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

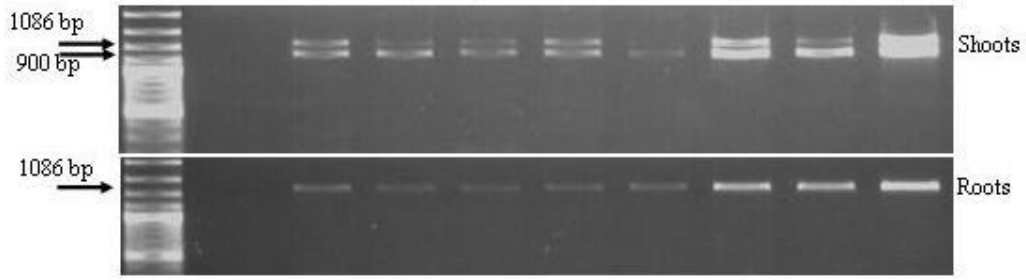
To identify the tissue specificity of the isolated *Musac;CyclinD2;1* in the banana plants, a semi-quantitative RT-PCR analysis was conducted on cDNA from different banana plant tissues. The band intensity was highest in the shoot tip, lower in young leaf and young fruit (14 days after flowering), while 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.

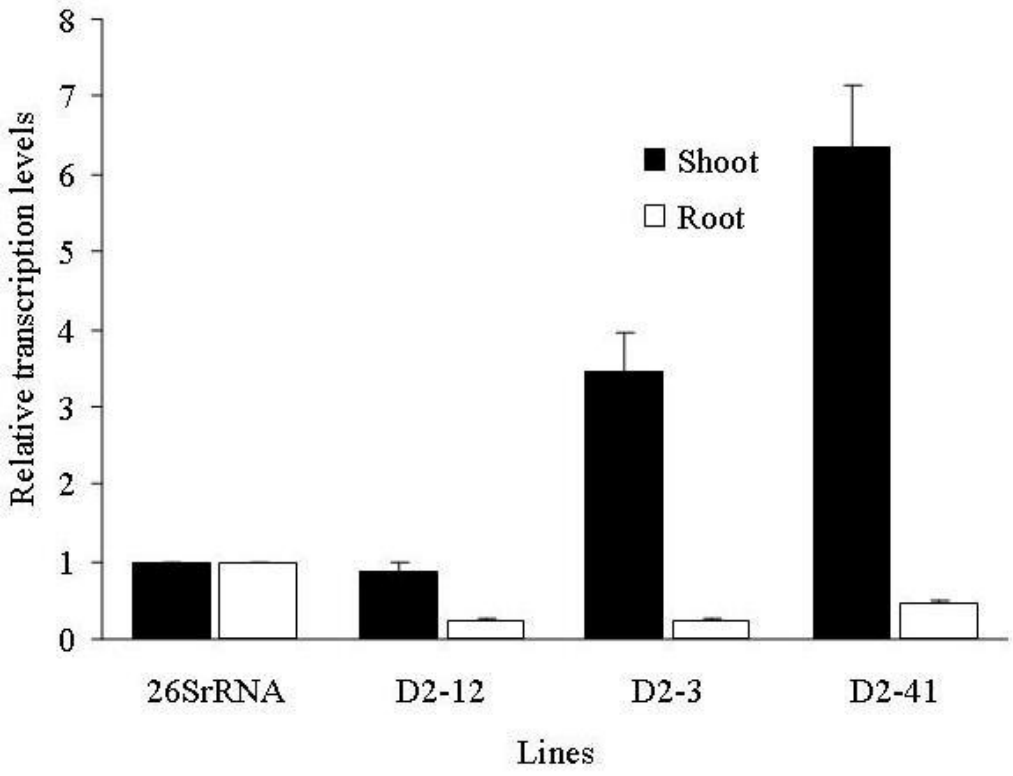


Fig. 4.3 Comparison of transcription of *Arath;CyclinD2;1* transgene in shoot and root apices of banana plants transformed with *Arath;CyclinD2;1* gene coding sequence. Transcription levels are relative to the transcription of the reference *Musa* 26S rRNA gene. Bars are means ± SE of three plants.

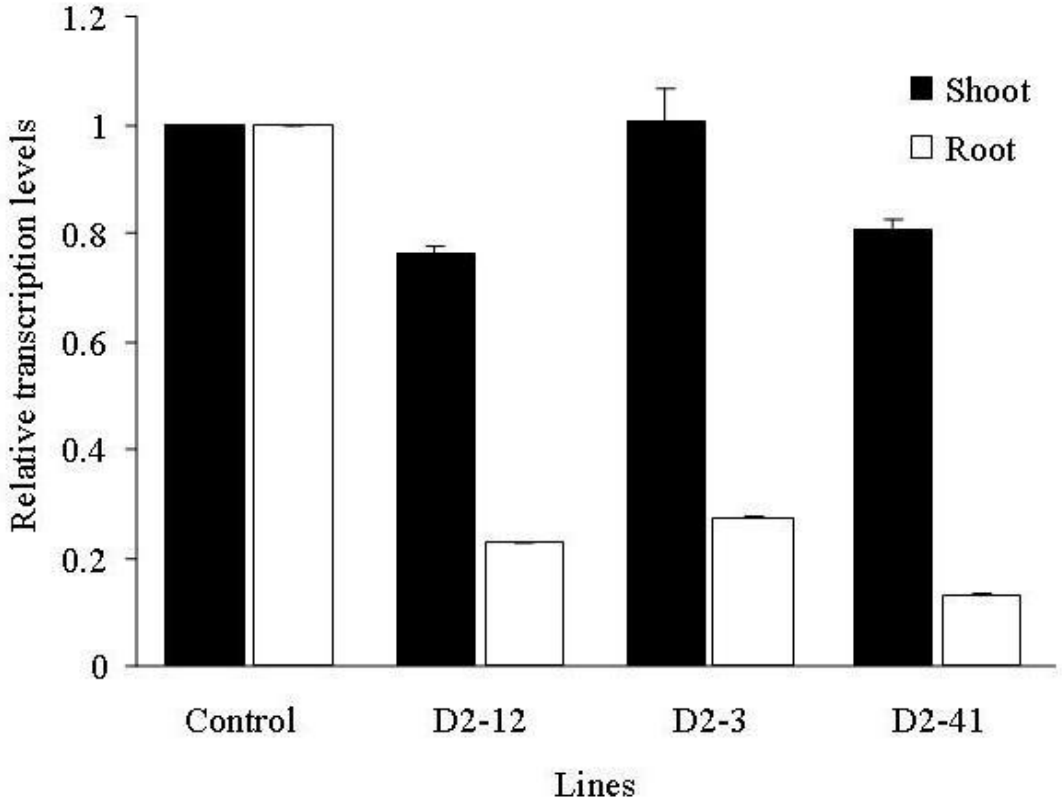


Fig. 4.4 Comparison of transcription of indigenous *Musac;CyclinD2;1* gene in shoots and root apices of banana plants transformed with *Arath;CyclinD;1* gene coding sequence. Transcription levels are relative to the transcription of the same gene in non-transgenic plants (control). Bars are means ± SE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

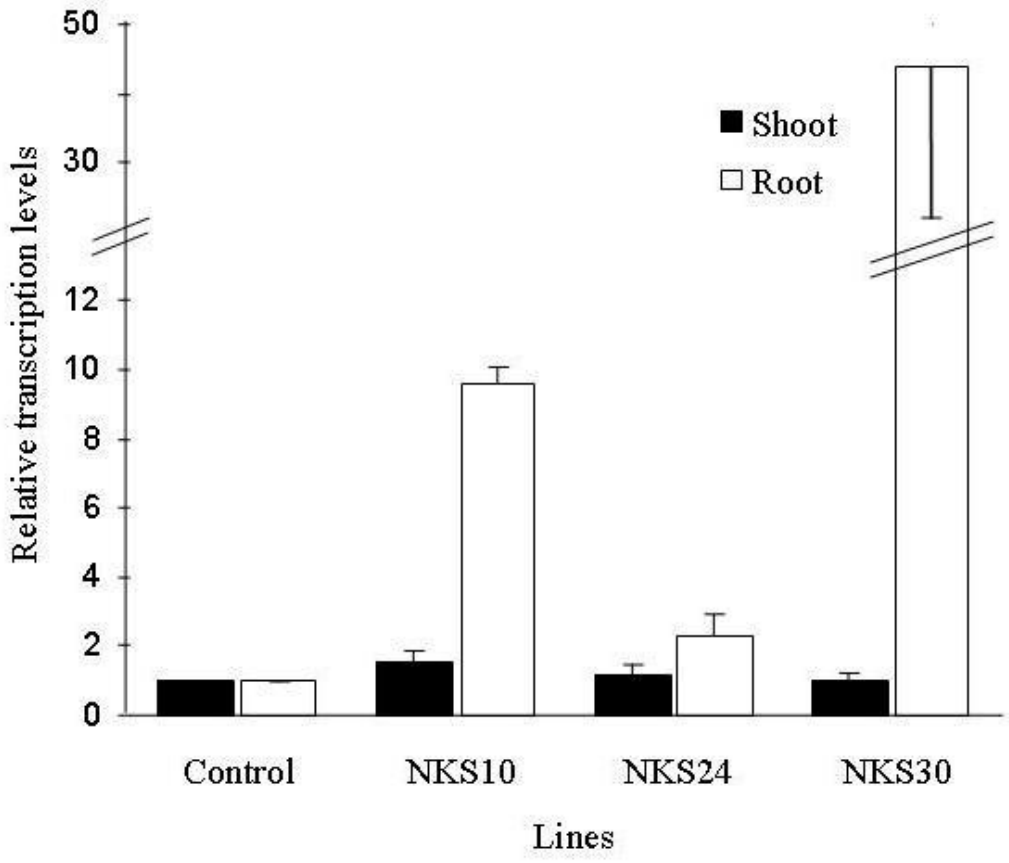


Fig. 4.5 Comparison of transcription of *Musac;CyclinD2;1* in shoot and root apices of banana plants transformed with a *Musac;CyclinD2;1* gene coding sequence. Transcription levels are relative to the expression of the same gene plants transformed with an empty vector, pBin19. Bars are means ± SE of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

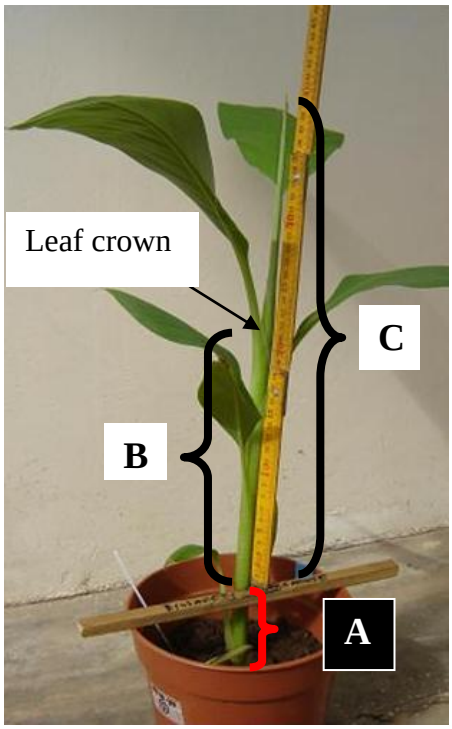
5.2.2 Establishment of transformed plantlets

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).

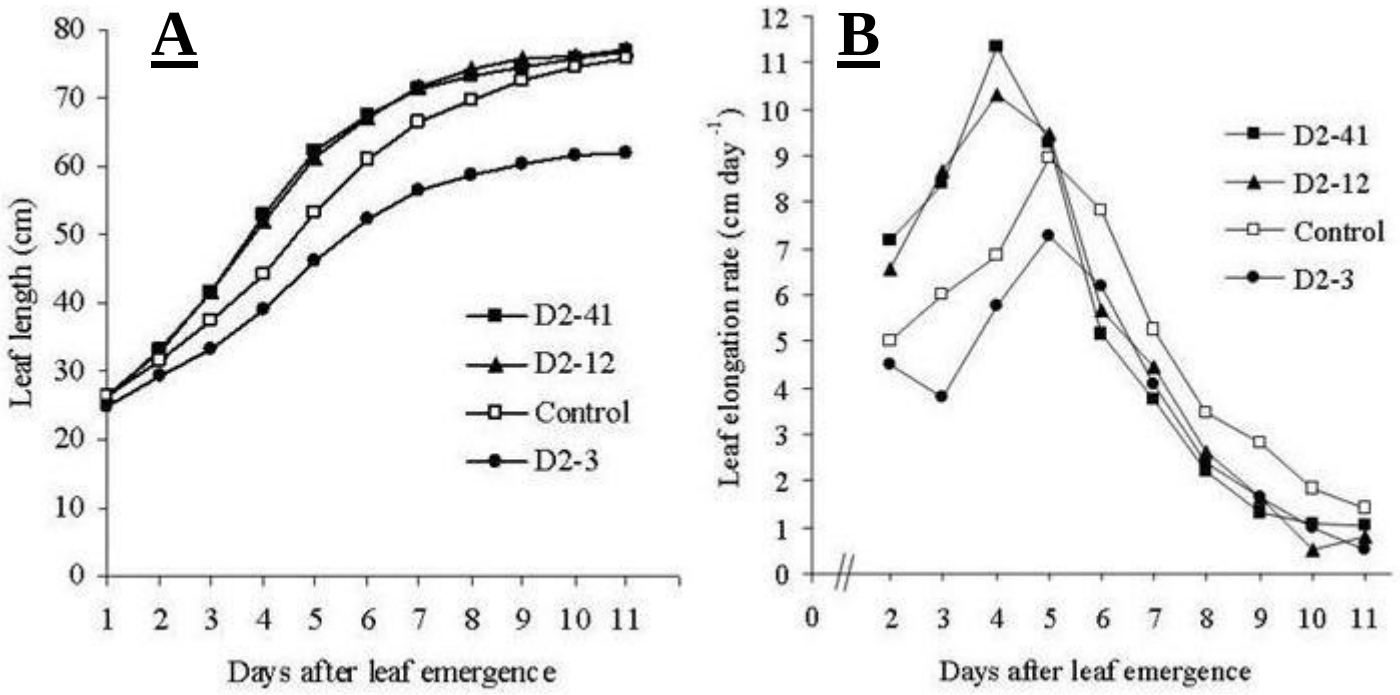


Fig. 5.2 Increase in leaf length over time (A) and leaf elongation rate (B) of banana plants transformed with *Arath;CyclinD2;1* and non-transformed plants (control). Data points are the mean of 3 plants.

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.

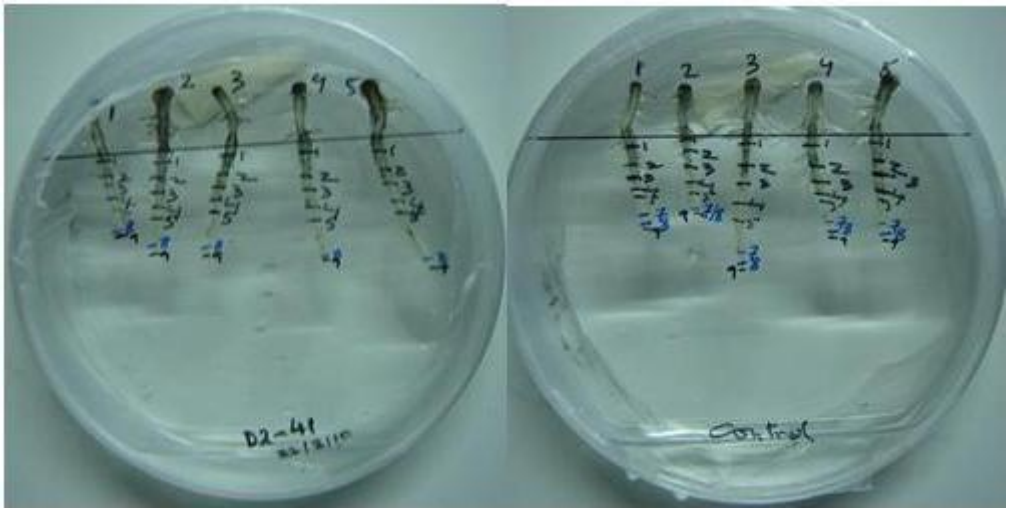


Fig. 5.3 *In vitro* growth of isolated roots of banana plants transformed with *Arath;CyclinD2;1* gene. (A) transformed line D2-41 and (B) non-transformed (control). Representative plates from five replicates are shown.

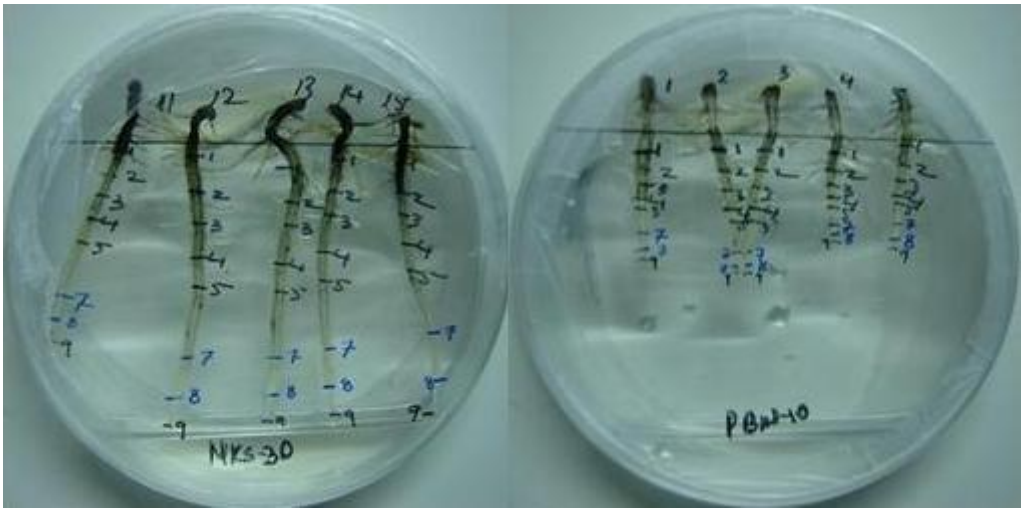


Fig. 5.4 *In vitro* growth of isolated roots of banana plants transformed with the *Musac;CyclinD2;1* gene. (A) transformed line NKS-30 and (B) control carrying an empty vector pBin19. Representative plates from five replicates are shown.

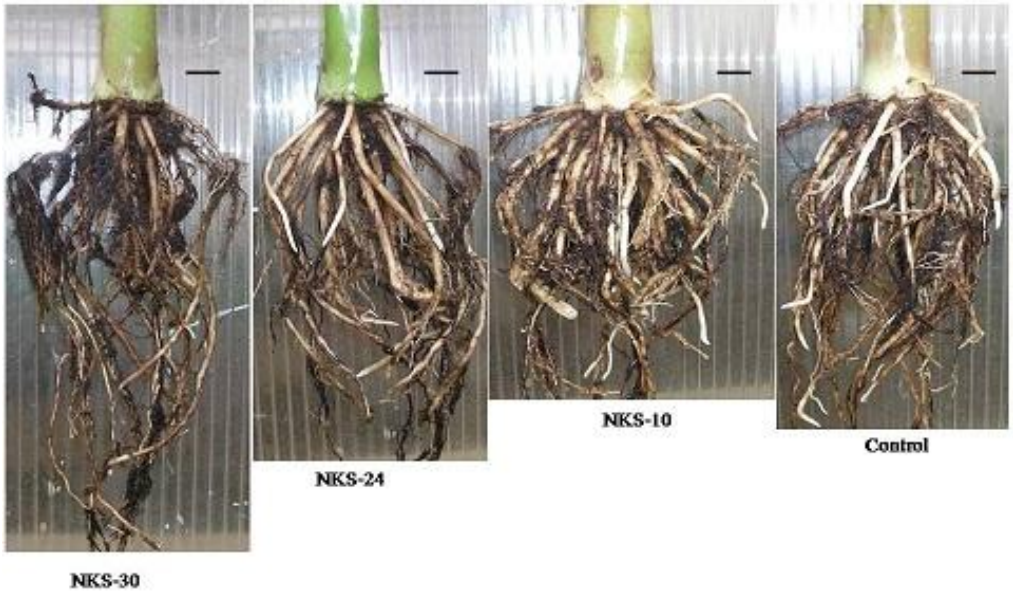


Fig. 5.5 Visual comparison of root systems of three banana lines over-expressing the *Musac;CyclinD2;1* and a control carrying an empty vector pBin19. Size bar = 2 cm.

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.