitte and Murray, 2003; Inzé and De Veylder, 2006; Brooker, 2009; Wayne *et al*., 2009; Fig. 1.2). According to these authors, decision for cells to resume division from a resting state (G0) or to continue division or to differentiate is made at the transition of Gap1 (G1) and synthesis (S) phase of the cell cycle. The initiation of the cell cycle at these points is regulated by the Cyclin Dependent Kinase (CDK)

proteins whose activity is modulated by the cyclin protein sub units. In the presence of CDK-activating kinases (CAKs), cyclins complexes with Cyclin Dependent Kinase (CDK) catalytic subunits to activate the phosphorylation process of the E2F transcription factor-retinoblastoma protein (E2F/Rb) dimers. Phosphorylated RBR is demobilized from promoter-bound Rb/E2Fa-DPa hetero-dimer transcription factor complex allowing the free E2Fa transcription factor to bind to the promoters of genes that are responsible for the S-phase entry phase. Among the transcribed genes are topo-isomerase that relaxes chromosomal strands, acetyltransferase responsible for the acetylation of the histone protein that causes chromatin relaxation and DNA polymerase (Vlieghe *et al*., 2003; Brooker, 2009). By contrast, the E2F-RBR pathway is inhibited by E2Fc through its competitive inhibiting the DPa subunit (Vannesta *et al.,* 2005). At the late S-phase and G2- to M-phase cyclins A- and B- types form complexes with CDKB, respectively (Soni *et al*., 1995; Mironov *at al*., 1999). The activated CDKB directly phosphorylate the histone proteins leading to chromosome condensation and the lamins that lead to disintegration of the nuclear membrane (Brooker, 2009). Cyclin D-type protein sub units effect intracellular transduction of signals as they pertain to the environment, mitogenic and nutrient status that influences the cell cycle progression (Weinert, 1998; Stals and Inzé 2001; Inzé, 2005; Inzé and De Veylder, 2006; Menges *at al*., 2007). Progression of the cell cycle is regulated so as to match cell division with the appropriate environment, to prevent replication of cells with damaged genomes and to regulate growth and development (Dewitte and Murray, 2003).



Overexpresssion of *CyclinD2;1*

Fig. 1.2 Eukaryote cell cycle. G1: Gap 1 phase; S: Synthesis phase; G2: Gap 2 phase; M: Mitosis phase. Cdk: Cyclin dependent kinase; ATP: Adenosine triphosphate; ADP: Adenosine monophosphate. Arrow indicates the intervention of this study. (Source: Wayne *et al*., 2009).

1.4.1 Classification of cyclins

From genome-wide analysis of Arabidopsis, rice and maize Cyclin A, B, C, D, F, H, J18, L, P, SDS, T, and U have been identified, with cyclins A, B, C, H and L being shared between the plants and animals (Wang *et al*., 2004; Hu *et al*., 2010). Cyclins A, B and D are the most important in mitotic cell division of plants (Reanudin *et al*., 1996; Wang *et al*., 2004). Cyclins contain a cyclin_N and cyclin_C domain; the cyclin_N domain that comprise the CDK binding site is more conserved (Wang *et al*., 2004; Hu *et al*., 2010).

1.4.2 Regulation of cyclins

Cyclins are regulated through synthesis and destruction. The transcription of cyclin A and B is induced by auxins, cytokinins and gibelleric acid (Stals and Inzé, 2001). Synthesis of cyclins D1, D2, D3 and D4 is induced by sucrose while cyclin D1 and D3 are stimulated by both sucrose and cytokinins (De Veylder *et al*., 1999; Riou-Khamlichi *et al*., 1999; Riou-Khamlichi *et al*., 2000; De Veylder *et al*., 2003). CyclinD3 is induced by auxin, cytokinin, gibberellins and brassinosteroids (Rossi and Vitoro, 2001). CyclinD2 is sequentially activated earlier than cyclinD3 in the cell cycle (Riou-Khamlichi *et al*., 2000; Masubelele *et al*., 2005). For their deregulation, cyclin A- and B- type contain a destruction box while some D- type cyclins have PEST motifs in their sequences that facilitate their degradation by ubiquitin-mediated proteolysis (Genschik *et al*., 1998; DeSalle and Pagano, 2001; Vandepoele *et al*., 2002).

Cell cycle progression can also be modulated by proteins that inhibit cyclin/CDK association or direct kinase activity. Inhibitor of cdc2 kinase (ICK1) or KIP related proteins (KRP) interfere with the formation of CDK/Cyclin complex (Oakafull *et al*., 2002, Stals *et al*., 2000). This inhibits the CDK kinase activity with a resultant reduction of cell division and decline in plant organ development (Wang *et al*., 2000; Barroco *et al*., 2006). In Arabidopsis, the two versions of CKI interact with CDKA (Wang *et al*., 1998) while the monocot ICKs/KPR inhibits both A- and D-type cyclin/CDK complexes (Coelho *et al*., 2005). Absisic acid (ABA) is reported to inhibit cell proliferation through upregulating

ICK1 transcription (Wang *et al*., 1998). Alternatively, Wee1 kinase has an inhibitory phosphorylation effect on the mitotic CDK activity that results into endoreduplication (Sun *et al*., 1999).

1.4.3 Role of the cell cycle in plant development

Plant developmental studies have correlated organ growth rate to the proliferation rate of the cells, their rate and duration of the expansion (MacAdam *et al*., 1989; Bultynck *et al*., 2003; Beemster *et al*., 2003; Beemster *et al*., 2005). The rate of cell production is determined by the meristem size and rate of cell division (Beemster and Baskin, 1998; Fiorani *et al*., 2000). Environmental stress factors have been shown to reduce plant growth through shortening the meristem size and prolonging the cell cycle duration (Granier *et al*., 2000; West *et al*., 2004), and by reducing cell division and expansion (Kavanova *et al*., 2006). The cell duration is prolonged by arresting the cell cycle at the G1 phase (Granier and Tardiue, 1999). On the contrary, the basis of enhanced growth of cyclinD transgenics has been attributed to shortened cell cycle duration and resultant increased rate of cell division (Cockroft *et al*., 2000).

1.4.4 Cyclin expression studies

Several over-expression studies of cyclins have been conducted to evaluate their effect on cell proliferation and plant development. Cases where the transgenes contributed to the phenotypes are summarized in Table 1.1. Over-expression of *CyclinB1;1* and *B1;2* enhanced cell proliferation that resulted into faster root growth in *Brassica* (Doerner *et al*., 1996) and in rice (Lee *et al*., 2003). In Arabidopsis, over-expression of *Arath;CyclinB1;2* induced cell division with a reduction in ploidy in trichomes (Schnittger *et al*., 2002). Tobacco BY-2 cells transformed with *CyclinD1* from *Antirrhinum majus* exhibited accelerated cell cycle (Koroleva *et al*., 2004). Upregulation of *Arath;CyclinD2;1* and *CyclinD3;1* in Arabidopsis increased cell proliferation but at the expense of differentiation (Dewitte *et al*., 2003). *Arath;CyclinD1;1* and *D2:1* increased cell proliferation in germinating Arabidopsis seeds with concomitant faster germination (Masubelele *et al*.,

2005). Similarly, over-expression of wheat *CycD2;1* in Arabidopsis resulted in increased cell proliferation and taller plants but the leaves were curled (Wang *et al*., 2006). In contrast, over-expression of Arabidopsis and tobacco *CycD2;1* resulted into plants with faster root and shoot growth, faster leaf emission and early flowering (Cockroft *et al*., 2000; Boucheron *et al*., 2005; Guo and Wang, 2008). Although the same cyclin enhanced shoot and root growth in rice *in vitro* (Oh *et al*., 2008), the effect was not evident in potted plants. Due to these contrasting reports, it is currently unpredictable if a particular overexpression construct of *cyclinD2* will yield growth stimulation in banana. Therefore, this study was conducted to evaluate the growth stimulating role by *cyclinD2* over-expression.

Banana plants reproduce continous through suckering consequently allowing flowering to happen on each mat throughout the year. This growth characteristic provides a continuous and stable source of food when the annual crops are out of production. And on the basis that productivity in banana is measured in terms of fruit weight per unit area per unit time (Stover, 2000; Hauser and Van Asten, 2010), accelerating banana plant growth and development will reduce the time a plant takes to flower, increase the rate of ratooning and offer more harvestable bunches in a year. Sweenen and De Langhe (1985) observed a positive relationship between growth, leaf emergence rates and early flowering in bananas. More practical, surveys carried out on banana production in Uganda (Karamura *et al*., 2004), Rwanda (Gaidashova *et al*., 2005) and Camerron (Efenden *et al*., 2003) identified the desire by farmers for early maturing banana cultivars. However, lack of a breeding scheme for faster growing bananas coupled with the low seed fertility and transfer of undesired characteristics of parental clones to hybrids preclude the application of the conventional crossing method to breed for quick maturing bananas. Given these limitations, it is imperative to evaluate genetic engineering of banana plants where a single trait can be introgressed while preserving other plant characteristic.

Table 1.1 Over-expression studies of cyclins and resultant phenotypes

Component	**Donor plant**	**Expressing plant**	**Phenotype modification**	**References**
CyclinB;1	*Arabidopsis thaliana*	*A. thaliana*	Enhanced root growth	Doerner *et al.*, 1996.
CyclinB2;2	Rice	Rice	Accelerated root growth	Lee *et al.*, 2003
CylinD2;1	*A. thaliana*	*Nicotiana tabacum*	Accelerated shoot and root growth *ex vitro*	Cockcroft *et al.*, 2000
CylinD2;1 and CycclinD3;1	*A. thaliana*	*N. tabacum*	Enhanced leaf production rate and plant height	Boucheron *et al.*, 2005
CylinD2;1	*Triticum aestivum*	*A. thaliana*	Retarded plant growth and curled plants	Wang *et al.*, 2006
CylinD2;1	*A. thaliana*	*Oryza sativa*	Enhanced shoot and root growth *in vitro.*	Oh *et al.*, 2008
CylinD3;1	*Arabidopsis thaliana*	*Arabidopsis thaliana*	Accelerated cell proliferation but low differentiation leading to retarded growth	Dewitte *et al.*, 2003
CylinD3;4	*N. tabacum*	*N. tabacum*	Accelerated growth *ex vitro*	Guo and Wang, 2008

1.5 Working hypothesis and objectives

Motivation to carry out the following study was based on results reported by Cockroft *et al*. (2000) that transformed tobacco plants over-expressing *CyclinD2;1* had accelerated rate of leaf initiation and stem growth rate attributed to shortened cell cycle duration and increased rate of cell division. It was therefore hypothesized that transformation of banana plants with a *Cyclin D2;1* gene would accelerate the cell cycle resulting in accelerated plant growth, and subsequently translate into shortened growth cycle of the banana crop. This would allow earlier fruit development and consequently earlier fruit harvesting that could offer more harvestable bunches in a year resulting in a significant economic benefit.

To find support for the set working hypothesis the following objectives were set to:

1. Isolate a *CyclinD2* gene from banana for over-expression of the coding sequence in a homologous host plant and characterize the isolated *CyclinD2* coding sequence to determine any homology with other *CyclinD*-type genes.
2. Transform banana plants with either the *Arabidopsis* or the *Musac;CyclinD2;1* transgene to generate transformed plants for phenotypic evaluation.
3. Determine the relative expression of the *Arabidopsis* and *Musac;CyclinD2;1* coding sequence in transformed banana plants allowing to relate transcription with phenotypic changes.
4. Evaluate growth and development of the transformed plants to determine if *CyclinD2;1* over-expression results in faster plant growth.

From this study, the first *CyclinD2;1* coding sequence was isolated from banana thus contribution towards assembling the banana genome information that is lacking. In addition, the structural characterization of the isolated sequence shed light on the genomic relationship of banana, other monocots and the model plants such as Arabidopsis. The thesis work also demonstrated the feasibility of transforming and overexpressing a *CyclinD2;1* gene in a more complex monocot species, banana, and provided support on the growth enhancement resulting from the *CyclinD2;1* transgenes in this crop.

CHAPTER TWO

ISOLATION OF A BANANA *CYCLIN D2; 1* GENE HOMOLOG

2.1 Introduction

Expression analyses of cyclins have shown their transcription abundance to coincide with active cell multiplication in cell suspension and plant organs (Soni *et al*., 1995; Dewitte *et al*., 2003; Jang *et al*., 2005). Subsequently, these stages have been targeted to isolate the respective genes (Hirt *et al*., 1992; Dahl *et al*., 1995; Magyar et al., 1997; Freeman *et al*., 2002). Since cyclins have a high conservation in their functional domains, such as the cyclin boxes (Renaudin *et al*., 1996; Wang *et al*., 2004; Menges *et al*., 2007), these functional domains have been used for isolating new plant cyclins. Initially, cyclin isolation relied on cDNA libraries that were screened using radioactively labeled probes generated with degenerated primers. These primers were designed within the conserved regions of characterized cyclins (Hata *et al*., 1991; Dhahl *et al*., 1995; Sorrel *et al*., 1999; Kvarnheden, *et al*., 2000; Freeman *et al*., 2003). Polymerase chain reaction (PCR) using degenerated primers have been recently used to amplify conserved regions of genes from reverse transcribed RNA sequences (Wang, *et al*., 2006; Primrose and Twyman, 2006). This method does not require construction of libraries or radioactive material and is also faster. Since all isolation techniques rely on conserved regions of the genes, they rarely generate full length sequences. As such, the flanking regions of the gene can be generated using the Rapid Amplification of cDNA Ends (RACE) technique (Frohman *et al*., 1988). Also when no introns exist in the targeted region, genome walking of uncloned genomic libraries has been used (Siebert *et al*., 1995; Rishi *et al*., 2004).

The objective of this study was to isolate a D-type cyclin from banana to carry out a sequence comparison with existing *cyclin* genes. Also the gene was isolated to be over-expressed in banana and to evaluate if over-expression changes the morphology of the banana plant. In general, *Musa* genomic information is still limited and existing data are in form of expressed sequence tags (ESTs) that are not related to plant development. In this part of the study, sequence information from other monocots was used to isolate a cyclinD from banana. Applying a combination of RT-PCR, RACE and genome walking a full genomic and cDNA of *cyclinD2;1* was isolated from an East African highland banana

(AAA) cultivar 'Nakasabira', a dessert (AAB) cultivar 'Sukalindiizi' and one of their progenitors, 'Calcutta 4' (*Musa acuminata* spp. *burmannicoides*).

2.2 Materials and Methods

2.2.1 RNA isolation

Due to the association of high transcription of *cyclins* with actively dividing cells, a highly proliferating embryogenic cell suspension of a Highland cooking banana ('Nakasabira'; *Musa* spp. AAA group) and of a dessert banana ('Sukalindiizi'; AAB group) were used as a source for cyclin cDNA. The cell suspension was established from callus that was initiated from young male flowers and was harvested six days after sub-culturing into a fresh MA2 liquid medium formulation according to Côte *et al*. (1996) supplemented with 1 mg/L 2,4-D. Two hundred micro liters of packed cell volume were transferred into a 2 ml Eppendorf tube and washed twice with sterile distilled water. The cells were frozen and ground using liquid nitrogen in a mortar with a pestle. Total RNA was isolated from 50 mg of the powder using the Nucleospin® plant kit (BD Biosciences Clontech) following the recommended protocol of the supplier. Similarly, total RNA was isolated from the shoot meristem of *in vitro* proliferating shoot tip cultures of one of the progenitors of bananas 'Calcutta-4' (*Musa acuminata*, AA, subspecies *burmannicoides*). The quality and concentration of the RNA was assessed on a 1% agarose gel and a spectrophotometer (Nanodrop®, ND 1000), respectively, hereafter the RNA was stored at -80^{o}C.

2.2.2 cDNA synthesis

The first strand cDNA was synthesized from 0.2 µg of the total RNA using the Improm-II™ Reverse Transcription system and by applying the recommended protocol of the supplier (Promega). Oligo dT15 (0.5µg) was annealed to the RNA at 70^{o}C for 5 min followed by chilling on ice for 5 min. Reverse transcription was performed in a 20µl reaction mixture containing 7.5 mM $MgCl_{2,}$ 0.215 mM dNTPs, 2.5 Units of reverse transcriptase and 10 Units of RNase inhibitor (RNasin). A PCR reaction was run for 5 min

at 25°C, 1 hr at 42°C, 15 min at 70°C and then the reaction was cooled at 4°C. The quality of cDNA was checked by using 1µl of cDNA in a PCR reaction with banana *Actin* specific primers BanActFw (5'-CTGGTGATGGTGTGAGCCAC-3') and BanActRv (5'-CAGGGCAACGTAGGCAAGCT-3') designed from *Musa Actin* (Genbank accessions AF285176 and AY904067) to give a 200 bp amplicon. PCR amplification was carried out for 3 min at 96°C followed by 35 cycles of 20 sec at 94°C, 20 sec at 58°C, 30 sec at 72°C and a final extension of DNA strands for 7 min at 72°C.

2.2.3 PCR reactions and purification

All PCR products were fractionated on 1% agarose gel using 1X TAE buffer. To enable viewing the amplification products, ethidium bromide was incorporated into the gel at 0.1µg/ml prior to casting. The stained gels were viewed under U.V.-light to identify amplification products and to isolate separated PCR products from the gel. Gel pictures were acquired with a photo documentation system.

2.2.4 Degenerated primer design

A pair of degenerated primers was designed using the consensus-degenerate hybrid oligonucleotide primers (CODEHOP) program (Rose *et al*., 1998) and based on the nucleotide sequence within the conserved region of the *cyclin* genes (Wang *et al*., 2004). The conserved region was obtained from a multiple alignment of monocot CycD2:1 amino acid sequences of *Zea mays* cultivar Mo17 (accession number AAL83926), *Triticum aestivum* (AAQ08041), *Oryza sativa* (EAZ43092) and *Saccharum officinarum* (AAV28532). Degenerated primers (5'-CGCCTCCATCCTGCTGtgygcngarga-3') and (5'-GAAGGAGCAGGGGTCacngcntgcat-3') produced a 600 bp amplicon. PCR was conducted with 3 µl cDNA in a 50 µl reaction mixture containing 1.5 mM $MgCl_2$, 0.2 mM dNTPs, 0.3 µM each of primer and 1 unit of Bio-Taq DNA polymerase enzyme. To increase the binding specificity of the primer, a touchdown PCR (Don *et al*., 1991) was applied. The PCR cycle program was 3 min at 95°C; 15 cycles of 45 sec at 94°C, 45 sec at

70°C with a reduction of 1°C per cycle, 1 min at 72°C; 25 cycles of 45 sec at 94°C, 45 sec at 58°C, 1 min at 72°C and a final extension of DNA strands for 7 min at 72°C.

2.2.5 Genome walking

To extend the flanking regions of the first fragment, primers were designed towards the ends of the first gene fragment. To obtain the 3' downstream region, forward gene specific primer MagwRv1 (5'-TCTGTCCCTGGATTTACAGGTTGGTG-3') and a gene specific nested primer MagwRv2 (5'-GAGAATGGAGCTTCTGGTGATGAG-3') were designed and used together with GeneRacer™ 3' primer (5'-GCTGTCAACGATACGCTACGTAA CG-3') and GeneRacer™ 3' nested primer (5'-CGCTACGTAACGGCATGACACAGTG-3'). The PCR reaction was conducted in a 25 µl reaction mixture with 3 µl of single strand cDNA and the PCR reaction components mentioned above. The only difference was that the concentration of the Generacer™ 3' primer was doubled (0.6 µM) but the gene specific primer maintained at 0.3 µM. The nested PCR reaction was conducted in a 50 µl reaction volume using 2 µl of the primary PCR product and the gene specific and Generacer™ 3' nested primer used at the same concentration of 0.3 µM. The PCR was performed for 4 min at 94°C followed by 5 cycles of 30 sec at 94°C, 30 sec at 70°C, 1.5 min at 72°C; 5 cycles of 30 sec at 94°C, 30 sec at 65°C, 1.5 min at 72°C; 25 cycles of 30 sec at 94°C, 30 sec at 60°C, 1.5 min at 72°C and a final extension of DNA strands for 10 min at 72°C.

A Blast search (Altschul *et al*., 1997) for the 5'-end of the *cyclin* gene indicated that no intron was to be expected. Thus, the upstream of the banana *cyclin* gene was generated by genome walking using restriction enzyme treated genomic DNA of the banana cultivar 'Nakasabira' (Section 1.3.6). The primary PCR was performed with gene specific primer MagwFw1 (5'-ATCTCTCCTGATGGAGGAGTCCAACG-3') and adapter specific primer AP1 (5'-GGATCCTAATACGACTCACTATAGGGC-3'). A nested PCR reaction was carried out with a gene specific primer MagwFw2 (GCTCTCTCGACCAACAAGCTCAA C) and a nested adapter specific primer AP2 (5'-AATAGGGCTCGAGCGGC-3'). The primary PCR reaction was conducted in a 25µl reaction mixture using gene specific primer1 and adapter primer1 with 2 µl of cut genomic DNA. The secondary PCR reaction

was carried out with 2 µl of the 100-times diluted first PCR amplification product using gene specific primer2 and adapter primer2. The PCR reaction included denaturing for 3 min at 94^{o}C; 10 cycles for 30 sec at 94^{o}C, 5 min at 68^{o}C, followed by 25 cycles of 20 sec at 94^{o}C, 5 min at 68^{o}C with 10 sec increment per cycle and a final DNA strand extension of 10 min at 68^{o}C.

2.2.6 Preparation of restriction enzyme-digested genomic DNA

Genomic DNA was extracted from the cigar leaf of the field-grown plants of the Highland banana cultivar 'Nakasabira' using the CTAB method (Ude *et al.*, 2002). DNA was purified with a CFX^{TM} DNA and gel band purification kit (Amersham Biosciences). Digestion and ligation of adapters were performed on 3.5 µg DNA following the protocol described by Siebert *et al* (1995). Briefly, the process involves digestion of the DNA for 18 hrs in separate 20 µl reaction mixtures containing either restriction enzymes *Pvu*II, *Sca*I or *StuI*. Reactions were terminated by heating for 20 min at 65^{o}C for enzyme *Sca*I and at 80^{o}C for *Pvu*II and *StuI*.

The digested DNA was precipitated by adding 20 µl of phenol/chloroform/isoamyl alcohol (25:24:1). This was followed by gentle vortexing of the mixture and centrifugation for 3 min at 13,000 rpm at room temperature. The top phase was transferred into a new Eppendorff tube to which 1/10 volume 3M NaOAc and 20 µg glycogen (Fermentas) were added. Two and half volume 95% ethanol was added, mixed by inverting the tube 6 times and the mixture was kept at -80^{o}C for 1 hr. The mixture was centrifuged for 15 min at 13,000 rpm and 4^{o}C. The recovered pellet was rinsed with 200µl of 70% ethanol and further precipitated by centrifugation for 5 min at 10,000 rpm and 4^{o}C. Ethanol was pipetted off and the pellet was vacuum-dried and suspended in 20 µl sterile distilled water. Adapter 1 (CTAATACGACTCACTATAGGGCTCGAGCGGCCGCCCGGGCAGGT) and adapter 2 (ACCTGCCC-NH_2) (Siebert *et al.*, 1995) were ligated to the blunt-ended digested DNA fragments. This involved mixing 5 µM of each adapter, heating the mixture at 95^{o}C for 3 min and allowing it to cool at room temperature. The adapter mixture was added to the DNA that had been relaxed by heating for 2 min at 50^{o}C and cooled on ice.

Three micro liters of ligase 10x buffer and 2 µg T4 DNA ligase (Roche Applied Science) were added to the mix and the final volume made to 30 µl with sterile distilled water. The ligation reaction was conducted at 20^{o}C for 17 hrs and terminated by heating at 65^{o}C for 10 min. The final volume was made to 100 µl and the adapter-ligated product stored at -20^{o}C.

2.2.7 DNA cloning

For cloning, PCR products were run on an agarose gel and the bands of interest were extracted from the gel, purified and cloned into the plasmid pCR®4-TOPO (Invitrogen). This involved ligating 30 ng DNA extracted from the gel with 10 ng of plasmid DNA in the presence of 1 µl NaCl in a 10 µl reaction mixture for 30 min at 25^{o}C. Ligated plasmid DNA was subsequently transformed into competent *Escherichia coli* cells using the heat shock method. This involved placing a 1.5 ml Eppendorf tube containing 50 µl of *E. coli* cells on ice for 30 min after which the TOPO vector was added, mixed gently and the mixture was maintained on ice for another 30 min. The Eppendorf tube containing the bacteria-plasmid mixture was immersed for 45 sec in a 42^{o}C water bath and immediately cooled on ice for 2 min. SOC medium (250 µl) was added to the cells and the mixture was incubated for 1 hr at 37^{o}C on a shaker (150 rpm). To select for transformed *E. coli* cells, 50 µl of the cell suspension was spread on a plate containing 25 ml Luria Bertani (LB) medium (g/liter: 10 tryptone, 5 yeast extract, 10 $NaCl_2$, 15 agar) supplemented with 50 µg/ml kanamycin. The plates were incubated for 17 hrs at 37^{o}C. Five bacterium colonies were selected and each inoculated in 5 ml of liquid LB in the presence of kanamycin and grown for 17 hrs under shaking at 200 rpm.

TOPO plasmid containing the cloned DNA insert was isolated from *E. coli* using the GeneJET^{TM} Plasmid miniprep kit (Fermentas Life Science). The presence of the insert was confirmed by restriction enzyme analysis digesting 1 µg of the plasmid with 1 unit of *Eco*R1 in a 20µl reaction mixture for 1 h at 37^{o}C.

2.2.8 DNA sequencing

After extraction from the gel, the PCR products were prepared for sequencing by running a sequencing PCR reaction in a 10 µl reaction mixture containing 400 ng TOPO plasmid DNA, 2 µl 5x Big-Dye buffer, 2 µl BigDye and 1 µM M13 forward or reverse primer. The PCR program comprised of 95^{o}C for 1 min, 30 cycles of 95^{o}C (10 sec), 52^{o}C (5 sec) and 60^{o}C (4 min). PCR products were purified with a 6.5% Sephadex® G-50 (Sigma cat # G5050-100G) column. PCR samples were loaded onto the centre of the Sephadex column and centrifuged at 2800 rpm for 2 min. The eluted products were dried in a concentrator and sequenced on an ABI377 DNA sequencer.

2.2.9 Isolation of *cyclin* sequences

Obtained sequences were used to search the nucleotide database of the National Center for Biotechnology (www.ncbi.nlm.nih.gov). The sequences that gave positive *cyclin* hits were identified and used in the ExPAsy online program for translation into amino acid sequences and checking for an uninterrupted open reading frame. The 5' end of the gene was determined by the ATG start codon while the TGA codon indicated the 3' termination. Based on the identified full nucleotide sequence, a forward primer (5'-ATGAGTCCAAGT TGTGACTGCG-3') and reverse primer (5'-TCATCTGGTTGTTTTCCTCCTCT-3') were designed to amplify the full cyclin coding region from a cDNA isolated from plants of cultivar 'Nakasabira'. This involved using 0.3 µM of primers and 1 Unit proof reading *pfu* Taq polymerase in a 50 µl reaction mixture containing 1.5 mM $MgCl_2$ and 0.2 mM dNTPs. The PCR program consisted of 94^{o}C (4 min), 35 cycles of 94^{o}C (30 sec), 60^{o}C (30 sec), 72^{o}C (1.5 min) and final extension of DNA strands 72^{o}C (10 min). The amplified PCR products were run on and isolated from a 1% agarose gel, cloned into the TOPO plasmid and *E. coli* cells were transformed with the plasmid. Because *pfu* Taq polymerase does not incorporate the 3'-terminal deoxyadenosine overhangs that facilitate ligation into the TOPO plasmid, an A-tailing reaction was performed on the PCR products. A 20 µl tailing reaction was conducted using 11 µl of gel extract to which 2 µl reaction buffer containing 1.5 mM $MgCl_2$, 0.1 mM dNTPs and 1.5 Units of Bio-Taq DNA polymerase enzyme was added. The

mixture was incubated for 10 min at 72^{o}C, immediately placed on ice and 4 µl of the reaction was ligated into the TOPO plasmid. The TOPO plasmid was purified from transformed *E. coli* cells and sequenced. The resultant sequences were aligned to obtain the consensus sequence. The same primers were used to amplify the corresponding coding sequences from cDNAs of plants of the banana cultivar 'Sukalindiizi' and from a diploid wild type 'Calcutta-4'. Furthermore, these primers were used to amplify the full genomic cyclin from genomic DNA of cultivar 'Nakasabira'.

2.2.10 Bioinformatics

Arabidopsis cyclin sequences were retrieved from the NCBI website (http://www.ncbi.nlm.nih.gov, Altschul *et al*., 1997). Rice cyclins were retrieved from the rice genome database (http://www.gramene.org/protein and http://www.iniprot.org), while the maize orthologs were downloaded from http://www.archieve.maizesequence.org. For tobacco and wheat cyclins and to verify the retrieved sequences, BLAST searches were performed on the GenBank of the NCBI website. PEST motifs in the protein were determined using the ePESTfind online program (http://www.EMBOSS.bioinformatics.nl/ cgi-bin/emboss/epestfind). The program was run at the default setting of five for the minimum number of amino acids between positive franks. To classify the isolated banana cyclin, phylogenetic analysis was performed using representative cyclin sequences from *Arabidopsis*, maize, rice, sugarcane, wheat, and a phylogram was constructed using Neighbor-Joining method with MEGA program version 3.1 (Kumar *et al*., 2004). The same MEGA program was employed to analyze the introns in the genes by aligning the genomic and cDNA sequences of the gene. Relatedness between the banana cyclin and *cyclinD2* of monocots and Arabidopsis was determined with ClustalW program (http://www.ebi.ac.uk/Tools/clustalw2/). Specificity of the isolated cyclin to banana was determined by perfrming a PCR reaction on genomic DNA of selected banana accessions using primers designed at the flanking regions of the cDNA. The accessions were Yalim (AA), Kahuti-Mshale (AA), Sukalindiizi (AAB), Ibwi (AAA), Mbwazirume (AAA), Gros Michel (AAA), their wild parents 'Calcutta-4' (*Musa acuminata* spp. *Burmannicoides*, AA), *M. balbisiana* (BB), *M. zebrina, M. malacensis* (AA), and wild relative Ensete.

2.3 Results

2.3.1 Cyclin isolation

Polymerase chain reaction (PCR) with degenerated primers generated a 586 bp amplicon (Fig. 2.1C-i). When the derived sequence was blasted against the NCBI Genbank, the sequence showed homology to N-termini of cyclin D2 type proteins. Thus, the 5' end of the gene was obtained by genome walking using digested genomic DNA generated by restriction with enzymes *ScaI* and *PvuII*. Application of a nested PCR reaction using primers MagwFw2 and adapter specific primer AP2 yielded in 400 bp fragments from cut DNA (Fig. 2.1C-ii) that comprised of 196 bases upstream the ATG start codon. For nested PCR, products from a primary PCR reaction using primers MagwFw1 and adapter specific primer AP1, which gave a smear on the gel, were diluted 100-times for the nested reaction. When this sequence was translated with the Expasy program it yielded a full N-terminus of the gene. The 3'-RACE PCR generated a 531 bp fragment (Fig. 2.1C-iii). Blast search with this sequence gave very strong hits of the cyclinD C-terminus. When the three fragments were joined (Fig. 2.1B) and submitted to the Expasy program for transformation, an open reading frame (ORF) with ATG and TGA as start and stop codon, respectively, was generated. Finally, primers that were designed at the 5' and 3' termini picked a full-length ORF of 1035 bp from cDNA (Fig. 2.2). The nucleotide sequence had a CG and AT content of 49 and 51%, respectively, an open reading frame of 345 amino acids and a protein size of 629,017 Daltons. A similar product was amplified using the same primer pair on cDNA from proliferating cell suspension of banana cultivar 'Sukalindiizi' (AAB) and shoot meristematic tissue of a wild diploid banana 'Calcutta-4' (AA).

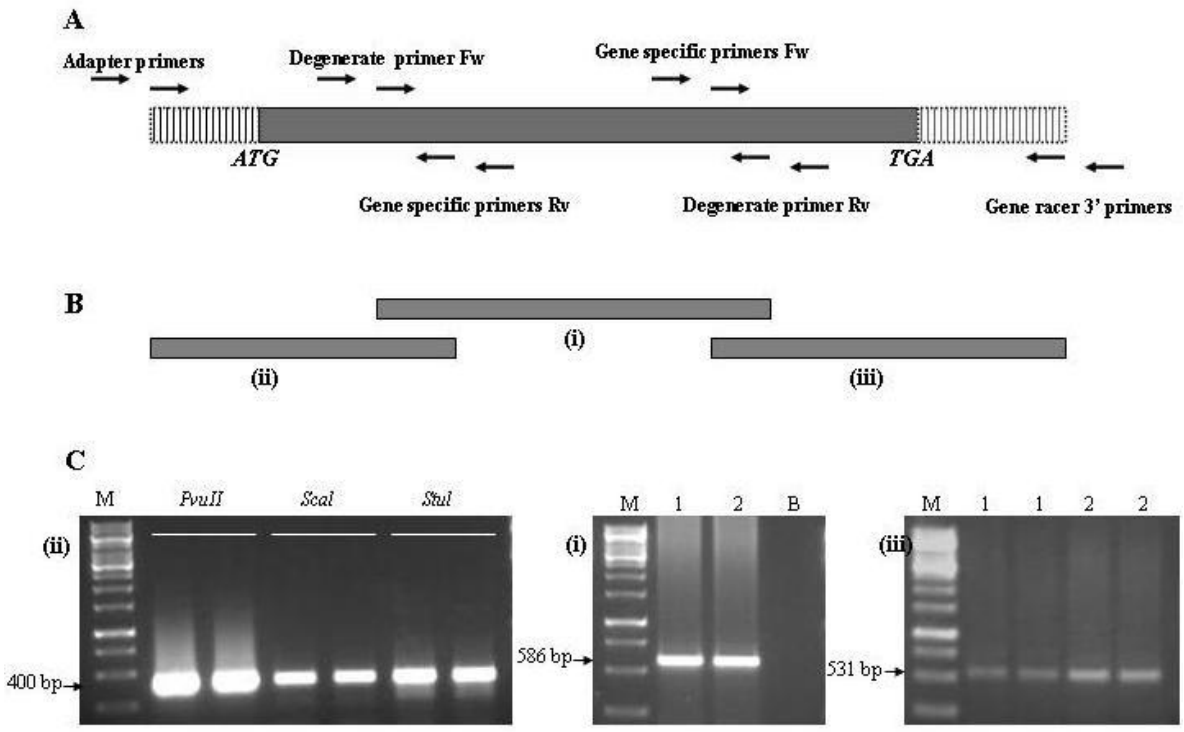


Fig. 2.1 Isolation of *CyclinD2;1* gene homolog from banana using PCR, RACE and genome walking. (A) Schematic overview of the primer positions. (B): (i) sequence of gene isolation where gene segments were amplified from the conserved region of the gene using degenerated primers and cDNA; (ii) 3' end of the gene generated by 3' RACE using forward primers designed in the first fragment together with Generacer 3' primers; (iii) 5' extension of the gene amplified through genome walking of genomic DNA using reverse gene specific and adapter primers. (C) Representative agarose gels for the respective steps: PCR products for (i), RACE secondary PCR reaction for (iii) and genome walk secondary PCR for (ii). M: 1Kb DNA ladder (GeneRuler™), 1 and are PCR samples, genome libraries were prepared with restriction enzymes *PVuII*, *ScaI* and *StuI*.

2.3.2 Molecular characterization of the coding sequence

Predicted amino acid residues of the three banana genotypes were 100% similar. Domain analysis of the protein established 134 and 87 amino acid of the cyclin_N and cyclin_C domains (Wang *et al*., 2004; Menges *et al*., 2007), respectively (Fig. 2.2). Furthermore, the banana *cyclin* gene was characterized by LxCxE motif (where x is any amino acid residue, i.e. LLCAE) and a retinoblastoma (RB) related protein binding motif (Fig. 2.3; Wang *et al*., 2004; Menges *et al*., 2007). The LxCxE motif was similar to that of maize, sugarcane and wheat but different from the *Arabidopsis* (LACGE) and tobacco (LLCTE). A unique motif, IWKVHAHY, was also identified within the cyclin N_domain (Fig. 2.2). In addition, the N_domain had the D, K, L and E residues that are highly conserved and required for cyclin function (Menges *et al*., 2007). One PEST motif was identified at amino acid position 1 to 33 (PESTFIND score of +2.13; Fig 2.2). PEST motifs are signals for rapid protein degradation and are an integral part of some cyclins (Renaudin *et al*., 1996; Wang *et al*., 2004). PEST motifs are characterized by a stretch of at least twelve negatively charged amino acids mainly proline (P), glutamic acid (E), serine (S), threonine (T), and aspartic acid (D) relative to the positively charged ones (Rechsteiner and Rogers, 1996). Based on the proportion of these amino acids to the positively charged residues, an algorithm computes the PEST score with a threshold of 5.0, below which the score is considered poor.

Phylogenic analysis between the full *Musa* cDNA amino acids sequences and the D-type cyclins from other plant species placed the *Musa cyclin* gene in a monocot specific clade (Fig. 2.4). Within this clade, the *Musa cyclin* had a 17% bootstrap support. At amino acid sequence levels, *Musac;CYCD2;1* was 40% identical to Arabidopsis, 54% to rice, 57% to maize, 54% to sugar cane, 57% to wheat cyclinD2.

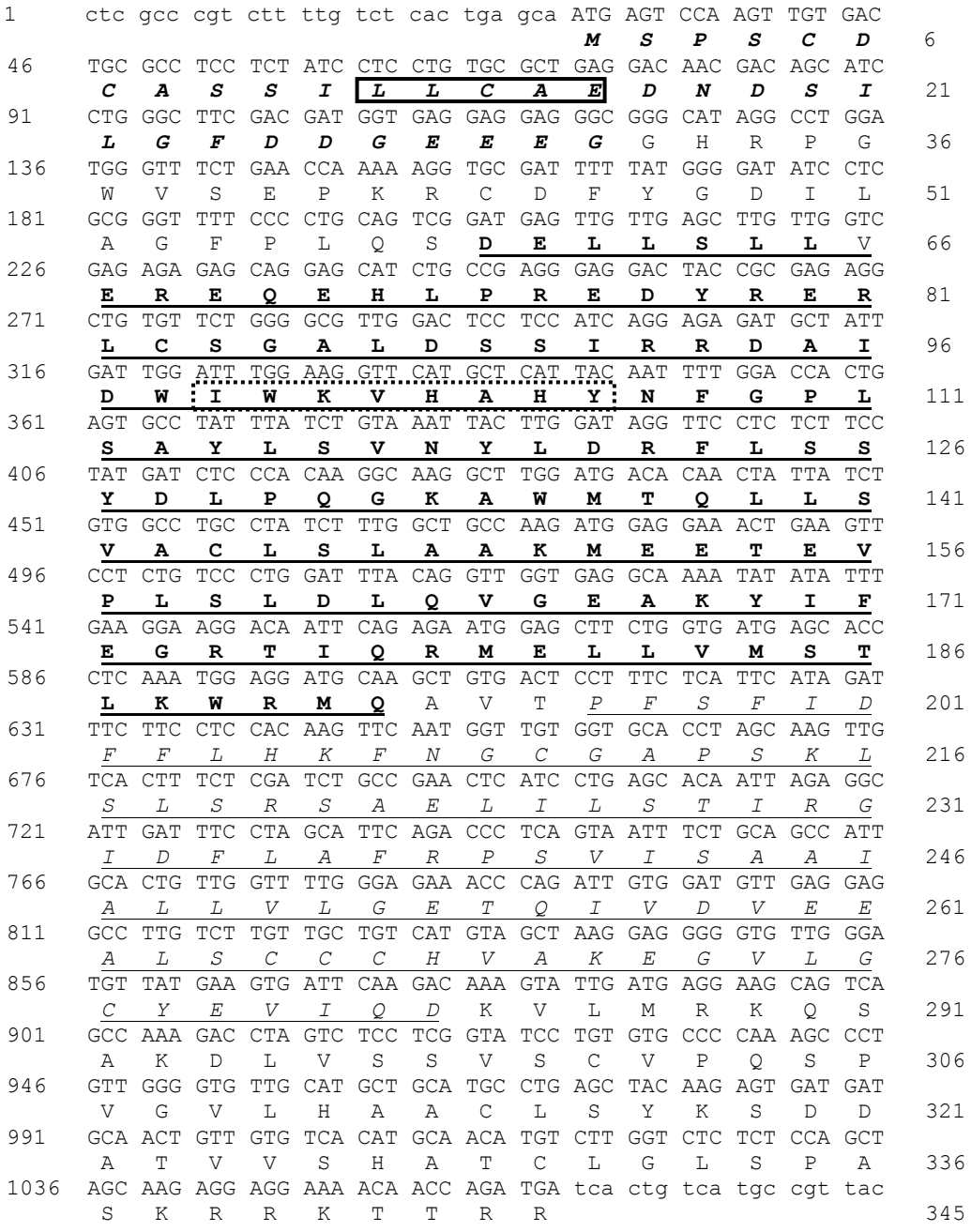


Fig. 2.2 Nucleotide and deduced amino acid sequences of *Musac;CyclinD2;1* cDNA. The PEST motif (amino acids 1 to 33) is bolded and in italics; retinoblastoma (RB) related protein binding motif LxCxE is boxed in solid; cyclin N_domain (134 amino acids) is bolded and underlined; Cyclin C_domain (87 amino acids) is in italics and underlined; signature motif is boxed in dotted line. Nucleotides are numbered on left and amino acid residues on the right.

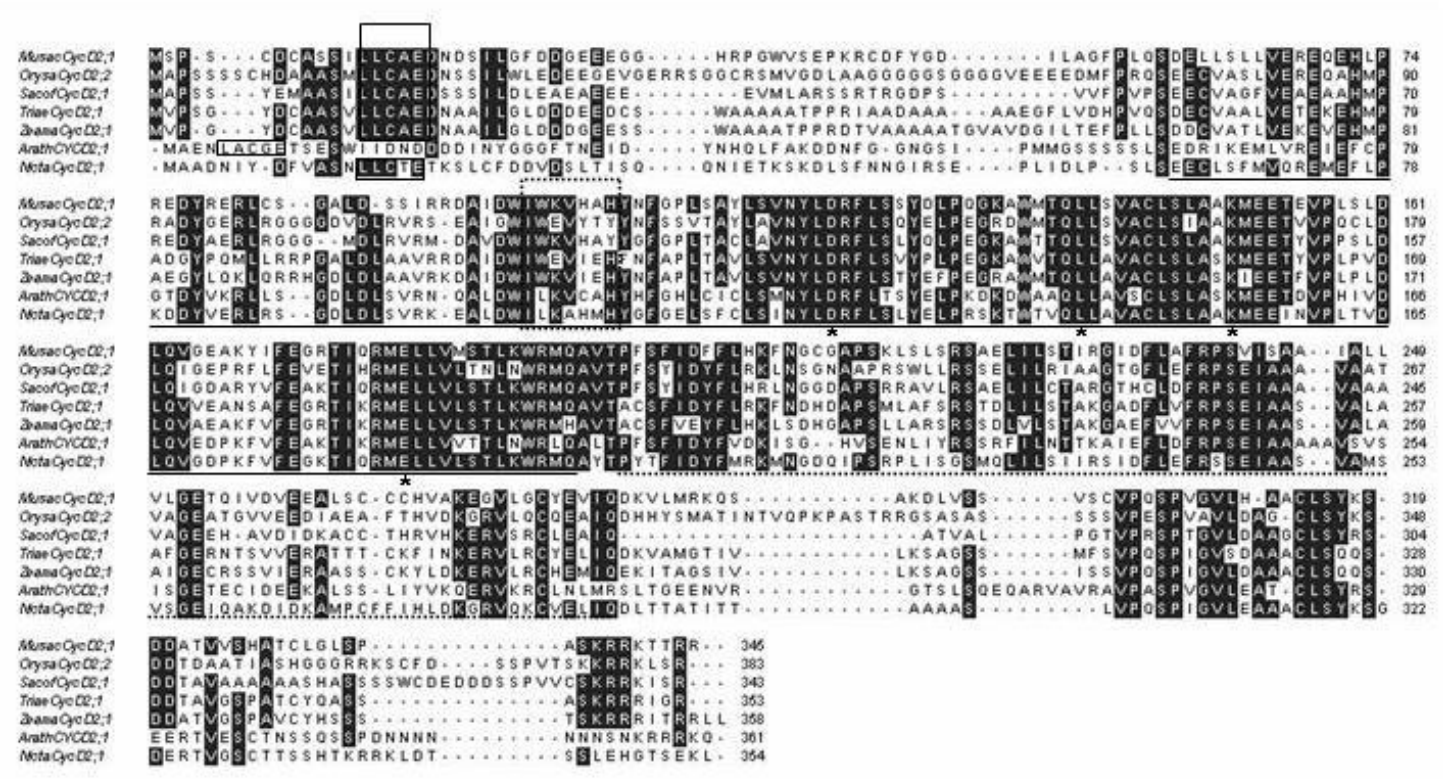


Fig. 2.3 Multiple alignment of *cyclinD2;1* amino acid sequence of banana with Arabidopsis (Arath, *Arabidopsis thaliana*), tobacco (Nicta, *Nicotiana tabacum*), rice (Orysa, *Oryza sativa*), sugarcane (Sacof, *Saccharum officinarum*), maize (Zeama, *Zea mays*) and wheat (Triae, *Triticum aestivum*). The LxCxE is presented in banana as LLCAE and similar to maize, sugarcane and wheat. The cyclin N_domain is underlined (solid line) and the C_domain with a dotted line; cyclin signature motifs are boxed (doted line), where IWKVHAHY is specific to banana. Four conserved residues required for cyclin function are underscored with stars. Dashes indicate gaps required to maximally align the sequences.

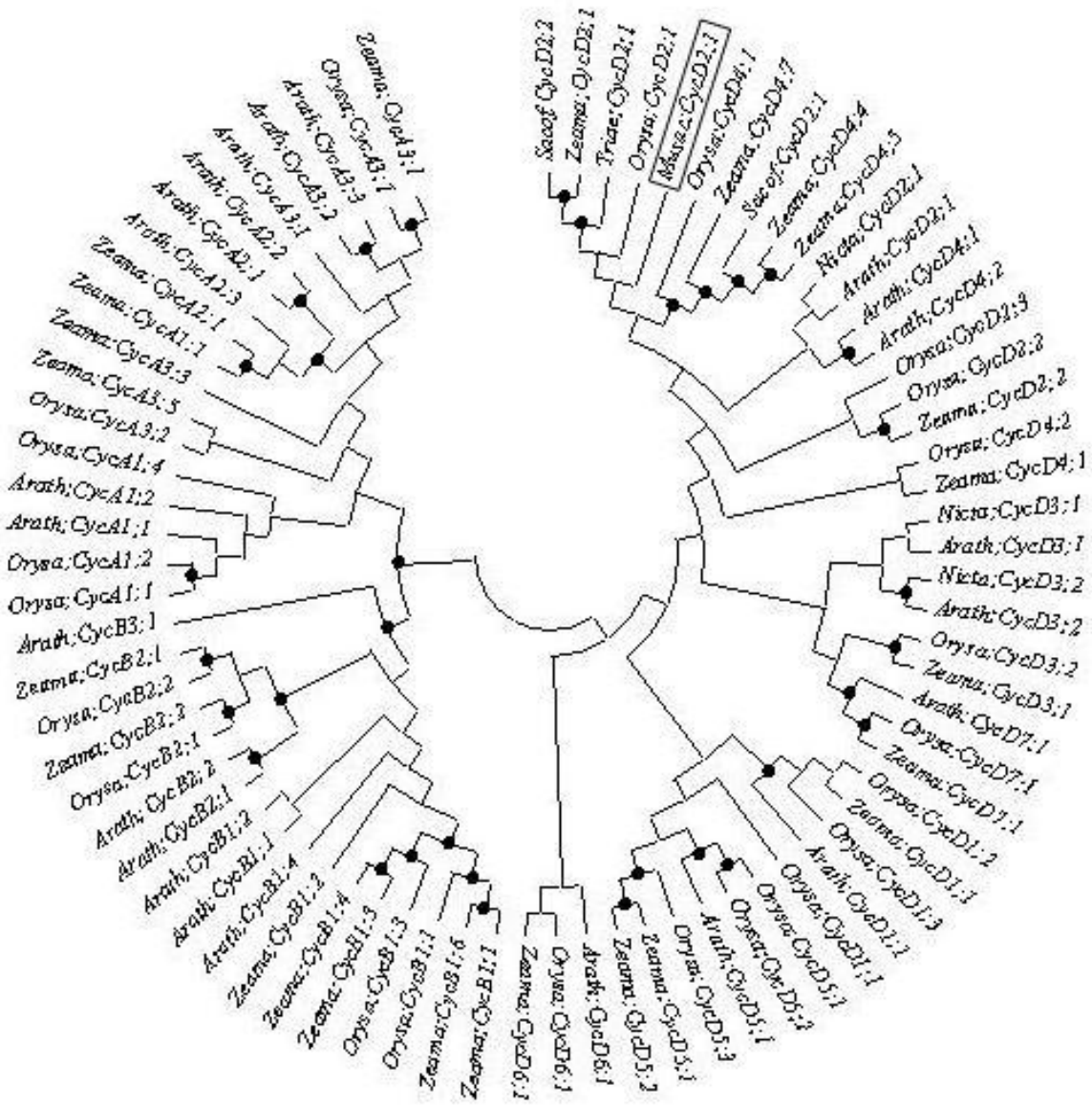


Fig. 2.4 Phylogenic relationship between *Musac;CyclinD2;1* (boxed) and other plant cyclins. Amino acid sequences were aligned using ClustalW program and the phylogram was constructed using the neighbour-joining method. Names are presented by the first three letters of the genus and two of species: Arath, *Arabidopsis thaliana*; Musac, *Musa acuminata*; Nicta, *Nicotiana tabacum*; Orysa, *Oryza sativa*; Sacof, *Saccharum officinarum*; Triae, *Triticum aestivum*; Zeama, *Zea mays*. Nodes with bootstrap values above 70 are shown and are indicated as bullets.

2.3.3 Molecular characterization of the genomic sequence

To test the specificity of the coding sequence of this cyclin homolog to *Musa*, a PCR reaction was conducted with gene specific primers designed for the flanking ends of the ORF. Genomic DNA from eleven banana accessions, their wild relative Calcutta-4, and wild relative, Enset was used. An amplicon of 2033 bp was generated that represented a full-length genomic *CyclinD* gene (Fig. 2.5). Alignment of genomic DNA of cultivar 'Nakasabira' and its cDNA and the cDNA from 'Sukalindiizi' and 'Calcutta-4' revealed 6 exons of length: 1, 304 bp; 2, 86 bp; 3, 101 bp; 4, 201 bp; 5, 122 bp; 6, 221 bp (Table 2.1). The introns were respectively spliced at GG/CG-GG/TT, CA/GT-AG/CA, GT/GA-GT/TG, GG/TA-GG/CA and GG/TA-GG/AG sites.

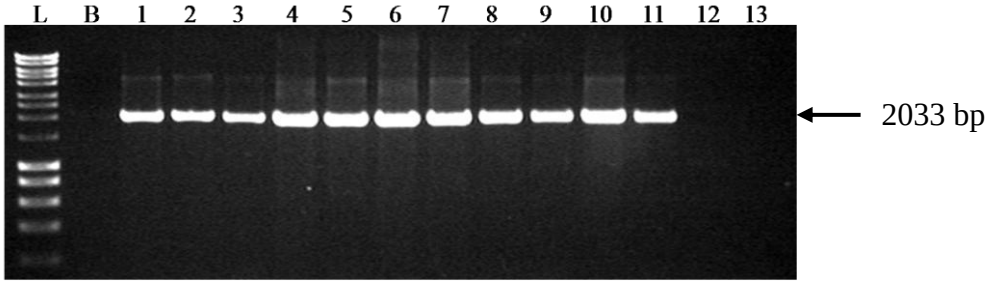


Fig. 2.5 Presence of *Musac;CyclinD2;1* coding region in selected *Musa* accessions after PCR reaction with genomic DNA. L: 200 bp HyperLadder™ (Bioline); B: Buffer; 1: Calcutta-4 (AA); 2: *Musa balbisiana (BB);* 3: *Zebrina;* 4: *M. ornata;* 5: *M. malaccensis (AA);* 6: Yalim (AA); 7: Kahuti (Mshale-AAA); 8: Ibwi (AAA); 9: Nakasabira (AAA); 10: Gros Michel (AAA); 11: Enset; 12: Maize; 13: Tobacco.

Table 2.1 Banana *CyclinD2;1* gene structure represented by size and composition of the exons and intron, and splicing sites.

Segment	Size (bp)	CG (%)	AT (%)	Splicing site
Exon 1	304	56	44	
Intron 1	534	33	67	GG/CG---GG/TT
Exon 2	123	40	60	
Intron 2	86	31	69	CA/GT---AG/CA
Exon 3	84	49	51	
Intron 3	101	36	64	GT/GA---GT/TG
Exon 4	99	44	56	
Intron 4	201	43	57	GG/TA---GC/CA
Exon 5	158	46	54	
Intron 5	122	32	68	GG/TA---GG/AG
Exon 6	221	49	51	

2.4 Discussion

A banana *CylinD* gene was isolated through a combination of genome walking and the 3' RACE technique. To our knowledge, this is the first cell cycle gene to be isolated in banana. The gene sequence isolated has a cyclin core of N and C domains constituting the binding site for the cyclin-dependent kinases (CDK) (Sorrell *et al*., 1999; Wang *et al*., 2004; Menges *et al*., 2007). The LxCxE protein sequence (in the form of LLCAE) at the N-terminal of the protein is essential for the cyclinD-associated kinase to bind and phosphorylate the retinoblastoma-related (RBR) protein. The LLCAE sequence is characteristic of cyclinD2 types (Sorrell *et al*., 1999; Menges *et al*., 2007) and this feature places the isolated banana cyclin into the D2-group. Further evidence for placing the banana cyclin into the D group is the six exons were identified in the isolated banana *cyclin*. This gene structure is in agreement with observations by Menges *et al*. (2007) on *cyclinD* genes in representative members of the angiosperm. Phylogenic analysis also placed the banana cyclin close to monocot cyclin D2 and D4, although comparison of the overall banana genome structure and organization placed banana closer to Arabidopsis than to rice (Dickman, 2004).

PCR further confirmed the presence of the gene in eleven banana accessions and their wild Enset relative (Fig. 2.6). According to the classification of banana (Simmonds, 1962; Stover and Simmonds, 1987), *Musa ornata* belongs to the section Rhodochlamys, while the rest of the bananas tested here belong to the section Eumusa; the two sections are of the genus *Musa.* Likewise, Enset is a member of the genus Ensete, which together with the genus *Musa* belongs to the family Musaceae. Therefore, the presence of amplification from banana and Enset further suggests that the detected cyclin sequence is conserved across the Musaceae family. Future sequencing the product from the distant banana relatives will further confirm this.

The *cyclin* gene is the first to be isolated from banana. Since the sequenced DNA was extracted from bananas of AA, AAA and AAB genomes, where the acuminata genome dominates, it can be denoted *Musa acuminata;Cyclin D2;1* or *Musac;CyclinD2;1* according

to the guidelines of the Commission on Plant Gene Nomenclature (Renaudin *et al*., 1996). The IWKVHAHY sequence found within the cyclin N-domain is so far unique for banana. This motif can be considered as a signature motif for the isolated banana cyclin, since it is variable and species-specific (Wang *et al*., 2004), Further, the PEST region that was identified had a low score. This low score has been reported in the majority of the CyclinD-type proteins (Sorrell *et al*., 1999; Wang *et al*., 2004; Menges *et al*., 2007). For the degradation of such proteins, Rechsteiner and Rogers (1996) proposed the phosphorylation of the serines and threonines as a possible mechanism for activating their latent PEST signals.

With regards to the splicing sites recognized in mRNA processing, only one *cyclin* intron out of the five had the conventional splicing border composition of AG---GU at the 5' and 3' ends reported for plants and vertebrates (Brown and Simpson, 1998; Reddy, 2001). A random sample in Genbank on genes that have been submitted as genomic DNA or cDNA identified this non-conformity to range between 50 and 100% in the Arabidopsis, popular and rice *cyclins* as well as banana *actins*.

In conclusion, a *Musac;CyclinD2;1* gene ortholog was isolated from banana, with higher sequence identity to monocot plants than *Arabidopsis*. This is the first cyclin to be isolated from banana and the sequence can contribute to the ongoing banana genome work (www.musagenomics.org). The sequence was deposited into a public database as *Musac;CYCD2;1* (GenBank accession number HQ839770). In chapter three an expression vector was constructed to facilitate the over-expression of this cyclin and in chapter five the resultant effect on banana plant growth is presented.

CHAPTER THREE

DESIGNING *CYCLIN* GENE CONSTRUCTS AND TRANSFORMATION OF BANANA

Part of this chapter has been published as: Talengera *et al*. 2010. Transformation of banana (*Musa* spp.) with a D-type cyclin gene from *Arabidopsis thaliana* (*Arath;CYCD2;1*). *Aspects of Applied Biology* 96:45-53.

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 mosaics 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 maker 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'-GGGGACAACTTTGT ATAGAAAAGTTGTGCAGCGTGACCCGGTCGT-3') and ATTB1Rv (5'-GGGGACTG CTTTTTTGTACAAACTTGAGAGGGTGTGGAGGGGGT-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. *Att*B 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). *Att*B 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 $MgCl_2$, 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^{o}C, followed by 35 cycles of

30 sec at 94^{o}C, 30 sec at 55^{o}C, 1 min at 68^{o}C and a final extension of DNA strands for 7 min at 72^{o}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^{o}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 $NaCl_2$, 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 plamids 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^{o}C. The reaction was stopped by addition of 1 µl of proteinase K and incubating the mixture for 10 min at 37^{o}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^{o}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^{o}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^{o}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^{o}C. This was followed by centrifugation for 15 min at 4^{o}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^{o}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^{o}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^{o}C after which the reaction was terminated by heating the reaction mixture for 10 min at 65^{o}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'-TCATCTGGTTGTTTTC CTCCTCT-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^{o}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^{o}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^{o}C and shaking at150 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^{o}C. Single colonies were inoculated into 5 ml of liquid LB medium and cultured for 16 hrs at 37^{o}C under 150 rpm shaking. Plasmids were isolated using the GeneJET^{TM} 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 µl competent cells from a -80^{o}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^{o}C) SOC medium (250 µl) was added and the mixture incubated for 4 hrs at 28^{o}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 µg/ml rifampicin and 250 µg/ml carbenicillin to select for transformed Agrobacterium cells. In addition, 100 µg/ml streptomycin and 300 µg/ml spectinomycin was used for the *Arabidopsis CYCD2;1* expression vector, and 100 µg/ml kanamycin for the *Arabidopsis CYCD2;1* expression vector. Cultures were incubated for 3 days at 28^{o}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^{o}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^{o}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 phytagel. The pH was adjusted to 5.8 before autoclaving the medium. Cultures were sealed with parafilm and incubated in the dark at 28^{o}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^{o}C. A single colony was inoculated into 25 ml of liquid YM medium and incubated at 28^{o}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^{o}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^{o}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^{o}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% Na_2SO_3 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^{o}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^{o}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^{o}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% Na_2SO_3 as an antioxidant pre-warmed to 65^{o}C was added and the mixture was thoroughly inverted ten-times. The suspension was incubated at 65^{o}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^{o}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 $MgCl_2$, 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 *ArabidopsiscycD2;1* coding sequence was performed with primers (5'-GCAAGCTCTAACTCCATTCTCC-3') and (5'-CCTGCTCCTGCGATAAACTA-3'). The PCR cycling program consisted of 3 min at 95^{o}C; 35 cycles of 30 sec at 94^{o}C, 30 sec at 60^{o}C, 30 sec at 72^{o}C and a final extension of DNA strands with 7 min at 72^{o}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 CTGGTGATTTCAGC-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^{o}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^{o}C that was calculated from the equation: Tm = 49.82 + 0.41 (% G+C) – (600/l) [where l = length of hybrid in base pairs] and T opt = Tm - (20 to 25^{o}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.

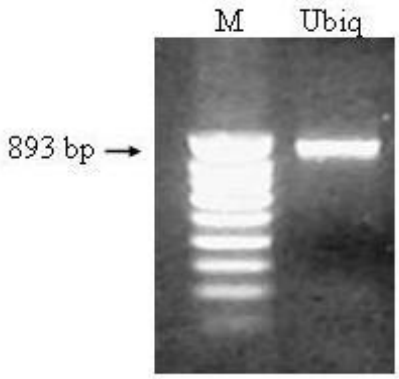
3.3 Results

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

TGCAGCGTGACCCGGTCGTGCCCCTCTCTAGAGATAATGAGCATTGCATGTCTAAGT
TATAAAAAATTACCACATATTTTTTTTGTCACACTTGTTTGAAGTGCAGTTTATCTA
TCTTTATACATATATTTAAACTTTACTCTACGAATAATATAATCTATAGTACTACAA
TAATATCAGTGTTTTAGAGAATCATATAAATGAACAGTTAGACATGGTCTAAAGGAC
AATTGAGTATTTTGACAACAGGACTCTACAGTTTTATCTTTTTAGTGTGCATGTGTT
CTCCTTTTTTTTTGCAAATAGCTTCACCTATATAATACTTCATCCATTTTATTAGTA
CATCCATTTAGGGTTTAGGGTTAATGGTTTTTATAGACTAATTTTTTTAGTACATCT
ATTTTATTCTATTTTAGCCTCTAAATTAAGAAAACTAAAACTCTATTTTAGTTTTTT
TATTTAATAATTTAGATATAAAATAGAATAAAATAAAGTGACTAAAAATTAAACAAA
TACCCTTTAAGAAATTAAAAAAACTAAGGAAACATTTTTCTTGTTTCGAGTAGATAA
TGCCAGCCTGTTAAACGCCGTCGACGAGTCTAACGGACACCAACCAGCGAACCAGCA
GCGTCGCGTCGGGCCAAGCGAAGCAGACGGCACGGCATCTCTGTCGCTGCCTCTGGA
CCCCTCTCGAGAGTTCCGCTCCACCGTTGGACTTGCTCCGCTGTCGGCATCCAGAAA
TTGCGTGGCGGAGCGGCAGACGTGAGCCGGCACGGCAGGCGGCCTCCTCCTCCTCTC
ACGGCACGGCAGCTACGGGGGATTCCTTTCCCACCGCTCCTTCGCTTTCCCTTCCTC
GCCCGCCGTAATAAATAGACACCCCCTCCACACCCTCT

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.

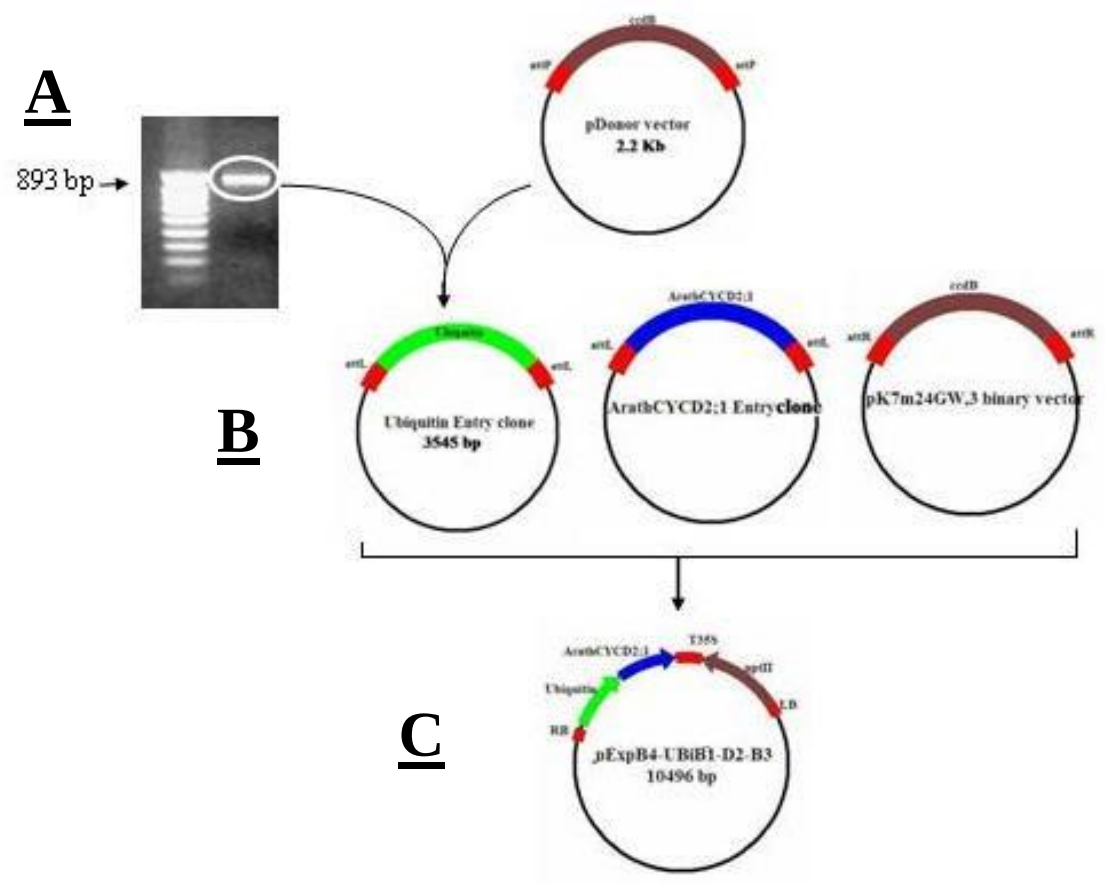


Fig. 3.2 Diagrammatical representation of the construction of the *Arabidopsis;CYCD2;1* expression vector using the Gateway™ cloning system. (A) ubiquitin promoter isolated from maize by PCR with primers containing *attB* site and ligated into pDONR vector by PB recombination reaction; (B) combination 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.

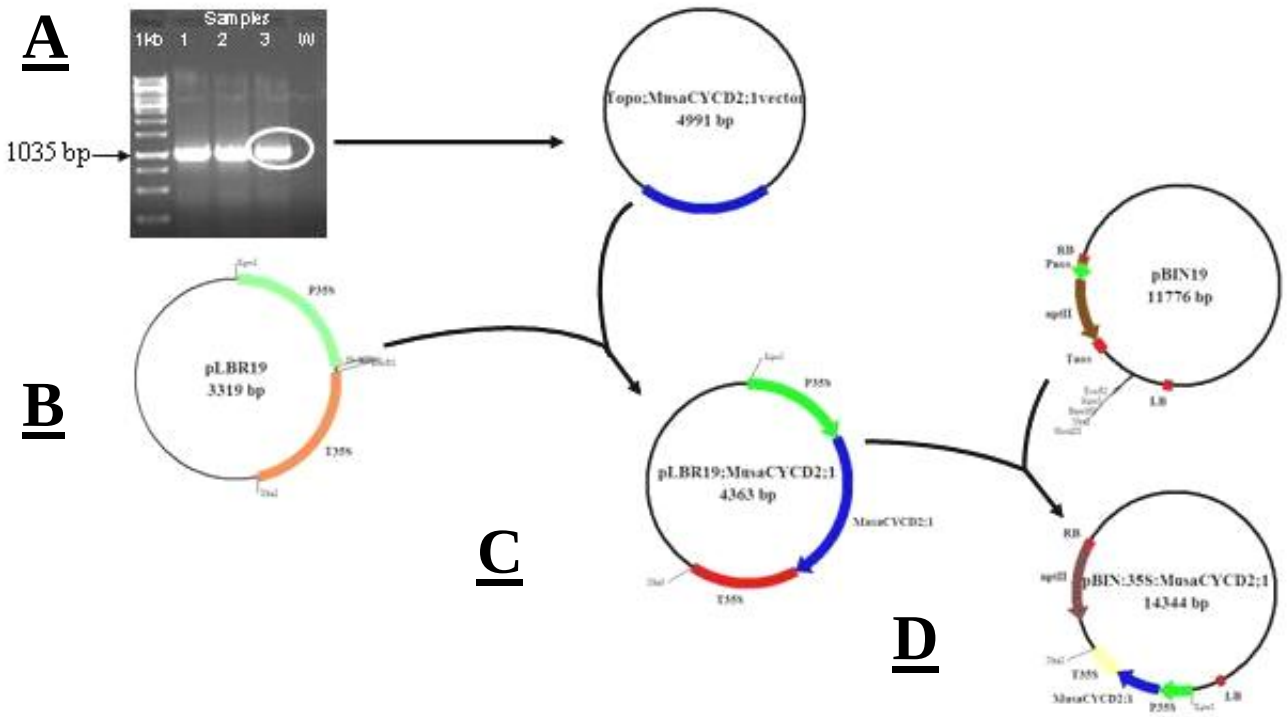


Fig. 3.3 Diagrammatical representation of the construction of the *Musac;CyclinD2;1* expression vector. (A) *Musac;CyclinD2;1* cDNA cloned into TOPO vector; (B) cDNA cloned between the 35S promoter and terminator of pLBR19 vector; (C) construct ligated into multiple cloning sites of expression vector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

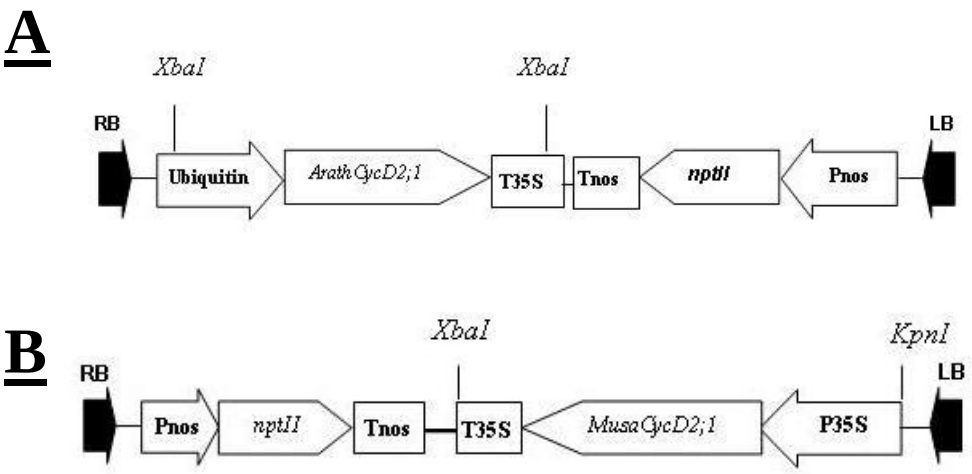


Fig. 3.4 (A) *Arath;CyclinD2;1* gene cassette in expression vector pExpression B4-UBiB1-D2-B3 vector. (B) *Musac;CyclinD2;1* gene cassette in expression vector pBIN:35S:*Musac;CyclinD2;1*. Restriction sites for gene insertion verification are indicated.

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).

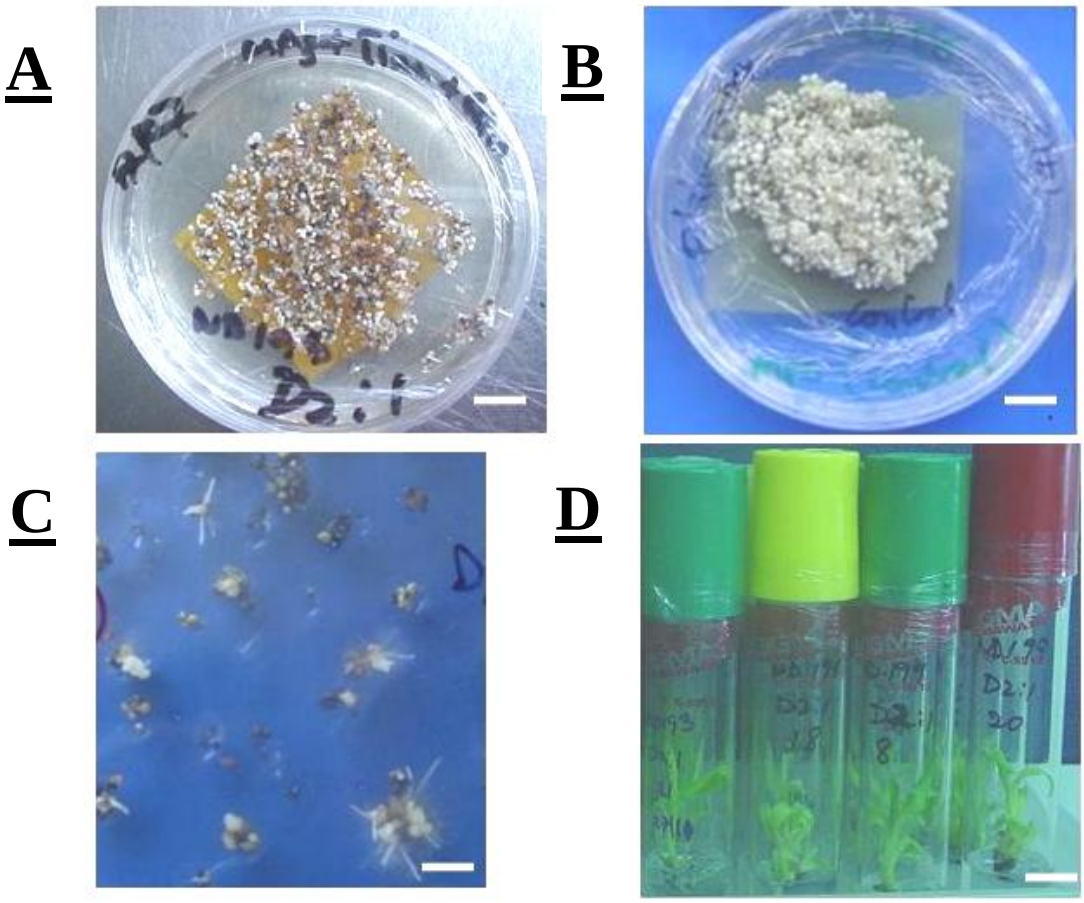


Fig. 3.5 Response of banana cells transformed with *Arath;CyclinD2;1* gene. (A) selection and regeneration of transformed cells, 8 weeks on selection medium, bar = 1.5 cm; (B) regeneration of non transformed cells, bar = 1.3 cm; (C) germinating embryos, bar = 0.8 mm; (D) rooted regenerants = 1.5 cm.

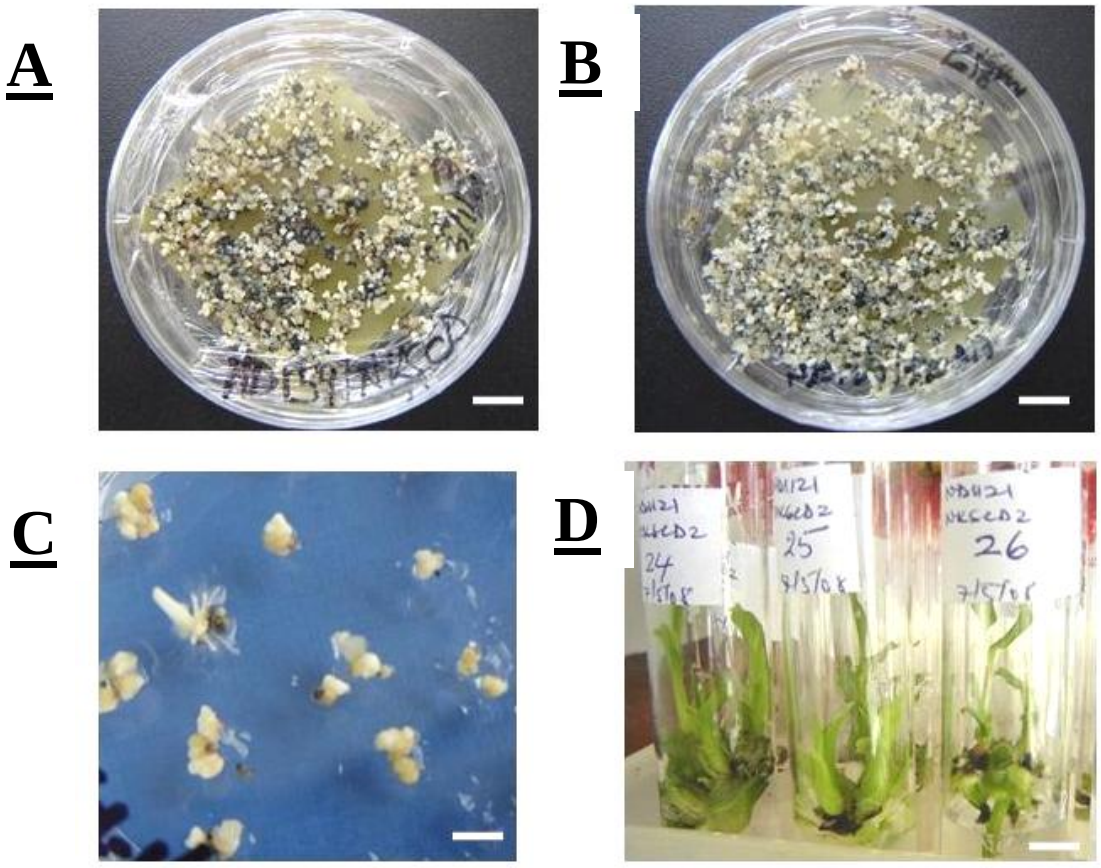


Fig. 3.6 Response of banana cells transformed with *Musac;CyclinD2;1* gene. (A) selection and regeneration of *Musac;CyclinD2;1* transformed cells, 8 wks on selection medium, bar = 1.5 cm; (B) selection of regeneration of cells transformed with empty vector pBIN19, 8 wks on selection medium, bar = 1.5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

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

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

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

Construct	Clones plated	Shoots	Regeneration frequency (%)
Musac;CYCD2;1	250	36	14.1 ± 1.7
pBIN19 (Control)	250	17	6.8 ± 0.8
T-test (*P* 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	*Arath;D2:1*	47	24	51.1
S. Ndizi: line 195	*Musac;D2;1*	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.

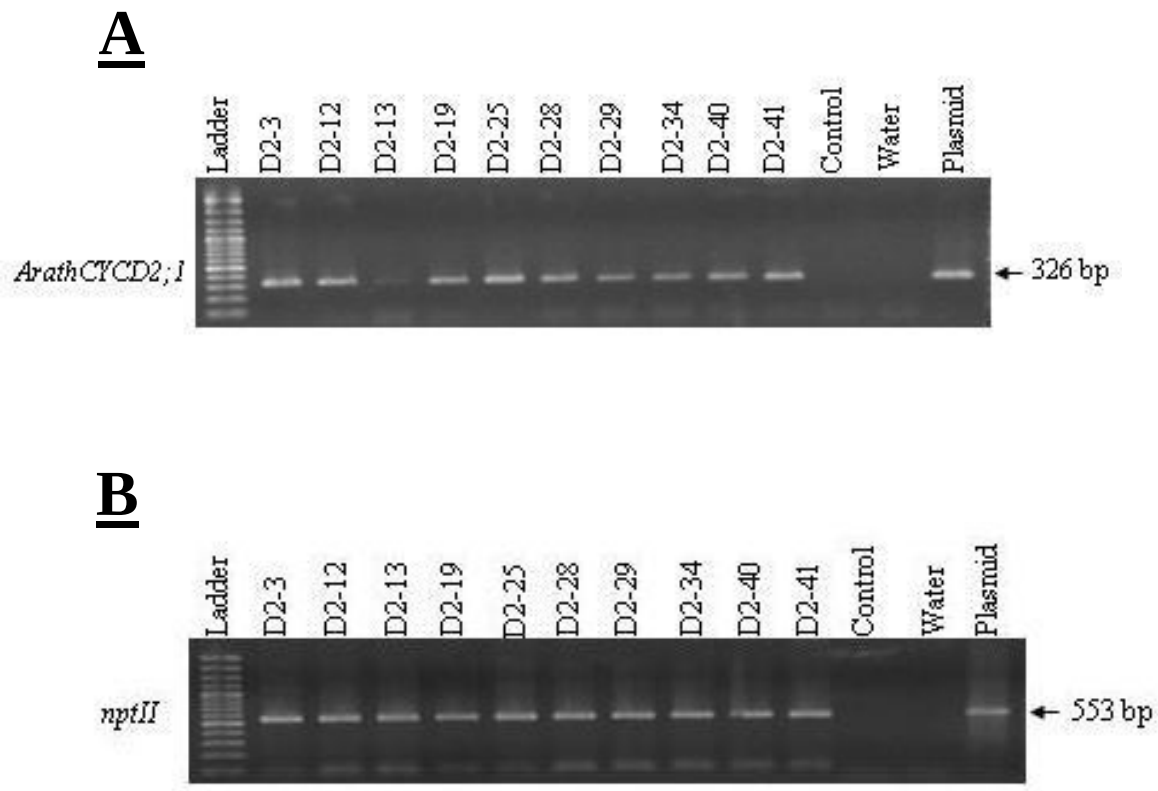


Fig. 3.7 PCR analysis of greenhouse potted *Arabidopsis;CyclinD2;1* regenerants. (A) PCR with *Arabidopsis;CyclinD2;1* specific primers; (B) PCR with neomycin phosphotransferase II (*nptII*) specific primers; L, 100bp DNA ladder; Control is a non-transformed plant, plasmid is vector pExpression B4-UBiB1-D2-B3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

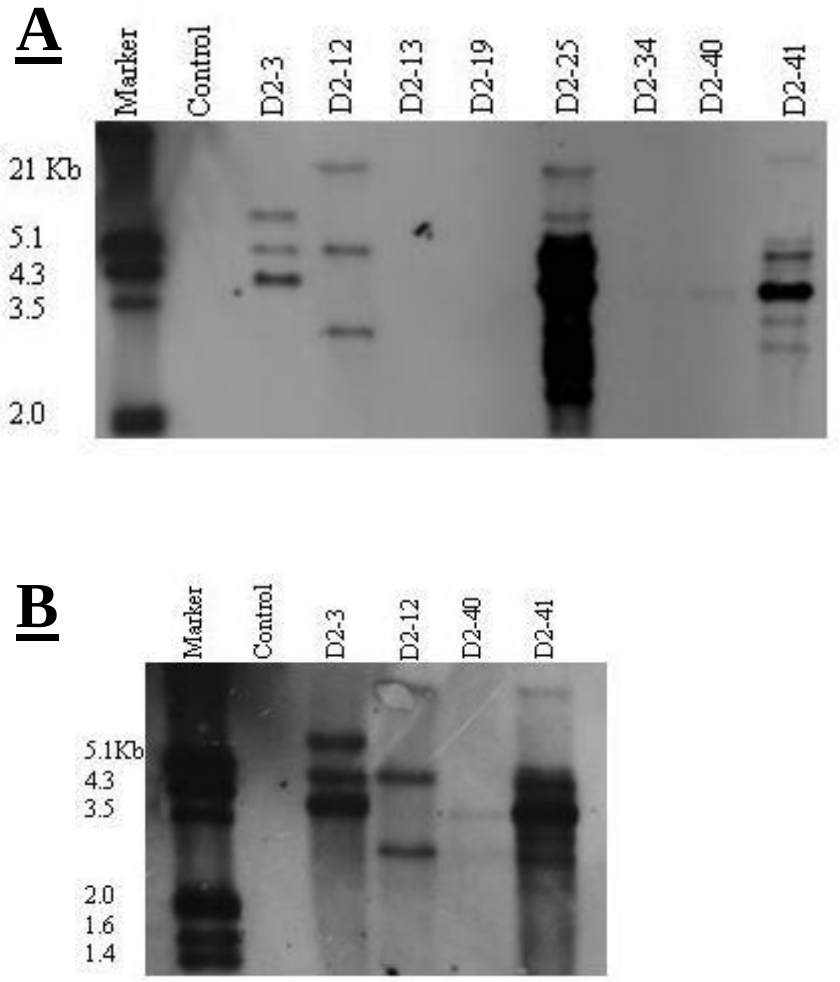


Fig. 3.8 (A and B) Southern blot of *Arath;CyclinD2;1* putative transformed bananas. Genomic DNA from selected transformed plants and a control (non transformed) were digested with *BamHI*, fractionated on 0.8% agarose gel and probed with a DIG-labeled 650 bp *Arath;CyclinD2;1* DNA fragment. Marker is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

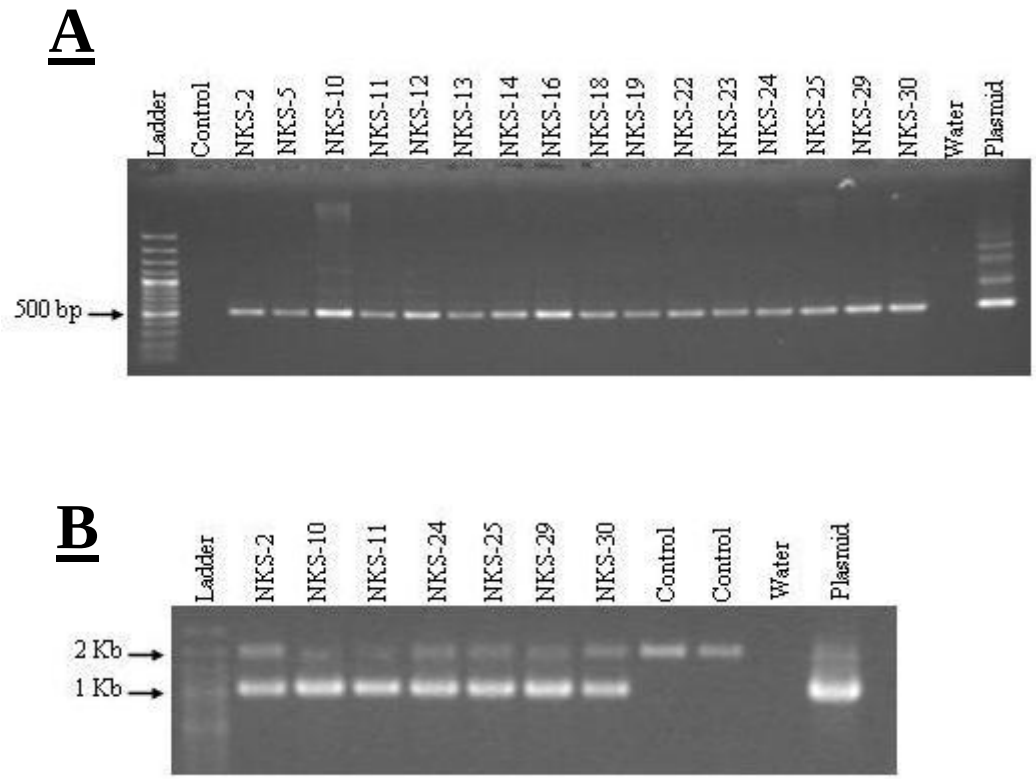


Fig. 3.9 PCR analysis of DNAs from greenhouse-grown *Musac*;*CyclinD2;1* transgenic plants. (A) PCR with a forward primer designed in the promoter and reverse primer in the cyclin gene coding regions; (B) PCR with primers flanking the gene coding sequences; L=100bp DNA ladder; NKS are lines that were 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.

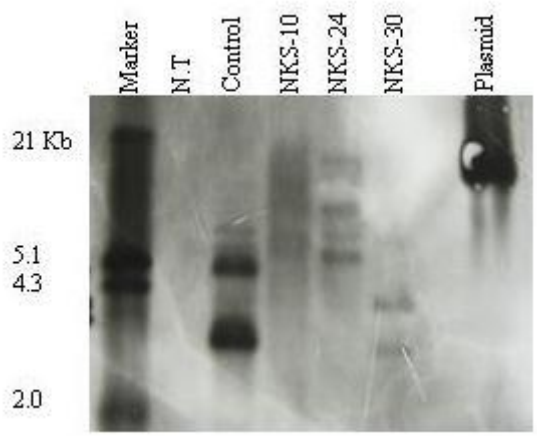


Fig. 3.10 Southern blot of banana plants transformed with *Musac*;*CyclinD2;1* coding sequence. Genomic DNA from selected transformed lines and non-transformed control were digested with *BamHI*, fractionated on 0.8% agarose gel and probed with DIG 750 bp labeled neomycin phosphotransferase II (*nptII*) 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 C*yclinD2;1* gene homologs. In the next part of the study, expression of the *cyclin* transgenes was investigated.

CHAPTER FOUR

TRANSCRIPTION ANALYSIS OF CYLIN TRANSGENES

4.1 Introduction

Transgene expression is influenced by factors such as the location of the transgene in the plant's genome, copy number, truncation, methylation, re-arrangement of the transgene and growth environment (Stam *et al*., 1997; Muskens *et al*., 2000; Yoshida and Shinmyo, 2000; Qi and John, 2007). In addition, the level of homology between the transgene and the endogenous ortholog may also influence the final expression of any or both genes. Gene activity manifests itself at the point of transcription into messenger RNA (mRNA) and the final product in form of protein. The relative abundance of mRNA of a gene in plant organs can provide information on the point of action of the gene as well as the level of transcription. Messenger RNA amounts in plant tissues were traditionally measured using Northern blot analysis. However, this procedure is quite laborious, not very quantitative, requires a minimum of 10 µg of mRNA per sample and may not detect genes that are expressed at low amounts (Huggett *et al*., 2005; Dombrowski and Martin, 2009). Alternative methods based on reverse transcription polymerase chain reaction (PCR) techniques that use mRNA after it has been reverse transcribed into a more stable complementally DNA (cDNA) form, offer therefore several advantages. Reverse transcription semi-quantitative PCR can amplify rare transcripts in samples, this method can be used with small amounts of cDNA and differences in cDNA amounts can be visualized on an agarose gel (Bustin, 2000; Marone *et al*., 2001). The more precise and sensitive quantitative real-time PCR method (Peirson *et al*., 2003; Ginzinger, 2003) has become the standard for studying gene expression in plants under different experimental conditions. In this method the expression of a target gene is compared relatively to the level of the expression of one or more "reference genes" and a calibrator (Livak and Schmittgen, 2001; Huggett *et al*., 2005).

The objective of this study was to determine the level of transcription of an additional copy of the banana *CyclinD2;1* (*Musac;CycD2;1*) gene driven by CaMV35S promoter in banana plants. In comparison, the integrity and expression of the *Arabidopsis;CyclinD2;1* (*Arath;CycD2;1*) transgene in transgenic banana and its influence on the expression of the endogenous banana *CyclinD2;1* were examined. Results obtained show that the two transgenes were transcribed in banana with higher

transcription of the Arabidopsis cyclin in the shoot tip than in the root apex and relatively high transcription of the banana cyclin in the root tip. Variability of transgene transcription was in particular evident in the root tip of plants over-expressing the banana cyclin despite using clonal banana material.

4.2 Materials and Methods

4.2.1 RNA isolation and cDNA synthesis

To determine the expression level of the *Musac;CycD;1* gene in the different banana plant tissues, total RNA was isolated from the plant shoot tip of field-grown non-transformed suckers. The leaf sheaths were removed to expose the shoot tip. Two centimeter cubes of the shoot tip comprising of the meristem dome, the surrounding leaf primordia and corm were then excised. RNA was also isolated from a mature and young rolled leaf and from pulp of young fruits (14 days after appearance of the inflorescence).

The expression levels of *Arath;CycD2;1* and *Musac;CycD2;1* genes in the shoot and root tips of transformed bananas and non-transformed control plants were determined using glasshouse-grown potted plants. Shoot samples were extracted the same way as for the field-grown plants. To obtain root samples, the plant root system was removed from the pots, cleared of the soil and washed under running water. Ten 1 cm root tips were isolated from each plant and pooled. All samples were wrapped in aluminum foil immediately after isolation, frozen in liquid nitrogen and kept at -80^{o}C. Samples were grinded using liquid nitrogen in a mortar with a pestle. Total RNA was extracted from 50 mg of the sample powder using the RNeasy Plant Mini Kit (Qiagen, Germany) following the recommended protocol of the supplier. Heating of the samples was omitted to avoid swelling of the samples that would result from the high polysaccharide content in banana tissue. RNA was treated with RNase-free DNAse I (Qiagen, Germany) on the column following the RNeasy Plant Mini Kit protocol. The integrity of the RNA was verified by heating 5 µl the RNA at 70^{o}C, followed by immediately cooling on ice and running it on a 1% agarose gel containing 0.1 µg/ml of ethidium bromide. Concentration was determined with a spectrophotometer (Nanodrop®, ND 1000).

First strand cDNA was synthesized from 0.5 µg of total RNA using the ImProm-II™ Reverse transcription Kit (Promega) random primers following the recommended protocol of the supplier. Oligo$(dT)_{15}$ primers were also used to synthesize cDNA that was used to study the integrity of *Arath;CycD2;1* transcripts. The quality of cDNA was checked by using 1 µl of the cDNA in a PCR with banana *Actin* specific primers (5'-CT GGTGATGGTGTGAGCCAC-3') and (5'-CAGGGCAACGTAGGCAAGCT-3') designed from *Musa* actin (Genbank accessions AF285176 and AY904067) to give a 200 bp amplicon. PCR amplification conditions were 3 min at 94^{o}C to denature DNA followed by 30 cycles of 20 sec at 94^{o}C, 20 sec at 60^{o}C for primer annealing, 30 sec at 72^{o}C for DNA extension and a final extension step for DNA of 2 min at 72^{o}C.

4.2.2 Semi-quantitative PCR

Semi-quantitative PCR was performed using 2 µl of the cDNA with *Musac;CycD2;1* and *Musa* actin specific primers using the PCR program indicated above. The *Musa* actin gene was amplified from the cDNA to confirm uniform cDNA template amplification. The PCR products were run on a 2% agarose gel containing ethidium. To pick the full length *Arath;CycD2;1* cDNA, forward primer (5'-ATGGCTGAGAATCT TGCTTGT-3') and reverse primer (5'-TCATTGTTTTCTCCTCCTCTTGT-3') annealing at the ends of the open reading frame (ORF) were used. Primers were used at 0.3 µM together with 0.5U proof reading *pfu* DNA polymerase in a 20 µl reaction mixture containing 1.5 mM $MgCl_2$ and 0.2 mM dNTPs. PCR was conducted for 3 min at 94^{o}C, 35 cycles of 30 sec at 94^{o}C, 30 sec at 56^{o}C, 1 min at 72^{o}C and final extension of DNA strands of 10 min at 72^{o}C. Amplified products were separated on a 1% agarose, stained with ethidium and viewed under U.V. light. For sequencing, the lower sized band in the shoot sample and the single band from the root sample were purified from the gel. The purified product was used in a PCR-based sequencing reaction with the forward and reverse primers in a forward and reverse reaction, respectively. Using MEGA version 3.1 software (Kumar *et al.*, 2004), the two sequences were aligned to locate the missing nucleotides.

4.2.3 Quantitative real-time PCR (qRT-PCR)

qRT-PCR was carried out to evaluate the expression levels of the *CyclinD* transgene and the endogenous banana *cyclinD*. The transcripts were quantified in triplicates on a LightCycler® 480 using SYBR-Green I chemistry in 384 well plate (Roche). The reactions were conducted in a 10 µl volume comprising of 50 ng of cDNA, 5 µl of preformed Sybr Green master mix and 0.5 µM of each primer. Cycling conditions consisted of an initial DNA denaturing for 10 min at 95^{o}C, followed by 45 cycles of 10 sec at 95^{o}C, 30 sec at 60^{o}C, and 20 sec at 72^{o}C. Melting curves of the PCR products were acquired by an extra cycle of 30 sec at 95^{o}C, 1 min at 59^{o}C, 10 sec acquisition at 95^{o}C and cooling for 30 sec at 40^{o}C. In the experiments the *Musa* 26S rRNA gene was used as a reference gene.

Three plants each with three technical replicates were used for qRT-PCR. Relative quantification of the transcription of the *Arath;CycD2;1* transgene and the endogenous *Musac;CycD2;1* were determined using the relative standard curve method (Applied Biosystems user Bulletin No. 2, 2001). Standard curves for the respective primers were constructed by regressing the quantification cycle (C_q; Bustin *et al*., 2009) data against the respective 1:5, 1:10, 1:20, 1:40 1:80 dilutions of the cDNA stock. The equation y = mx + b, where b = y-intercept of the standard curve line and m as the slope of the standard curve line was derived. The C_q-values were substituted into the equation to derive the corresponding log amount of the transcripts in the cDNA in the samples:
Log transcripts = (C_q value – b)/m

The transcript amounts were normalized by dividing with the values of the reference gene, *Musa* 26SrRNA. Relative transcription levels of *Arabidopsis;CyclinD2;1* in the transgenic plants were derived by dividing the expression of the transgene by the expression of the reference gene, *Musa* 26SrRNA. For the *Musac;CyclinD2;1* expression, the relative levels were computed by dividing the normalized expression of the gene by the normalized expression of the same gene in the control plants. T-tests between the relative transcript levels were carried out with SAS 9.1 program.

4.2.4 Primer design

Primers were designed using the online software Primer3 (http://www-genome.wi.mit.edu) to anneal at 60^{o}C and were further analyzed with OligoAnalyzer3.1 (http://eu.idtdna.com). Amplicon size was maintained below 500 bp as recommended for SYBR Green I (LightCycler® 480 SYBR Green I Master, Roche manual; www.roche-applied-science.com). Primers for the *Arath;CycD2;1* gene were designed within the less conserved C-terminus of the cyclins to prevent amplification of the endogenous banana cyclins. Primer specificity was validated by semi-quantitative RT-PCR and checking the products on 2% agarose for absence of dimers. Likewise, primer-dimers were checked on qPCR products and by analyzing the dissociation curves for single peaks.

Table 4.1 Primers used in real-time qRT-PCR.

Gene	Accession	Oligonucleotides sequence (5'-3')	Size (bp)
Musac;CycD2;1		Forward: GGTTGTGGTGCACCTAGCA	
		Reverse: GCTGACTGCTTCCTCATCAA	251
Arath;CycD2;1	X83370	Forward: GCAAGCTCTAACTCCATTCTC	
		Reverse: CCTGCTCCTGCGATAAACTA	326
Musa 26SrRNA	AF399949	Forward: ACATTGTCAGGTGGGGAGTT	
		Reverse: CCTTTTGTTCCACAGCAGATT	107

4.3 Results

4.3.1 *Musac;CyclinD2;1* gene expression profiling

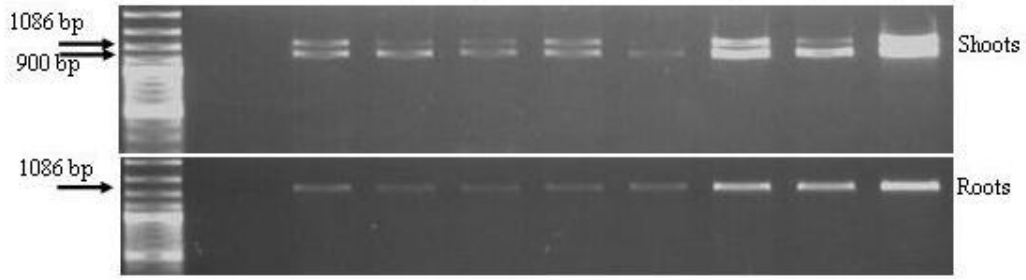
To identify the tissue specificity of the isolated *Musac;CyclinD2;1* in the banana plants, a semi-quantitative RT-PCR analysis was conducted on cDNA from different banana plant tissues. The band intensity was highest in the shoot tip, lower in young leaf and young fruit (14 days after flowering), while no transcripts were detected in the mature leaf (Fig. 4.1).

Fig. 4.1 Expression of *Musac;CyclinD2;1* in different banana plant tissues. AM: Apical shoot meristem; YL: young folded leaf at emergence; ML: mature leaf; YF: young fruit, 14 days after flowering.

4.3.2 Integrity of *Arath;CyclinD2;1* transcripts

Two sizes of *Arath;CyclinD2;1* transcripts were identified. The root tip had a full length mRNA while the shoot tip had the full mRNA and a shorter truncated version (Fig. 4.2A.). Sequencing of the two RT-PCR products established an internal truncation of 186 bases (Fig. 4.2B).

A



B

ATGATATCAACTATGGCGGCGGATTTACGAACGAGATTGATTACAATCACCAACTTTTTGC
TAAAGACGACAACTTTGGCGGCAACGGATCAATTCCGATGATGGGTTCTTCTTCATCGTCC
TTGAGTGAAGACAGAATCAAAGAGATGTTGGTGAGAGAGATTGAGTTTTGCCCTGGAACTG
ATTATGTTAAGAGATTGCTTTCTGGTGATTTGGATTTGTCTGTTCGAAACCAAGCTCTTGA
TTGGATTCTAAAGGT**TTGTGCTCATTACCATTTTGGACATCTGTGCATATGCCTATCCATG**
AACTACTTGGATCGGTTCTTAACATCCTATGAATTGCCGAAAGACAAGGATTGGGCTGCTC
AGTTACTAGCTGTGTCTTGCTTATCATTAGCATCCAAAATGGAAGAAACTGATGTGCCTCA
CATTGTTGATTTACAGGTGGAAGATCCCAAGTTTGTTTTTGAGGCCAAAACAATAAAAAGG
ATGGAGCTTTTGGTTGTCACCACTTTGAATTGGAGATTGCAAGCTCTAACTCCATTCTCCT
TCATTGATTATTTCGTTGACAAGATCAGTGGTCACGTGTCGGAGAATTTGATCTATAGATC
GTCAAGATTCATCTTAAACACCACCAAAGCAATTGAATTCTTAGACTTCAGGCCTTCTGAG
ATAGCTGCAGCTGCTGCAGTGTCTGTTTCCATTTCAGGAGAAACAGAATGCATTGATGAGG
AAAAGGCACTGTCTAGTCTCATATATGTAAAACAGGAGAGGGTGAAGAGATGTTTGAATCT
GATGAGAAGTCTCACTGGGGAGGAGAATGTGCGGGGAACTAGTTTATCGCAGGAGCAGGCG
CGAGTTGCGGTAAGAGCTGTACCTGCAAGTCCAGTTGGAGTGTTGGAAGCAACATGTTTGA
GCTATAGGAGTGAAGAGAGAACAGTTGAGTCATGTACAAATTCCTCACAGAGTAGTCCAGA
CAACAACAACAACAACAACAACAGCAACAAGAGGAGGAGAAAACAATGA

Fig. 4.2 Truncation of the *Arath;CyclinD2;1* transgene in transgenic banana. (A) qRT-PCR products showing the full 1,086 bp ORF and the truncated 900 bp cDNA product in the shoot tip (S) and the full-length product in the root tip (R). M = 100bp DNA ladder; Control is a non transgenic wild-type plant; D2-3, D2-12 and D2-41 are products from independent transgenic lines; 1-3 are representative plants of each line. (B) *Arath;CyclinD2;1* transgene cDNA showing the truncated 186 bp region (bold letters) and demarcated by bent arrows.

4.3.3 Gene expression analysis

In relation to the transcription of the *Musa 26SrRNA* reference gene, transcription of the transgene in the shoot tip was significantly ($p<0.0001$) higher in line D2-41(seven-fold) and in line D2-3 (four-fold) than in line D2-12 (identical to reference gene; Fig. 4.3). In the root tips, transcription was lower than the transcription of the reference gene, but the relative expression levels between the lines were approximately retained, with relative transcription in D2-41 (0.4-fold) and 0.2-fold in both D2-3 and D2-12.

With the exception of line D2-3, transcription of the endogenous banana *CycD2;1* was reduced in the *Arath;CycD2;1* transformed plants (Fig. 4.4). The reduction in transcription of the endogenous banana *CycD2;1* gene was remarkably (4-5 fold) in the roots, with a difference in C_q value of 4 compared to the non-transformed plants. In contrast, in banana plants transformed with a *Musac;CycD2;1* gene, there was no difference in the total expression levels of the banana *CycD2;1* gene in shoot tips of transformed and non-transformed or empty-vector transformed plants (Fig. 4.5). However, significant differences ($p = 0.024$) in transcription of the *Musac;CycD2;1* gene were observed in the root tips when transformed and non-transformed plants were compared. Banana plants transformed with the *Musac;CycD2;1* gene showed a very high transcription level, 66-fold higher in line NKS-30 followed by line NKS-10 (10-fold), with the least in NKS-24 (2-fold). Of the studied *Musac;CycD2;1* transformants, high variability between the sampled plants was found in the NKS-30 line where plants exhibited a 0.6, 2.9 and 73-fold expression. The relationship between the transcription of the two up-regulated cyclins in the shoot and root apices of is summarized in Table 4.1.

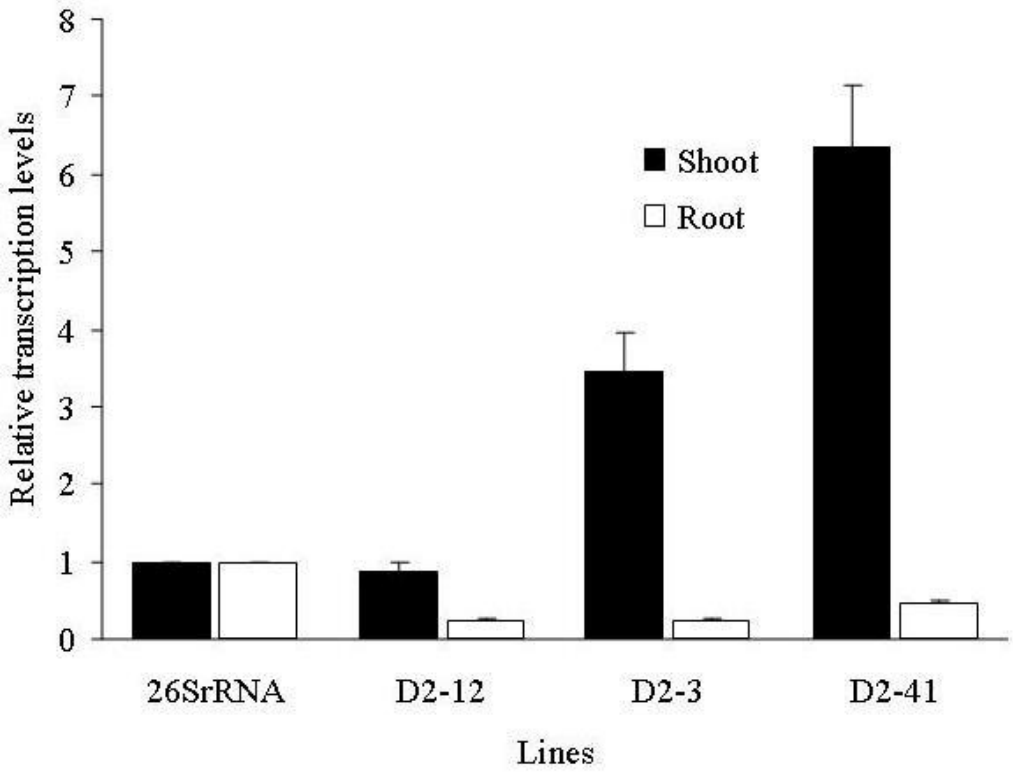


Fig. 4.3 Comparison of transcription of *Arath;CyclinD2;1* transgene in shoot and root apices of banana plants transformed with *Arath;CyclinD2;1* gene coding sequence. Transcription levels are relative to the transcription of the reference *Musa* 26S rRNA gene. Bars are means ± SE of three plants.

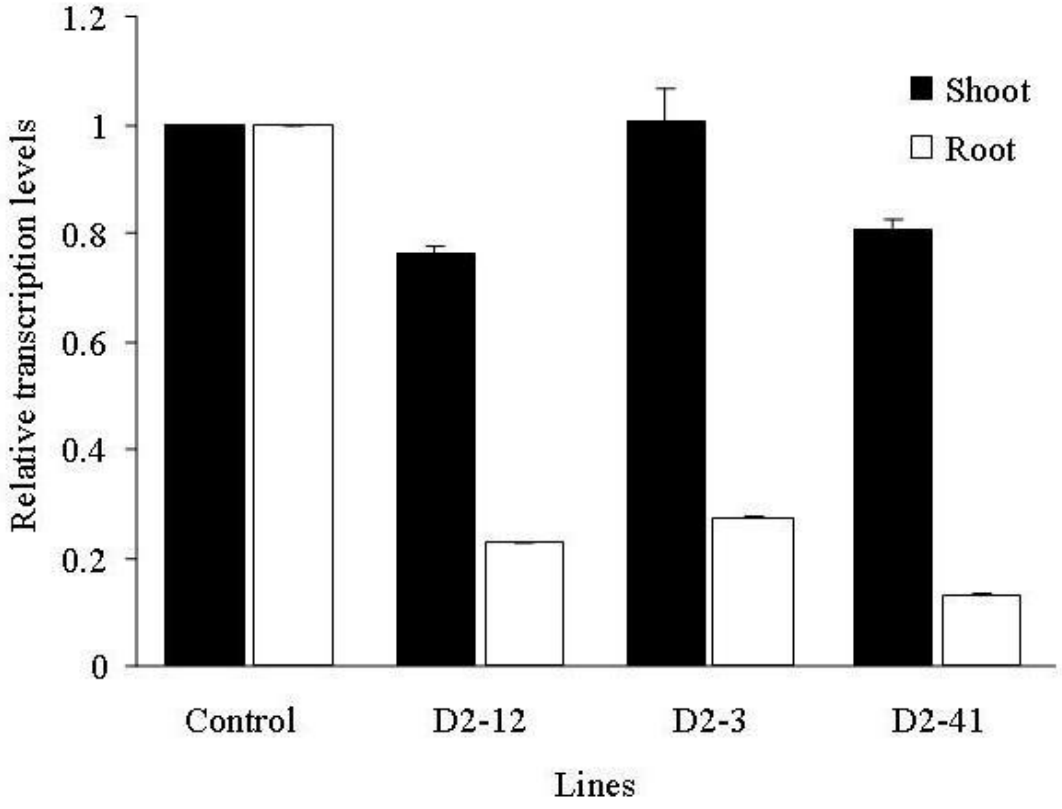


Fig. 4.4 Comparison of transcription of indigenous *Musac;CyclinD2;1* gene in shoots and root apices of banana plants transformed with *Arath;CyclinD;1* gene coding sequence. Transcription levels are relative to the transcription of the same gene in non-transgenic plants (control). Bars are means ± SE of three plants.

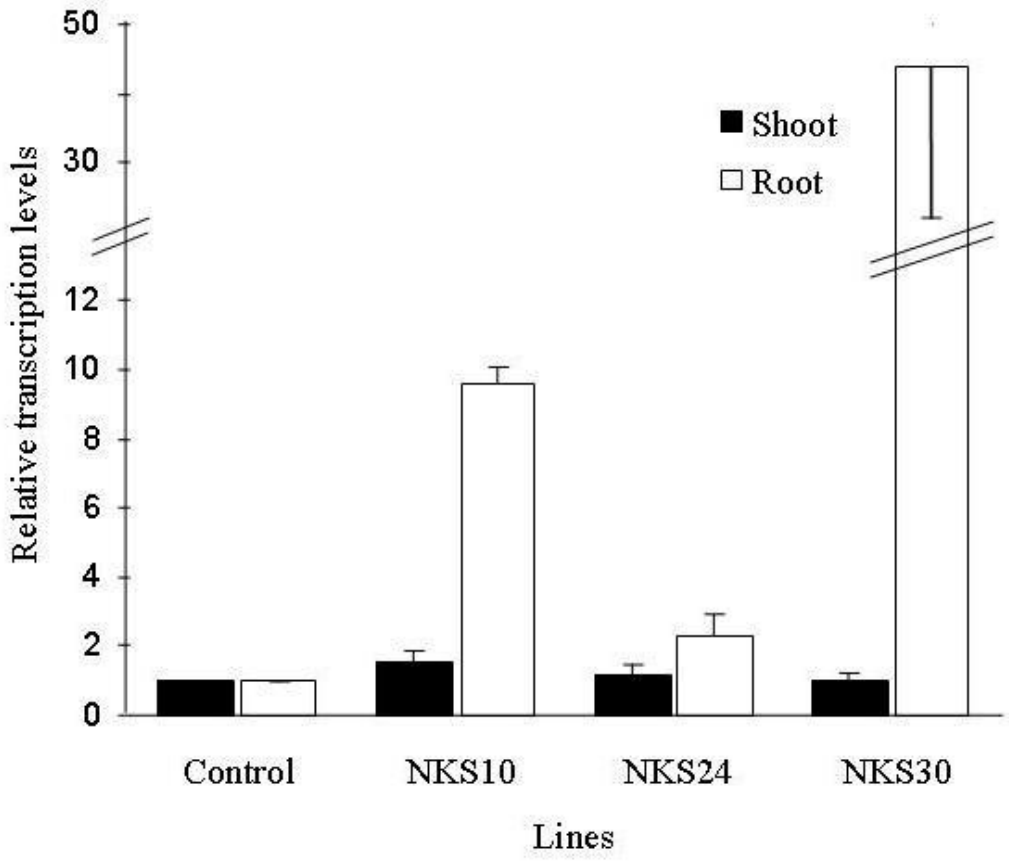


Fig. 4.5 Comparison of transcription of *Musac;CyclinD2;1* in shoot and root apices of banana plants transformed with a *Musac;CyclinD2;1* gene coding sequence. Transcription levels are relative to the expression of the same gene plants transformed with an empty vector, pBin19. Bars are means ± SE of three plants.

Table 4.1 Summary of cyclin transcription in shoots and root apices.

	Arath;CycD2;1 transformants		*Musac;CycD2;1* transformants
Tissue	*Arath;CycD2;1* transcripts	*Musac;CycD2;1* transcripts	*Musac;CycD2;1* transcripts
Shoot	+ **up-regulated**	-	-
Root	-	- **down-regulated**	+ **up-regulated**

+: response

-: no response.

4.4 Discussion

This study showed that transcription of the endogenous banana *cyclin*, *Musac;CycD2;1*, is higher in the shoot tip than in younger leaves or fruits. This is consistent with the role of cyclinD in cell division in meristematic tissue (Gaudin *et al*., 2000; Freeman *et al*., 2002). The shoot apical meristem is a region of active cell division to form leaf primordia (Stover and Simmonds, 1987). For the young unfurled leaf, active cell division is associated with the formation of the stomata complex from the meristemoid cells. Similarly, early fruit development is characterized by rapid cell division that precedes cell expansion to form the storage tissue (Stover and Simmonds, 1987; Kvarnheden *et al*., 2000). Since the shoot tip had the highest transcripts among the three tissues tested, the tip was used for the experiments to monitor the transcription of exogenous cyclins in transgenic banana plants.

In this study, a difference in transcript sizes of *Arath;CycD2;1* was found which was very likely a result of internal truncation of mRNA. Similar truncation has previously been found in Arabidopsis plants that were transformed with the *Arath;CycD2;1* coding sequence (Qi and John, 2007). According to Brendel *et al.* (1998), sequences, such as AGGT, located at the intron boundary act as splicing signal. This sequence occurs in the Arabidopsis cyclin as well as in the third intron of the banana *cyclinD2;1* genomic sequence. It is therefore possible that this sequence also initiated the splicing mechanism in banana for the *Arath;CycD2;1* transgene mRNA. However, unclear is why such truncation was only found with the shoot apex but not with root tips. Since the primers used in real-time PCR amplified both the intact and also the truncated form of the mRNA, the higher abundance of transcripts in the shoot compared to the root might have been caused by amplification of the intact and truncated mRNA in the shoot apex.

In the *Arath;CycD2;1* transformants, transcription of the endogenous banana *cyclin* genes was higher in the shoot apex than the root tip. This difference could partly be due to anatomical differences in the sampled tissues. The banana shoot apex is comprised of the main shoot meristematic tip and auxiliary leaf meristems (Simmonds and Stover, 1987). Therefore, in comparison to a root with a defined meristematic tip, pooling several root tips might not have equated the shoots meristematic tissue and might also

have contained non-meristematic tissue. Further, expression of both Arabidopsis and banana *cyclinD* gene in transformed banana revealed variability in the amount of transcripts for these genes although the experimental materials were micro-propagated clones. This interplant variability of transgenic plants has been previously reported for commercially seed-derived transgenic plants (Greenplate, 1999; Martins *et al*., 2008) and also for vegetatively propagated potato plants (Down *et al*., 2001). Such variability has been attributed to environmental factors that can influence gene expression in individual plants even in a controlled environment (Meyer, 1995; Down *et al*., 2001).

Lines carrying the Arabidopsis *cyclin* gene had a relatively low amount of transcripts in the root compared to the shoot. In contrast, plants transformed with the banana *cyclin* gene had higher cumulative amounts of total banana *cyclin* transcripts (exogenous and endogenous) in the roots than in the shoots. Over-expression of Arabidopsis c*yclinD2;1* further significantly reduced transcription of the endogenous banana cyclinD2;1 in the root apices although the Arabidopsis *cyclin* gene was transcribed in the root (Table 4.1). Transcription studies in Arabidopsis have shown that accumulation of *Arath;CycD2;1* transcripts causes activation of the cell cycle in the root apical meristem (Masubelele *et al*., 2005).

Since the root meristematic tissue seems to be more responsive to changes in *cyclinD2;1* gene content than the shoot meristem, cumulative transcription of both *Musa cyclin* genes might have also resulted in cell cycle activation in this study. This could possibly be the reason for faster root growth in banana transformed with the banana *cyclin* gene. Also, the observed faster leaf growth of transformed banana transcribing the *Arath;CycD2;1* gene might be due to the relatively high Arabidopsis *cyclin* gene transcription found in the shoot apex.

In conclusion, two cyclin genes (Arabidopsis and banana) could be expressed in transformed banana. The transcription of these genes was different with relatively high amounts of Arabidopsis gene in the shoot apex and a relatively high amount of the banana gene in the root apex. To be able to relate the observed transcript amounts to phenotype, plant growth measurements were conducted in the next chapter (Chapter five).

CHAPTER FIVE

PHENOTYPIC EVALUATION OF TRANSFORMED BANANA PLANTS EXPRESSING D-TYPE CYCLINS

5.1 Introduction

The D-type cyclins through their activation of cyclin dependent kinase A (CDKA) play a major role of modulating the progression of the cell cycle at the G1/S transition. In plants, cyclin expression is associated with meristematic tissues (Soni *et al*., 1995; Freeman *et al*. 2002; Dewitte *et al*., 2003; Inzé and De Veylder). Over-expression of the *Arath;CycD2;1* transgene in *Arabidopsis* plants resulted in faster seed germination (Masubelele *et al*., 2005). Similar up-regulation of *Arath;CycD2;1* in tobacco plants enhanced shoot and root growth (Cockroft *et al*., 2000; Boucheron *et al*., 2005). In rice, over-expression of the *Arath;CycD2;1* gene enhanced both shoot and root growth at the *in vitro* stage of plants, but not in potted plants (Oh *et al*., 2008), suggesting a culture stage related transgenic plant response.

Several non-destructive techniques have been devised to measure and monitor leaf and root growth. In banana, a technique developed by Kumar *et al*., (2002) estimates leaf blade area by multiplying the blade length and width with by factor of 0.8. A more sophisticated digital photographic technology is used in *Arabidopsis* studies (Cookson *et al*., 2005). Roots growth of *in vitro* cultured plants can be measured by monitoring the advancement of the tips on petri dishes (Beemster *et al*., 1998). Cytological methods are used to measure cells and meristem sizes microscopically (Beemster and Baskin, 1998; Baskin, 2000; Fiorani *et al*., 2000). Alternatively, for thick tissues the surfaces can be printed to facilitate measuring of their epidermal cells (Reuveni, 1988). Using the kinematic approach, leaf growth velocity, meristem and mature cell size are measured and the values are used to derive cell division rates (Beemster and Baskin, 1998; Baskin, 2000; Fiorani *et al*., 2000). In roots and plants with distinct apical meristems, growth is measured *in situ* as a gain in length and height, respectively. For grasses species that have a concealed shoot apical meristem, leaf elongation rate (LER) of a representative leaf has been used to estimate growth of the whole plant (Fiorani *et al*., 2000; Arrendondo and Schnyder, 2003; Bultynck *et al*., 2003). However, this technique has not been used in banana. Instead, growth of field grown banana plants is commonly measured as number of days a plant takes to flower and fill the fruits (Vuylsteke *et al*., 1993; Tenkouano *et al*., 1998).

In this chapter, experiments were conducted to evaluate the effect of constitutive overexpression of an *Arath;CycD2;1* and a *Musac;CycD2;1* transgene on plant growth of transgenic banana plants. In particular, the growth of leaves and roots was examined. Banana plants transformed with the Arabidopsis *cyclin* gene exhibited faster leaf growth in two lines with one line showing higher root growth. Banana plants transformed with *Musac;CyclinD2;1* had longer roots than non-transgenic control plants.

5.2 Materials and methods

5.2.1 Transgenic lines evaluated in the study

Phenotypic comparison of bananas transformed with *Arath;CyclinD2;1* was made between the transgenic lines and a non-transgenic regenerant. For the bananas transformed with a *Musac;CyclinD2;1*, a regenerant carrying an empty vector, pBin19, was used as a control. The gene constructs and procedures are detailed in chapter three.

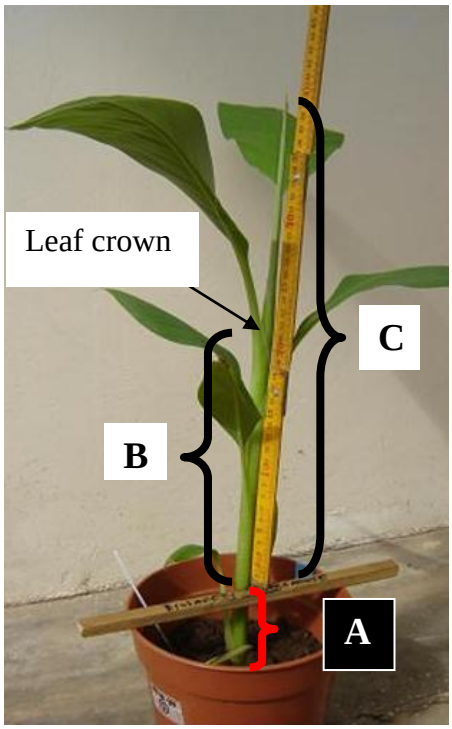
5.2.2 Establishment of transformed plantlets

Weaning, potting and growth evaluation of plants were done in a Level 3 containment glasshouse at the National Agricultural Laboratories Institute (NARL) (Kawanda, Uganda, $0^{o}25$'N, $32^{o}32$'E, 1190 masl). *In vitro* raised banana regenerants were potted in 200 mL plastic cups containing a pasteurized forest top soil and farm yard manure mixed at a ratio of 12:1 (Vuylsteke and Talengera, 1998). The potting substrate was analyzed at NARL's soil laboratory and had the following properties as determined by the methods described by Okalebo et al (2002): sandy loam texture (67.8% sand, 19.6% clay, 12.6% silt), pH of 7.2, 2.3% organic C and 0.21% total N. Other nutrients in the potting mix, as determined by the Mehlich 3 extraction method (Mehlich 1984), were 62.5 ppm of P, 2370.9 K, 4300.4 Ca, 1415.9 Mg, 2.4 Cu, 12.6 Zn, 151.3 Fe and 474.4 Mn. Plants were hardened under a low transparent plastic tent for three weeks after which the humidity was reduced by gradual opening of the sides of the tent during the fourth week. Subsequently, the plants were transferred into 3 L pots containing two kilograms of the same potting substrate. Watering was done daily and the temperature was maintained at 27-32^{o}C and humidity at 30-60% through intermittent misting.

5.2.3 Phenotypic evaluation of transgenic plants

5.2.3.1 Measurements of leaf length and plant height

Leaf elongation was used to estimate the aerial plant growth of potted transgenic plants. Growth evaluation was performed on potted plants three months after they had gone through hardening and establishment in pots (Fig. 5.1). At this stage, the plants were emitting the ninth leaf and this leaf was selected for measurement. Leaf length was measured daily at 9 am using a ruler. Measurements were started at the time the leaf emerged from the plant crown, through unfurling, until leaf growth ceased. On the first day of measurement, leaf length was taken from the base of the plant at the point where the top-most roots emerge (collar) to the tip of the leaf (Fig. 5.1). Accessing the collar region involved disturbing the soil. To avoid this, the distance from the collar to the rim of the pot was recorded on the first day and the rim was used as a reference point for the subsequent measurements. Plant height was therefore measured from the rim of the pot to the junction of the petioles of the top-most leaves. Leaf growth data included (i) the time taken for the leaf to unfurl, (ii) leaf blade width and length, (iii) final leaf length and (iv) time taken to obtain this length. The laminar area was derived by multiplying the blade length and width at the widest point by a factor of 0.8 (Kumar *et al*., 2002). Post emergence increase in leaf length with time was exponential for at least the first four days. Thus, growth rates were computed for this exponential growth and these values were used to compare growth between the plants.



C = Distance from rim to tip of cigar leaf.
Cigar length = A + C

B = Distance from rim to junction between top most open leaves.

A = Distance from collar to rim of pot

Fig. 5.1 Illustration of measuring banana plant height and leaf growth.

5.2.3.2 *Determination of epidermal cell size*

To determine the size of mature epidermal cells, prints of epidermal cells were prepared from the adaxial surface at the middle of the lamina of the fully open ninth leaf. About 2 cm area of the leaf surface was cleaned with a water-soaked cotton wool and left to dry. A thin layer of translucent nail polish was then painted onto the area and after drying, a piece of translucent adhesive tape was applied to the painted area. When the tape was peeled off, the tape and the polish imprint adhering to it was mounted onto a glass slide. Epidermal cells were counted under a light microscope (Leitz Orthoplan large field, Wetzer-Germany) at a magnification of 160-times. A 0.175 sq mm field in the 10x eye piece (Leiz) was used as a counting guide and only the cells that were within this field and partially at the top and left edge of the field were counted. Three fields were counted and the means calculated. Counts were multiplied by a factor of 5.17 to derive the number of cells per square mm. The number of epidermal cells making up the adaxial leaf area was calculated by multiplying the total leaf area (mm^2) by the number of cells in a square mm. Cell density considered to be inversely proportional to the cell number was also used to estimate cell size.

5.2.3.3 *Measuring root growth*

Root growth was evaluated using intact roots on *in vitro* cultured shoots as well as isolated root cultures. Shoots of the transgenic lines and controls were multiplied on MS medium (Murashige and Skoog, 1962) supplemented with 5 mg/L BAP and 30 g/L sucrose. The pH of all culture media was adjusted to 5.8 before autoclaving the medium for 15 min at 121^{o}C. Cultures were maintained at 27^{o}C and 16 hrs of light supplied by 40 W cool white fluorescent tubes. Twenty single shoots from each line were isolated and cultured in sterilized 200 ml glass baby food jars (Sigma) containing 25 ml of growth regulator free MS medium. After 4 wks, the plantlets were removed from the jars and the medium washed off the roots. The number of roots and the length of the longest root on each plantlet were recorded.

To measure root growth of isolated root cultures, single shoots were cultured for 2 weeks in 200 ml glass baby food jars containing MS medium (Murashige and Skoog,

1962). The medium was supplemented with 0.186 mg/L NAA to induce primary roots but with minimal secondary roots (Pierik, 1987). One cm root tips were aseptically isolated and plated onto 10 cm petri dishes containing 25 ml of culture medium composed of N6 basal salts (Nitsch and Nitsch, 1969), MS vitamins and supplemented with: 20 g/L ascorbic acid, 40 g/L sucrose, 0.2 g/L yeast extract, 0.189 mg/L NAA and 2.3 g/L phytagel (Duchefa Biochemie). Five roots were cultured on each petri dish in five replicates. The dishes were placed vertically and incubated in the dark at 27^{o}C. To monitor root growth, the position of the root tip was marked at the bottom of the petri dish on the first day and after every two days. By measuring the distance between the marks, the average daily growth was computed. After 18 days of culture, the increase in root length and the numbers of secondary roots formed were recorded. The root structure of the potted plants was also examined. This involved lifting the 6 months old plant out of the pots with their intact roots and potting substrate. The roots were freed of the soil, washed under running tap water and photographed.

5.2.4 Data analysis

Data on the aerial growth included plant height, leaf elongation rate during the first four days post emergence, days it took the leaf to open, the final leaf length, days it took the visible part of the leaf to reach the final length and the final leaf blade area. The epidermal cell and stomata density of the fully opened leaf was also recorded. The visible length of the leaf after its appearance and the rate of increase in length were plotted over time. Data on root growth included the number of primary roots produced on *in vitro* rooted shoots and the length of the longest root. On isolated root cultures, records were made on the daily root growth rate and final length as well as the number of secondary roots that were formed.

Data were analyzed using the SAS statistical package (SAS, 2002). All data were subjected to analysis of variance using the Proc ANOVA program and the significance level was set at $P = 0.05$. The mean separation was performed using the Duncan-Weller multiple range test. The extension of the root system was evaluated visually on 6 months old potted plants.

5.3 Results

5.3.1 Leaf growth of *Arath;Cyclin D2;1* expressing banana

Five transgenic lines that showed integrated *Arath;CyclinD2;1* transgene in their genome (Chapter four) were selected for growth evaluation. From the preliminary evaluation, three lines with the highest, intermediary and lowest leaf elongation rate were selected. From post emergence elongation measurement of leaf number nine, the leaves exhibited a sigmoid growth with an increasing rate of elongation immediately after emergence that declined and finally stopped (Fig. 5.2A and B). Increase in rate of elongation was initially exponential and therefore, an exponential leaf elongation rate was calculated between the first and fourth day and used for comparing the different transformed plants. The leaf elongation rate was significantly higher in lines D2-12 and D2-41 and lasted for four days after leaf emission before it declined (Fig. 5.2B).

In general growth parameters of transformed and un-transformed control plants did not significantly differ (Table 5.1) except for plants of lines D2-41 and D2-12. Plants of these two lines showed a significantly higher leaf elongation rate than non-transformed plants and further after leaf emergence, leaves of plants of these lines opened significantly earlier (6 days after emergence, while the leaves and of control D2-3 lines opened after 7 days) (Table 5.1).

Plants of line D2-41, which had the fastest leaf elongation rate, were tested again with much higher number of test plants for a detailed leaf growth study (Table 5.2). As already found in the experiment outlined in Table 5.1, plants of line D-41 exhibited a significantly higher leaf elongation rate than non-transformed control plants and the leaves also opened significantly earlier (Table 5.2). Further in this experiment, significantly higher final leaf length and blade area were found for plants of line D2-41 when compared to non-transformed control plants. Moreover, the lamina of line D2-41 had a significantly lower epidermal cell density compared to the control. However, when the cell density was multiplied with the leaf area to determine the total number of epidermal cells per leaf, no significant difference was found between transformed and non-transformed plants indicating that the epidermal cells of plants of line D2-41 were

larger than that of the control plants. Therefore, cell expansion was primarily responsible for the leaf size.

5.3.2 Root growth of *Arath*;*CyclinD2;1* expressing banana

There was no difference in root production of *in vitro* rooted transformed banana and non-transformed banana control plants when the *Arath;CyclinD2;*1 transgene was expressed in transformed plants (Table 5.3). However, plants of line D2-41 produced significantly longer roots (Table 5.3; Fig. 5.3) than plants of all other lines. Consistent with the increased root length of the seedling roots, the *in vitro* analysis of isolated roots showed that plants of line D2-41 also had a significantly faster root growth and produced the highest number of secondary roots (Table 5.3).

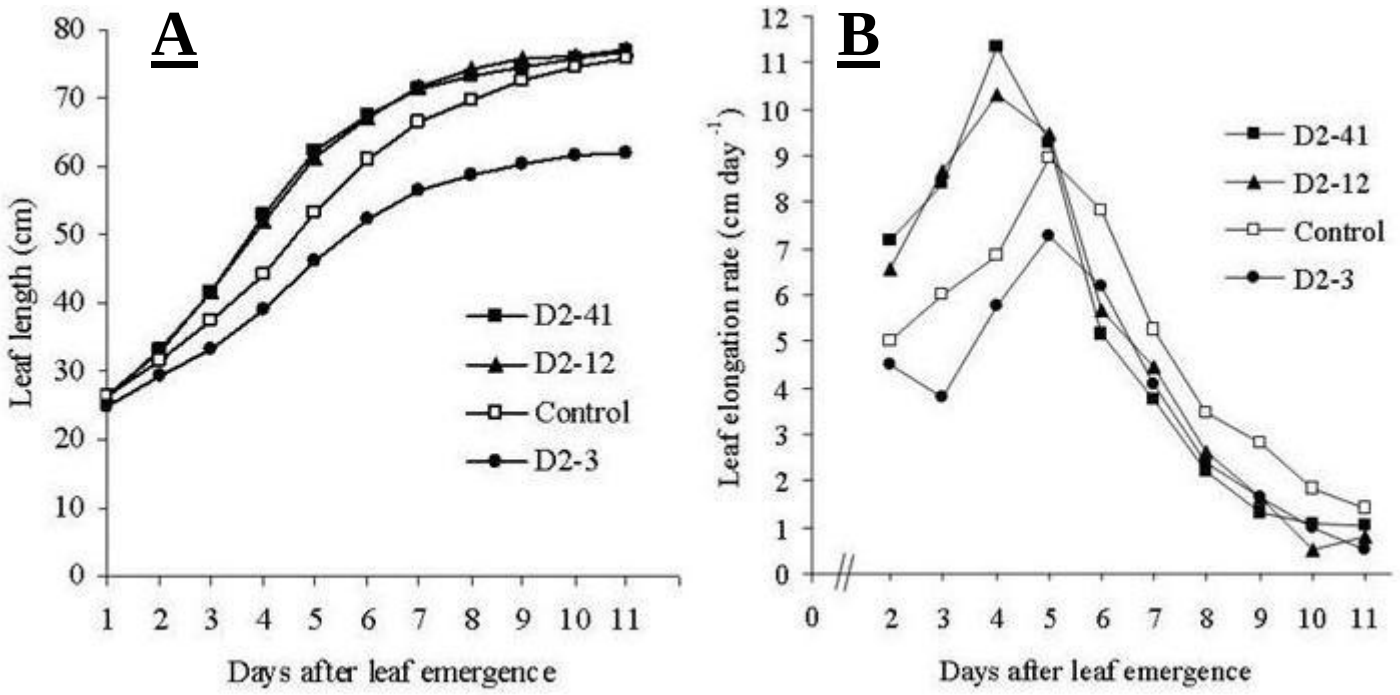


Fig. 5.2 Increase in leaf length over time (A) and leaf elongation rate (B) of banana plants transformed with *Arath;CyclinD2;1* and non-transformed plants (control). Data points are the mean of 3 plants.

Table 5.1 Leaf growth of transformed banana plants expressing *Arath;CycD2;1* and non-transformed control plants.

Line	Plant height (cm)	Leaf elongation rate (cm/day)	Days for leaf to open	Duration of leaf elongation	Mature leaf length (cm)	Leaf blade area (cm^2)
Control	27.0±2.1a	0.16±0.01bc	7.0±0.0ab	12.0±0.0a	77.5±3.8a	472±50a
D2-3	24.8±0.7a	0.15±0.01c	7.3± 0.3a	12.0±0.6a	62.7±1.0a	314±50a
D2-12	26.3±0.9a	0.23±0.01ab	6.3±0.3bc	11.7±0.3a	77.2±4.3a	468±52a
D2-41	26.0±2.5a	0.23±0.03a	6.0±0.0c	11.0±0.0a	77.3±9.6a	466±96a
P-value	*0.819*	*0.044*	*0.034*	*0.142*	*0.125*	*0.158*

Values are mean ± SE of 3 individual plants. Parameters were measured 8 wks after potting the plants. Duration of elongation, leaf length and leaf blade size were determined for mature leaves. Letters denote significance determined using ANOVA at $P = 0.05$ within the column. Means followed by the same letter within the column are not significantly different.

Table 5.2 Leaf growth of transformed banana plants expressing *Arath*;*CyclinD2;1* and non-transformed control plants.

Line	Plant height (cm)	Leaf elongation rate (cm/day)	Days for leaf to open	Duration of leaf elongation	Mature leaf length (cm)	Leaf blade area (cm^{-2})	Epidermal cell density (mm^{-2})	Number of cells per leaf (10^6)
Control	39.0±1.3a	0.11±0.01b	9.3± 0.2a	15.3±0.6a	95.7±2.4b	533.5±24.2b	939.1±23.7b	51.2±1.9a
D2-41	41.9±1.1a	0.14±0.01a	8.5± 0.1b	13.9±0.5a	105.6±2.5a	641.5±23.1a	806.8±24.5a	50.7±2.3a
P-value	*0.119*	*0.024*	*0.003*	*0.086*	*0.007*	*0.003*	*<0.000*	*0.877*

Values are the means ± SE of 15 plants and except for adaxial epidermal cells where values are the means ± SE of 13 plants. Parameters were measured 8 wks after potting the plants. Duration of elongation, leaf length and leaf blade size were determined for mature leaves. Letters denote significance determined via the Student's *t*-test within the column. Means followed by the same letter within the column are not significantly different.

Table 5.3 Root growth of banana transformed plants expressing *Arath;CyclinD2;1* and non-transformed control plants.

Line	Rooting of *in vitro* shoots n = 20		*In vitro* root cultures n = 25		
	Number of roots	Longest root length (mm)	Length (mm)	Growth rate (mm/day)	Number of secondary roots
Control	4.8±0.4a	99±5b	16.0±0.6bc	1.0±0.0b	10.8±0.8b
D2-3	4.0±0.5a	87±5b	17.1±1.0bc	1.0±0.1b	12.5±1.1b
D2-12	4.5±0.3a	87±5b	18.2±0.7b	1.1±0.0b	12.1±1.2b
D2-41	4.5±0.3a	120±9a	21.9±0.8a	1.3±0.1a	21.2±2.8a
P-value	*0.428*	*0.001*	*<0.0001*	*<0.0001*	*<0.0001*

Values are the means ± SE of 20 intact roots and 25 isolated roots. Data on intact roots were taken after 3 wks of shoot culturing. Final length of cultured roots was recorded after 18 days. Letters denote significance determined using ANOVA at $P = 0.05$ within the column. Means followed by the same letters within the column are not significantly different.

5.3.3 Leaf growth of *Musac;CyclinD2;1*expressing banana

In addition to the effect of the heterologous Arabidopsis *CycD2* gene, the effect of over-expressing the native banana homolog was also investigated. Thirty six lines (Chapter three) were created of which plants of 3 lines were analyzed (NKS-10, 24 and 30) in more detail. Plants of these lines had the greatest plant height when compared to plants of all other lines. With the exception of the mature leaf area that was significantly higher in line NKS-24, other leaf growth parameters were not significantly different between transformed and non-transformed control plants (Table 5.4).

5.3.4 Root growth of *Musac;CyclinD2;1*expressing banana

Plants of line NKS-10 and 24 exhibited the lowest number of roots and the shortest roots on *in vitro* shoots. In isolated root assays, plants of line NKS-10 had the lowest root growth rate. Shoots of line NKS-30 produced significantly more and longer roots when compared to all other lines tested including the control (Table 5.5). Also in isolated root cultures, roots of line NKS-30 had a significantly faster growth, which was almost two-fold higher than in the control (Table 5.5 and Fig. 5.4). Number of secondary roots was also highest in line NKS-30 but not significantly different to line NKS-10 and the control. When the root system of six month old potted plants was visually examined, line NKS-30 exhibited a longer but thinner root system compared to the other transgenic lines and control plants (Fig. 5.5).

Table 5.4 Comparison of plant and leaf growth parameters of *Musac*;*CycD2;1* transformed and non-transformed control plants.

Line	Plant height (cm)	Leaf elongation rate	Days for leaf to open	Duration of leaf elongation	Leaf length (cm)	Mature leaf area (cm^2)
Control	29.1±0.6a	0.11±0.01a	9.2±0.2a	14.0±0.4a	75.2±1.7a	417.5±13.1 a
NKS-10	29.2±0.5a	0.11±0.01a	8.8±0.3a	13.7±0.4a	73.9±1.2a	396.4±10.2 a
NKS-24	30.3±1.1a	0.10±0.01a	8.6±0.2a	14.0±0.3a	76.5±2.3a	338.7±15.0 b
NKS-30	28.6±1.2a	0.13±0.01a	8.8±0.3a	13.2±0.4a	74.8±2.4a	384.5±16.8 a
P-value	*0.513*	*0.069*	*0.431*	*0.261*	*0.794*	*0.001*

Values are the means ± SE of 12 plants. Plant height was taken 8 weeks after potting the plants at which time other growth measurements were started. Duration of elongation, leaf length and leaf blade size were determined for mature leaves. Letters denote significance determined using ANOVA at $P = 0.05$ within the column. Means followed by the same letter within the column are not significantly different.

Table 5.5 Root growth of transgenic banana plants expressing *Musac;CyclinD2;1* and non-transgenic control plants.

Line	Rooting of *in vitro* shoots n = 20		*In vitro* root cultures n = 25		
	Number of Roots	Longest root length (mm)	Length (mm)	Growth rate (mm/day)	Number of Secondary roots
Control	6.3±0.4b	145±7ab	17.1±1.1c	1.1±0.1 c	15.5±1.1a
NKS-10	5.2±0.4c	128±7bc	18.1±1.2c	1.1±0.1 c	14.2±1.1a
NKS-24	4.0±0.2c	112±9c	26.1±2.2b	1.5±0.1 b	6.4±1.6b
NKS-30	7.9±0.2a	156±9a	35.3±1.9a	2.1±0.1 a	21.5±1.8a
P- value	<0.0001	0.002	<0.0001	<0.0001	<0.0001

Values are the means ± SE of 20 intact roots and 25 isolated roots. Data on intact roots was taken 3 wks of culturing the shoots, while for cultured roots final length was recorded after 18 days. Letters denote significance determined using ANOVA at $P = 0.05$ within the column using. Means followed by the same letter within the column are not significantly different. Means followed by the same letters within the column are not significantly different.

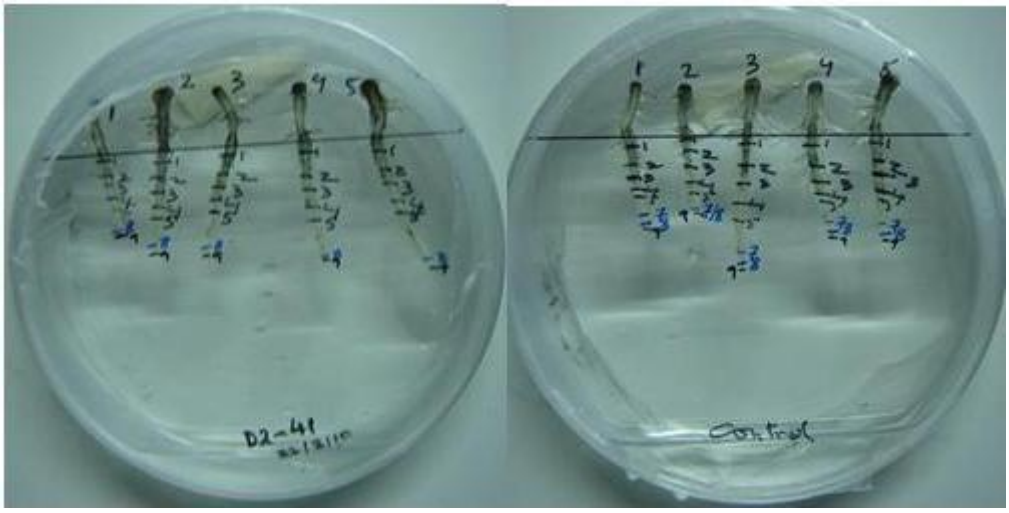


Fig. 5.3 *In vitro* growth of isolated roots of banana plants transformed with *Arath;CyclinD2;1* gene. (A) transformed line D2-41 and (B) non-transformed (control). Representative plates from five replicates are shown.

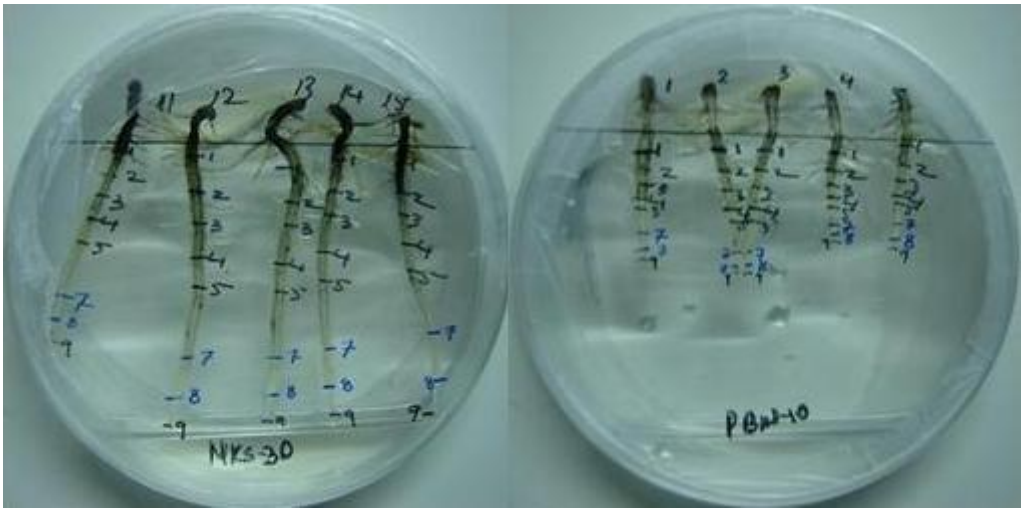


Fig. 5.4 *In vitro* growth of isolated roots of banana plants transformed with the *Musac;CyclinD2;1* gene. (A) transformed line NKS-30 and (B) control carrying an empty vector pBin19. Representative plates from five replicates are shown.

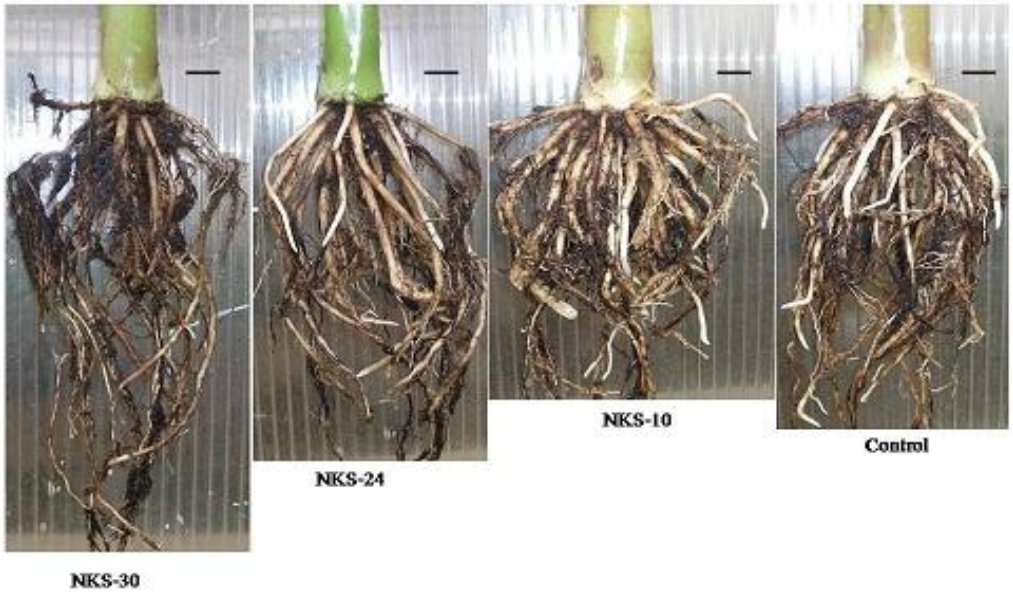


Fig. 5.5 Visual comparison of root systems of three banana lines over-expressing the *Musac;CyclinD2;1* and a control carrying an empty vector pBin19. Size bar = 2 cm.

5.4 Discussion

Results obtained in this study showed differences in growth phenotype among the transgenic lines. Faster leaf growth in banana was observed in lines D2-12 and D2-41 carrying the *Arath;CyclinD2;1* gene and faster root growth in line D2-41. In comparison, notable phenotype from overexpression of *Musac;CyclinD2;1* gene was in root growth that was observed in line NKS-30. The *Arath;CyclinD2;1* transgenic line D2-41 exhibited faster leaf elongation in the first four days after emergence, enrolled earlier and had a bigger lamina. Based on the higher transcript levels of *Arath;CycD2;1*, the faster leaf development together with a significantly faster root growth observed in this transgenic line might have resulted from the transgene. Similar enhanced growth from *Arath;CyclinD2;1* were reported in tobacco (Cockcroft *et al*., 2000; Boucheron *et al*., 2005) and in rice (Oh *et al*., 2008). From the root cultures, the higher number of secondary roots observed in line D2-41 could also possibly be attributed to the *Arath;CyclinD2;1* gene. This *CyclinD2;1* is a close homolog of *CyclinD4;1* whose overexpression in Arabidopsis induced lateral root formation (De Veylder *et al*., 1999).

In banana plants transformed with *Musac;CyclinD2;1*, no difference in aerial shoot growth was observed. Instead, more *in vitro* root initiation and enhanced root growth was observed in line NKS-30. A longer root system was also maintained in potted plants of line NKS-30. For this line, root growth could be attributed to the transgene as in Arabidopsis where overexpression of *Arath;CyclinD2;1* increased the meristematic region in root apices (Masubelele *et al*., 2005). A positive correlation exists between root meristem size and root growth rates and D-type cyclins activate division in the root apex to promote seed germination (Rost and Bryant 1996; Beemster and Baskin 1998). The difference in growth response between the roots and shoots could be attributed to the response of the two organs to the growth conditions in the glass house. According to Walter *et al*. (2009), root growth responds more strongly to temperature and soil moisture.

With reference to the high levels of *Musac;CyclinD2;1* transcripts, the enhanced root development may be attributed to the transgene. Regarding the differentially higher expression of *Musac;CyclinD2;1* in the roots compared to the shoots, it is possible that

the 35S promoter used could be more active in the banana root than in the shoots. Compared to other lines, the exceptionally higher root growth observed in line NKS-30 could be attributed to the site of insersion of *Musac;CyclinD2;1* transgene in this line. Gelvin (2003), reported positional effects where a transgene inserted in a transcriptionally active region of the recipient genome would be highly expressed.

A constant leaf elongation rate in the first four to five days after leaf emergence has been reported in monocotyledonous grass species (Fiorani *et al*., 2000; Bultynck *et al*., 2003). In contrast, banana leaf growth was exponential for this period. It was also noted that the leaf blade did not change in length and width after its emergence, implying that the observed elongation growth was due to the elongation growth of the petiole. This was similar to observations on field grown bananas by Stover and Simmonds, (1998) where the laminar was fully formed by the time of emergence. The difference in elongation growth between the transgenic and the control could be partly attributed to the enhanced meristematic activity by the transgene. Elongation growth, as observed in monocot leaves, is a result of cell division in the basal meristem followed by linear cell elongation (Green, 1976; Bultynck *et al*., 2003). Similarly, cell elongation is reported to proceed more rapidly in roots than leaves (Walter *et al*., 2009) and this could partly explain the higher growth response in roots compared to the above-ground parts of the transgenic plants.

Line D2-41 had bigger laminar and bigger epidermal cells compared to the control. It is likely that the studed banana cultivar has an inherent stable cell cycle mechanism whose enhancement level was not offset. Naturally, banana are distinctively diploids, triploids or tetraploid with no mixoploidy reported (Doleze *et al*., 1997; Pillay *et al*., 2001). Cyclin kinase inhibitors (CKI) that interact with cyclins and CDKs and influence endocytosis are reported to vary with species (Ruhu and John, 2007). This also implies that the cell expansion contributed to the observed difference in leaf size.

In conclusion, *Arath;CyclinD2;1* transgene showed substantial effect on shoot growth in lines D2-12 and D2-41. Transformation with *Musac;CyclinD2;1* caused faster root growth in line NKS-30. Compared to other monocotyledonous species, banana leaf growth displayed a unique exponential growth trend.

CHAPTER SIX

GENERAL CONCLUSION AND RECOMMENDATIONS

In general, studies with transformed banana have the disadvantage that transformed shoots represent the final product of a transformed plant. Banana plants are triploids and predominantly sterile disallowing any self-fertilization and therefore the resultant phenotypes are fixed. In particular, somaclonal variation, caused by the tissue culture process, cannot be excluded which might result in phenotypic changes that are not related to the expression of a transgene (Filipecki and Malepszy, 2006).

A first new aspect of this study was the successful isolation of a *CyclinD2;1* gene coding sequence from banana. This addressed the objective set to isolate a cell cycle (*CyclinD*-type) gene homologue from banana and to determine the level of homology of banana cyclins with those of other plant species. Phylogenic analysis provided strong evidence that the banana cyclin is more related to known monocot cyclinD-types than to the Arabidopsis homolog. This phylogenic grouping was based on the overall amino acid sequence which is reported to be less conserved in cyclins (Vandepoele *et al*., 2002; Menges *et al*., 2007). Further, functional homology between the Arabidopsis and banana *CyclinD* was also possibly sufficient to cause interactive effects of the two orthologs in transformed plants. Particularly in roots, high *Arath;CyclinD2;1* transcription resulted in much lowered transcription of the endogenous banana *CyclinD2;1*.

In a second new aspect, transformed banana were produced over-expressing either the Arabidopsis or the banana *CyclinD* coding sequence to allow phenotypic evaluation. This addressed the set objectives to over-express *Arath;CycD2;1* and *Musac;CycD2;1* in banana and to evaluate the phenotypic effect on the expressed *CyclinD2;1* on the growth and development of banana plants. An interesting observation was that *CyclinD2;1* transformed banana plants had a higher regeneration and transformation rate compared to cells only co-cultured with an empty vector pBin19. Transformation and regeneration competence is reported to be high in cells when transformed while in the actively dividing stage (Arias *et al*., 2006). The banana *CyclinD2;1* was isolated from an actively proliferating cell suspension and was also confined to the banana meristematic shoot and fruit tissue linking it to the cell cycle. *CyclinD* is associated with cell proliferation where it plays a regulatory role at the G1/S transition phase of the cell cycle (Dewitte and Murray, 2003; Inzé and De Veylder, 2006). A future study might

confirm whether over-expression of *CyclinD* can indeed improve banana transformation and *in vitro* shoot regeneration.

A third new aspect of the study was the lack of any relation between various transcript amounts and phenotypic changes found in transformed plants. Plants of one line, D2-41, showed a high leaf elongation rate with an equally high *Arath;CyclinD2;1* transcription. In contrast, plants of line D2-12 with a similar leaf growth phenotype had the lowest transcript amount whereas plants of line D2-3, with an intermediary transcript amount of the *Arath;CycD2;1* and the least affected endogenous *cyclin* genes, showed the lowest leaf growth rate. Several factors may explain the lack of any direct relation between leaf growth phenotype and transcript amounts of a transgene. First, unlike growth measurements that were taken over time, transcription analysis was carried out by a one-time sampling. Thus, any difference in the pot environment could affect the cell cycle and therefore cyclin transcript amounts. Roots growth is reported to be more responsive to temperature and soil water potential (Walter *et al*., 2009). These environmental factors affect growth by restricting cell division through down regulating cyclin genes (Sacks *et al*., 1997; Rymen *et al*., 2007). Such differences can also partly explain the variability in transgene transcription exhibited by plants of the same line. Environmental influence on gene expression has been well-documented (Meyer, 1995; Down *et al*., 2001) and specifically a high response of *CyclinD2;1* transcription to cell cycle stimuli has been, for example, reported for Arabidopsis (Riou-Khamlichi *et al*., 2000; Dewitte and Murray, 2003). Such variability could have been reduced by mass propagation of plants of individual lines and pooling at least three plants as a biological sample instead of using only individual plants for analysis. However, such study would require extensive growth space to house a considerable number of plants with replicates, which is impossible to be carried out in the present growth facilities in Uganda.

A fourth new aspect was that only marginal enhanced leaf growth rates were found for transformed plants. This observation is probably attributed to gene redundancy that could have confirred resilience in the genome of the banana used in the study. Only one *CyclinD2;1* gene was overexpressed out of the large family of cyclins identified in Arabidopsis, maize and rice genomes (Menge *et al*., 2007; Guo *et al*.., 2007; Hu *et al*., 2010). Thus, unlike in tobacco where overexpression of Arabidopsis *CyclinD2;1* gave

remarkable phenotype changes, upregulating only one gene member in a more complex plant like banana might have had limited effect on the growth phenotype. Another explanation could be that the glasshouse conditions under which the aerial growth was evaluated might have limited transformed plants from exhibiting their full growth potential. For example, a better growing root system in a transformed plant, as found in this study for transformed plants, can quickly outgrow the pot volume limiting the growth rate of such plant. Dosselaere *et al.* (2003) observed restricted root growth and development of potted banana plantlets determined by pot size. During the present study, restricted growth was observed when attempts were made to grow banana plants in 50 L potting substrate to maturity. The plants grew bigger but both transformed and non-transformed plants flowered after two years compared to the nine months it would take in field conditions. It is known that final plant yield is determined by developmental and physiological processes, for which a single gene could play a major role (Van Camp, 2005). Therefore, to determine the effect of over-expression of cyclin on yield, a detailed evaluation of the produced transformed plants in a confined field trial will provide a more reliable assessment of the performance of the gene. Such studies will also consider the vegetative and reproductive aspects of the transformed plants. For example floral initiation in banana has been reported to be induced after a given number of leaves have been produced by a banana plant (Stover and Simmonds, 1997; Swennen and De Langhe, 1985). Thus, a plant with faster leaf growth, as found for plants of line D2-41, is likely to flower earlier. In addition, a positive correlation was reported in banana between the number of leaves produced and final bunch weight (Swennen and De Langhe, 1985). Both aspects should also be evaluated in more detail in future studies.

A fifth new aspect of the study showed that roots expressing the banana *CyclinD* exhibited faster *in vitro* root growth and the root system of potted plants of one line, NKS-30, was also visually longer. An extensive root system determines the plant's ability to obtain water and mineral nutrient (Taiz and Zeiger, 2006). The presented study provided first evidence that expression of *CyclinD* gene can be an interesting strategy to change root architecture and to obtain a better developed root system with longer roots. This could be a valuable trait for improving banana productivity in particular to improve drought tolerance. Blomme *et al* (2001) reported a positive

relationship between the banana root system and aerial plant growth. Breeding for extensive root systems was further suggested as one of the strategies to prevent nematode damage (Gowen, 1996). In addition, such root architecture can improve plant anchorage and can prevent plants from toppling under the weight of big bunches and during windy and wet seasons (Tenkouano *et al*., 1998). However, non-transformed plants of the same cultivar that were used to establish transformed plants should be tested if the root phenotype found in transformed plants also exists in a natural population. Therefore, more transformed and non-transformed banana lines should be produced in the future to evaluate in greater detail if *CyclinD* expression is a valuable strategy to improve banana rooting and improve performance against stressful conditions. Also, such studies should involve assessment if any effect of *CyclinD* on the root biomass also directly affects aerial parts.

At the formulation of this study, it was hypothesized that transformation of banana plants with a *CyclinD2;1* gene would accelerate the cell cycle that would result in accelerated banana plant growth. This study found some support for this original working hypothesis. In particular, expression of Arabidopsis *CyclinD2;1* caused faster leaf growth in one transformed banana line (D2-41) while the banana *CyclinD2;1* induced remarkable root growth in plants of line NKS-30. However, future evaluation of these transformed plants under natural growth conditions should be conducted to further support the hypothesis when plants exhibit their full potential and at the same time evaluate their vegetative and flowering phases.

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APPENDICES

Appendix I. Medium (MA2) used for maintaining banana cell suspension.

Group	**Component**	**mg/L**
MS macro nutrients	NH_2NO_2	1,650
	KNO_3	1,900
	$CaCl_2$	332.2
	$MgSO_4$	180.7
	KH_2PO_4	170
MS micro nutrients	$MnSO_4$	15.1
	H_3BO_3	6.2
	$ZnSO_4.7H_2O$	8.6
	KI	0.83
	$Na_2MoO_4.2H_2O$	0.25
	$CuSO_4.5H_2O$	0.025
	$CoCl_2.6H_2O$	0.025
Iron complex	$FeSO_4.7H_2O$	27.9
	$Na_2EDTA.2H_2O$	37.3
Vitamins	Glycine	2.0
	Thiamine-HCl	0.5
	Pyridoxine-HCl	0.5
	Nicotinic acid	0.5
	Ascorbic acid	40
	Myo-inostol	100
	L-Glutamine	99.4
	Malt extract	100
	Biotin	1.0
Phytohormones	2,4-D	1.0
Carbon source	Sucrose	45,000
	pH	5.3

Appendix II. Medium used for re-suspending the Agrobacterium.

Group	Component	mg/L
Macro nutrients	NH_2NO_2	165.0
	KNO_3	190.0
	$CaCl_2$	33.2
	$MgSO_4$	18.1
	KH_2PO_4	17.0
Micro nutrients	$MnSO_4$	15.1
	H_3BO_3	6.2
	$ZnSO_4.7H_2O$	8.6
	KI	0.83
	$Na_2MoO_4.2H_2O$	0.25
	$CuSO_4.5H_2O$	0.025
	$CoCl_2.6H_2O$	0.025
Iron complex	$FeSO_4.7H_2O$	27.9
	$Na_2EDTA.2H_2O$	37.3
Vitamins	Glycine	2.0
	Thiamine-HCl	10
	Pyridoxine-HCl	0.5
	Nicotinic acid	0.5
	Myo-inostol	50
	L-cystein	400
Carbon source	Sucrose	68,500
	Glucose	36,000
Gelling agent	Phytagel	2.3
Acetosyringone		49.0
	pH	5.3

Appendix III. Medium (MA3) used for inducing embryos development.

Group	Component	(mg/L)
SH macro nutrients	$NH_2H_2PO_4$	300
	KNO_3	12,500
	$CaCl_2.2H_2O$	200
	$MgSO_4.7H_2O$	400
SH micro nutrients	$MnSO_4.4H_2O$	10.0
	H_3BO_3	5.0
	$ZnSO_4.7H_2O$	1.0
	KI	1.0
	$Na_2MoO_4.2H_2O$	0.1
	$CuSO_4.5H_2O$	0.2
	$CoCl_2.6H_2O$	0.1
Iron complex	$FeSO_4.7H_2O$	15.0
	$Na_2EDTA.2H_2O$	20.0
Vitamins	Glycine	2.0
	Thiamine-HCl	0.5
	Pyridoxine-HCl	0.5
	Nicotinic acid	0.5
	Ascorbic acid	40
	Myo-inostol	100
	L-Glutamine	100
	Malt extract	100
	Biotin	1.0
	L-Proline	230
Carbon source	Sucrose	45,000
	A-Lactose monohydrate	10
Phytohormones	NAA	0.2
	Zeatin	0.05
	Kinetin	0.1
	2ip	0.2
Gelling agent	Phytagel	3.0
	pH	5.8