African Journal of Biotechnology Vol. 10(65), pp. 14254-14263, 24 October, 2011
Available online at http://www.academicjournals.org/AJB
DOI: 10.5897/AJB11.1169
ISSN 1684–5315 © 2011 Academic Journals

Full Length Research Paper

Analysis of gene expression by promoter reporter constructs and knockout plants: A case study under phosphorus stress

Kefyalew Negisho[1]*, Tesfaye Balemi[2] and Belayneh Admassu[1]

[1]Holetta Agricultural Research Center, National Agricultural Biotechnology Research Programme, Ethiopian Institute of Agricultural Research, Ethiopia.
[2]Department of Plant Science and Horticulture, College of Agricultural and Veterinary Science, Ambo University, Ethiopia.

Accepted 21 July, 2011

Promoter reporter constructs were developed using Gateway Cloning Technology for plant non-specific lipid transport proteins (nsLTP1, AT4g12545 and nsLTP2, AT5g46900), MADS-Box (AT4g37940) and leucine rich repeat (LRR, AT2g28970) genes. The purpose of the construct was to investigate gene expression and function under phosphorus (P) stress. *Agrobacterium tumefaciens* was used to transform *Arabidopsis thaliana*. Transformed (T_1), knockout and wild type ecotype Colombia-0 *A. thaliana* were investigated. To monitor the gene activity, plants were grown under different conditions: fully inside solid media, on the surface of solid media and hydroponically with 0.5 mM l^{-1} phosphorus and without phosphorus. Plants were also grown on MS media with selectable marker containing 50 mM l^{-1} Kanamycin. Morphological root assessment was also done by using knockout plants for the respective promoters. The promoters under study were induced promoter. GUS staining was observed on T_1 plants when planted fully inside solid media and on the selectable marker for all the constructs regardless of phosphorus treatments. Mechanical impedance, oxygen deficiency and stress due to selectable marker were the probable reasons for the root specific gene expression. This suggests that these genes were involved in adaptation mechanism for plants under such stress conditions.

Key words: *Arabidopsis thaliana,* gene expression, kanamycin, knockout plants, LRR (leucine rich repeat), MADS-Box, nsLTPs (non-specific lipid transport proteins), selectable marker.

INTRODUCTION

Analysis of promoter reporter gene fusions is one of the most widely used techniques for identifying sequences that control temporal and spatial regulation of cloned genes. Gene fusion under which promoter controls the open reading frame of reporter gene(s) helps to study the expression and function of a given gene depending on internal and external signals. Promoter, which is usually the upstream of the coding sequence of a gene, plays a pivotal role in controlling gene expression and directs the RNA polymerase to the correct transcriptional start site. Thus, permits the initiation of transcription in plants (Galune and Breiman, 1997; Buchanan et al., 2000; Smale and Kadonaga, 2003). Promoter can be of two types: constitutive promoter that controls gene expression throughout the life cycle of the plant, and induced type of promoter that controls temporal and spatial gene expression depending on the environmental factor(s) (Werner, 1999). Transcriptional and translational fusions of a promoter or gene of interest to the GUS reporter allows assay of gene expression in a quantitative and qualitative manner. By using gene fusions to individual members of gene families and introducing these fusions into a germline, one can study the expression of individual genes separate from the background of the members of the gene family (Jefferson, 1987).

*Corresponding author. E-mail: kefalg@yahoo.com. Tel: 251-(0)-912861872. Fax: 251-(0)-112370377.

Plants interact with abiotic and biotic factors for the completion of developmental and sexual maturation. Genes are expressed in response to these environmental factors at different parts and ages of the plant and many of the responses require transcription and translation of specific genes (Schmid et al., 2005). Therefore, an understanding of how plants can cope with a fluctuating environment has a practical importance in gene expression study and helps explore the function the genes.

Not all genes are turned on at all times during the life cycle of plant but different genes are expressed at certain degree. Genes having similarity with gene of plant non-specific lipid transfer proteins (nsLTPs), MADS-Box and leucine-rich repeat (LRR) of the model plant *Arabidopsis thaliana*, were differentially expressed in roots of Ethiopian mustard (*Brassica carinata)* genotypes: efficient ($Bale_1$) and inefficient ($Bacho_2$) under phosphorus deficiency (Bremer and Schenk, 2009).

The suggested novel roles of plant non-specific lipid transfer proteins are: participation in defence reactions against phytopathogens, cutin formation, embryogenesis, symbiosis, and adaptation of plants to various environmental conditions (Sterk et al., 1991; Kader, 1997; Nieuwland et al., 2005; Stanislava, 2007). MADS-Box genes encode a family of transcription factor genes that mediate the binding of RNA polymerase and the initiation of transcription and changes in them are likely to be key molecular determinants of the morphological evolution of plants (Schwechheimer and Bevan, 1998; Doebley and Lukens, 1998). Leucine rich repeat (LRR) domains are involved in protein -protein interactions and are found in many proteins. LRR involves in disease resistance, signalling pathways, or the regulation of extracellular enzymes (Forsthoefel et al., 2005).

To illustrate response of plants to abiotic stresses, promoter regions of the differentially expressed plant non-specific lipid transfer proteins (nsLTPs), transcription factor gene MADS-Box and Leucine-rich repeat (LRR) genes were fused with reporter gene. Transformed plants (T_1) and wild type plants were investigated. Additionally, seeds of knockout plants for nsLTPs and MADS-Box genes were obtained from the Nottingham University, UK for the study. Hence, the study was aimed to analyze the expression of these differentially expressed genes by promoter::GUS (β-glucuronidase gene) and the knockout plants under phosphorus stress.

MATERIALS AND METHODS

Preparation of promoter constructs

To analyse promoter activity, the region upstream from the coding regions of nsLTP1 (AT4G12545), nsLTP2 (AT5G46900), MADS-Box (AT4G37940) and LRR (AT2G28970) genes were taken from the National Centre for Biotechnology Information (NCBI). The forward and reverse primers were designed from the promoter region using Primer3 (Rozen and Skaletsky, 2000). Four nucleotides (CACC) were added to 5' end of the forward primers to aid the directional pENTR™/D-TOPO® cloning.

Plant materials and genomic DNA isolation

Seeds of *A. thaliana* ecotype Colombia-0 were obtained from Prof. Dr. G.F.E. Scherer, University of Hanover. Seeds were surface sterilized using 70% ethanol and 6% sodium hypochlorite + 0.02% Triton ×-100. To break dormancy and to synchronize germination, seeds were imbibed in sterile distilled water and placed in dark room for 3 days at 4°C. The seeds were grown in a greenhouse under long day (16/8 h day/night) condition at 23°C. To isolate genomic DNA, few leaves were frozen in liquid nitrogen and grinded (Dolferus, 1991). Shorty buffer and 10 mg ml^{-1} RNAse A were added to release the nucleic acid, maintain the pH and digest RNA. The mixture was centrifuged at 16,000 ×*g* in a Biofuge Pico (Germany) at room temperature for 5 min. The resulting 500 µl supernatant was taken to a new 1.5 ml reaction tube and 550 µl of a 25:24:1 mix of phenol chloroform and isoamyl alcohol, respectively was added to precipitate protein and cellular debris. Protein and cellular debris were pelleted by centrifugation for 5 min at 16,000 ×*g*. 480 µl of supernatant was taken and 500 µl isopropanol was added and further centrifuged at 16,000 ×*g* for 5 min to precipitate the DNA. The supernatant was discarded and the DNA pellet was washed with 70% ethanol. The DNA pellet was dissolved with sterile double distilled water and used as template.

PCR condition

PCR reaction mixture contained 0.5 µM of each primers, 25 ng template DNA, 10 mM each dNTP, 2.5 units of DNA polymerase and 1× PCR buffer. The PCR profile included an initial step at 94°C for 3 min, followed by 35 cycles at 94°C for 30 s at respective primer annealing (at 64°C for nsLTP 1 and nsLTP2, 58°C for MADS-Box, LRR and Actin, and 65°C for GUS), 72°C for 2 min, and final extension at 72°C for 7 min. Gel documentation was done on 1% agarose containing final concentration of 0.5 µg/ml ethidium bromide and visualized on an INTAS Gel Jet Imager (Germany).

Cloning

TOPO TA and entry clones were undertaken following the Invitrogen kits (Invitrogen). QIAGEN purified PCR product was sent for sequencing to IIT Biotech (Bielefeled, Germany). Plasmids having 100% similarity with genes of interest after BLAST analysis were used to undertake the LR reaction to produce the expression clones. The expression clones were produced by LR reaction performed between entry clone (promoter + pENTR™/DTOPO® Vector) and destination vector (pKGWFS7). The recombination reaction were done in 1.5 ml reaction tube at room temperature by mixing 5 µl of 100 ng $µl^{-1}$ entry clone, 1 µl of 150 ng $µl^{-1}$ destination vector, 8 µl of pH 8.0 TE buffer and 2 µl of LR clonase II enzyme mix. The reaction mixture was initially incubated for 4 h at 25°C and later for 10 min at 37°C by adding 1 µl of proteinase K solution.

Transformation of competent bacterial cells

One shot chemically competent TOP10 *E. coli* cells (Invitrogen) were transformed by 2 µl of the ligation reaction by heat shock transformation method. The transformed *E. coli* cells were grown on Lauria Bertani (LB) medium (10 g of NaCl, 5 g of Bacto-yeast extract and 10 g Bacto-trypton per L at pH 7.5) at 37°C for 16 h. Selection of the transformed cells was done by growing on LB medium containing 50 µg/ml Kanamycin after transforming *E. coli*

with pCR®2.1-TOPO® Vector and pENTR™/D-TOPO® Vector. This is because both vectors carry gene region that is resistance to the selectable marker kanamycin. Whereas, selection of the transformed cells was done on LB medium containing 100 µg/ml Spectinomycin after transforming *E. coli* with destination vector (pKGWFS7).

The chemically competent *Agrobacterium tumefaciens* strain GV3101 was prepared. The competent cells were transformed with the expression clones (promoter + pKGWFS7) by the freeze-thaw method (Höfgen and Willmizer, 1988). The transformed cells were spread on LB medium containing 100 µg/ml spectinomycin, and 100 µg/ml rifampicin, and incubated for three days at 28°C. Since *A. tumefaciens* strain GV3101 has resistance gene to rfampicin and the destination vector used to transform agrobactium carries spectinomycin resistance gene. Thus, rifampicin and spectinomycin were used as selectable markers to select the transformed agrobacterium cells before plant transformation. Plant transformation was done by floral dip and vacuum infiltration (Bechtold et al., 1993; Bent, 2000).

Screening of transgenic *Arabidopsis thaliana*

First harvested seeds after transformation of plant (T_0) were surface sterilized with 70% ethanol by shaking them vigorously for 10 minutes. The ethanol was removed and the seeds were washed three times with sterile distilled water. MS medium (1% phytoagar + 2% sucrose) was supplemented with 50 µg/ml kanamycin and plates were kept under long day in growth chamber at 23°C for 10 days to distinguish between transgenic and non-transgenic seedlings. Clearly distinguished transgenic seedlings were grown in the greenhouse and self pollinated to harvest T_1 seeds.

Hydroponic growing of T_1, wild type and knockout to investigate the activity of genes

Simple technique was established to grow the model plant hydroponically. Horizontal none transparent plastic pots with volume of 5 L were used for growing *Arabidopsis* hydroponically. Styrofoam was cut to fit into the size of the plastic pot and 2 cm diameter holes were punched out using cork borer on the Styrofoam with the distance of 4 cm between two holes. Using the same cork borer ,rock wool was cut and placed in the punched holes of Styrofoam. The pierced Styrofoam was filled with rock wool, and fitted into the plastic pots containing nutrient solution. *Arabidopsis* nutrient solution contained the following macronutrients (4 mM $Ca(NO_3)_2.4H_2O$, 0.5 mM $(NH_4)2SO_4$, 2 mM $Ca(NO_3)_2.4H_2O$, 0.5 mM $NaH_2PO_4.2H_2O$, 1.5 mM K_2SO_4, 0.5 mM $MgSO_4.7H_2O$), and micronutrients (0.5 µM $CuSO_4$, 25 µM H_3BO_3, 1.5 µM $MnSO_4.H_2O$, 0.025 µM $Na_2MoO_4.2H_2O$, 1.5 µM $ZnSO_4.7H_2O$ and 20 µM FeEDTA). Sterilized wild type, T_1 and the knockout seeds were placed inside the rock wool, which was inserted in the hole of Styrofoam for germination. After 5 days of growth in full nutrient solution, the seedlings were transferred to pots containing 0.5 mM phosphorus L^{-1} and to pots without phosphorus. The nutrient solution was changed in each subsequent week until flowering. The experiment was replicated three times. GUS staining and visualization was undertaken after 8 and 15 days of P starvation and at the time of flowering.

Growing T_1 and wild type on the surface of solid media to investigate the activity of genes

Wild type and T_1 seeds were sterilized and grown on the MS media. T_1 seeds were grown MS media for containing 50 µg/ml kanamycin for T_1 for 10 days. Ten clearly distinguished transformed plants were transferred to MS media containing 0.5 mM phosphorus L^{-1} and without phosphorus. The seedlings were grown on the surface of MS without selectable markers for 10 and 15 days and were stained. Similarly, T_1 seeds were grown on the MS media containing 50 µg/ml kanamycin for 21 days and were stained. The experiment was replicated three times.

Growing T_1 and wild type fully inside the solid media to investigate the activity of genes

The solid media was prepared by adding 1% agarose into *Arabidopsis* nutrient solution. Autoclaved media was poured onto 100 mm SQ PS Petri dish (Australia) and when solidified, 4 cm portion of the media was cut out under sterile condition. Ten T_1 from each construct and wild type seeds were placed on the cut part inside the thick solid media. The experiment was replicated three times treated with phosphorus and without phosphorus. The seedlings were grown in the growth chamber under long 16/8 h day at 23°C.

Plant tissue assay for gene activity

Histochemical analysis of GUS activity was performed as described by Jefferson et al. (1987). Whole plant part and excised root tissues were incubated at 25°C for 4 to 24 h in a 50 mM $NaPO_4$ (pH 7.5, 5 mM $K_3Fe(CN)_6$, 5 mM $K_4Fe(CN)_6$, 0.2% Triton ×-100) containing 2 mM 5-bromo-4-chloro-3-indoyl glucuronide (X-Gluc). The gene expression for stained plants and tissue parts were visualized with AxioSkop2 mounted with Axiocam MRC5 (Germany).

RESULTS AND DISCUSSION

Defining promoter region and primer design

Promoter sizes taken from the data base were 1,218, 1,381, 1,768, and 1,690 bp for nsLTP1, nsLTP2, MADS-Box and LRR, respectively. Primers used to amplify the respective promoter region are indicated in Table 1. The desired promoter size was confirmed by amplification after genomic DNA isolation, after each cloning, and before plant transformation. The promoter sizes were compared with DNA ladder, positive (actin) and negative (sdH_2O) controls. The size of each promoter was as it was expected (Figure 1A and B). The existence of the GUS gene was confirmed after *Agrobacterium tumefaciens* transformation Figure 1B. This helps to know the existence of both promoters and reporter gene in the vector before plant transformation.

Cloning and sub-cloning of DNA fragments into different vectors

The amplified promoter fragment was sub-cloned into Gateway cloning vectors and subsequently to expression vector. This forms a new novel recombined DNA molecule at which the promoter and the GUS reporter gene had fused. The sequencing results in this study have shown 100% identity for all the cloned and sequenced promoter regions after entry cloning. The key to cloning a

Table 1. Accession number, forward (FP) and reverse (RP) primers, primers length in base pair (bp), sequence of forward and reverse primers and promoters and reporter GUS product size (bp).

Accession number	FP/RP	Primer length	Primer sequence	Product size (bp)
At4G12545/nsLTP1	FP	20	5' *CACC*TTGTGGTGAACTGGTG 3'	1,218
	RP	20	5' TGCAAGAGAGGTTCTTGGAG 3'	
At5G46900/nsLTP2	FP	21	5' CACCTGCAGATATTGGGCCTA 3'	1,381
	RP	23	5' GATGAGGAGAAGAGCAATCTTAG 3'	
At4G37940/MADS-B	FP	20	5' CACCGGGATTTGGTCACTTG 3'	1,768
	RP	20	5' TTGGATCACAATCTTCCCTC 3'	
At2G28970/LRR	FP	20	5' CACCGAGCTTGAACAAGATG 3'	1,690
	RP	20	5' GGCAAAGGTTCCAATTATGG 3'	
GUS	FP	20	5' GGCACAGCACATCAAAGAGA 3'	300
	RP	20	5' TGCTGTCGGCTTTAACCTCT 3'	

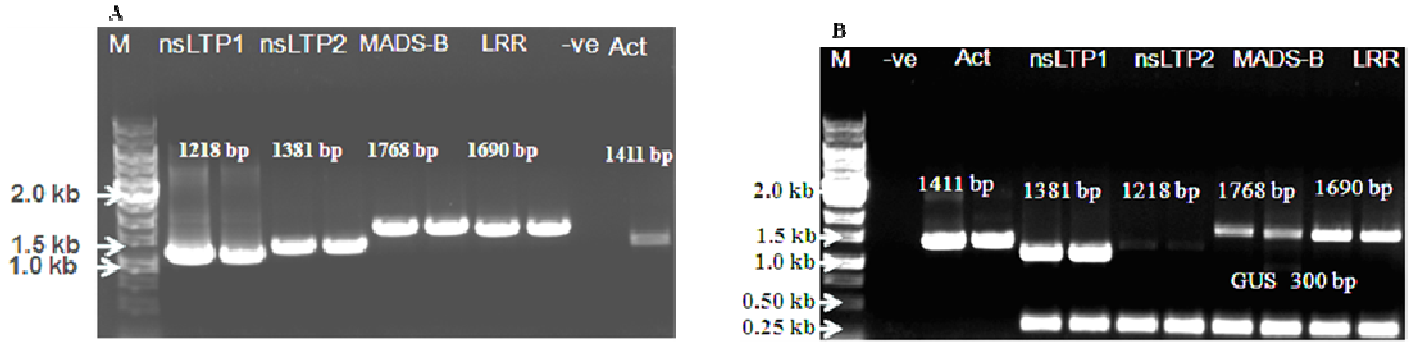

Figure 1. PCR detection of promoter region of nsLTP1, nsLTP2, MADS-Box, LRR, sterile double distilled water (sdH_2O) as a template for the negative control and actin primers were used as positive control. Promoter region amplified using plasmid as a template after entry clone and promoter region amplified using plasmid as a template after transformation of *Agrobacterium tumefaciens* (A); 300 bp reporter gene was amplified before plant transformation. Two lanes were used for each promoter. M: 1 kb DNA ladder (B).

DNA fragment of interest is to link it to a plasmid vector DNA molecule, which helps to replicate within a host cell. DNA fragments with either sticky ends or blunt ends can be inserted into plasmid vectors through Gateway cloning. Further, clonase enzyme II mix (Invitrogen) was responsible for recombination of *att*L with *att*R sites and the insert can then be mobilized into any destination vector having *att*R sites via an LR reaction (Magnani et al., 2006).

Plant transformation mediated *Agrobacterium tumefaciens*

Healthy plants were ready for transformation at the time of flower initiation and transformed plants were covered with plastic to maintain moisture (Figure 2A and B). Results reveal that several individual transgenic lines carrying promoter-GUS construct were produced with 5 to 7% transformation efficiency (Figure 2C). Bacterial suspension carrying right construct, healthy plants, early flowering stage and repeated transformation help to get enough T_0 seeds and eventually increase the transformation efficiency (Desfeux et al., 2000). T_0 plants were self pollinated to produce T_1 seeds (Figure 2D). Genotypic ratio of T_1 after screening on selectable marker was 3:1 for transformed and untransformed plants, respectively (Table 2). This suggests that the transformation was efficient, stable and inheritable according to the Mendele's law of segregation.

Plant tissue assay for gene activity

Transgenic (T_1), wild type and knockout plants of *A. thaliana* were grown hydroponically with and without

Table 2. Chi-square result of genotypic ratio of T_1 and wild type after screening the seeds on selectable marker to estimate efficiency transformation, heritability and stability of the gene of interest.

Gene	Type	O	E	X^2_{cal}	$X^2_{1(0.05)}$
nsLTP1	T_1	762	740.25	2.6	3.8
	WT	225	246.75		
nsLTP2	T_1	749	747.75	0.01	
	WT	248	249.25		
MADS-Box	T_1	750	741.75	0.37	
	WT	239	247.25		
LRR	T_1	756	750	0.19	
	WT	244	250		

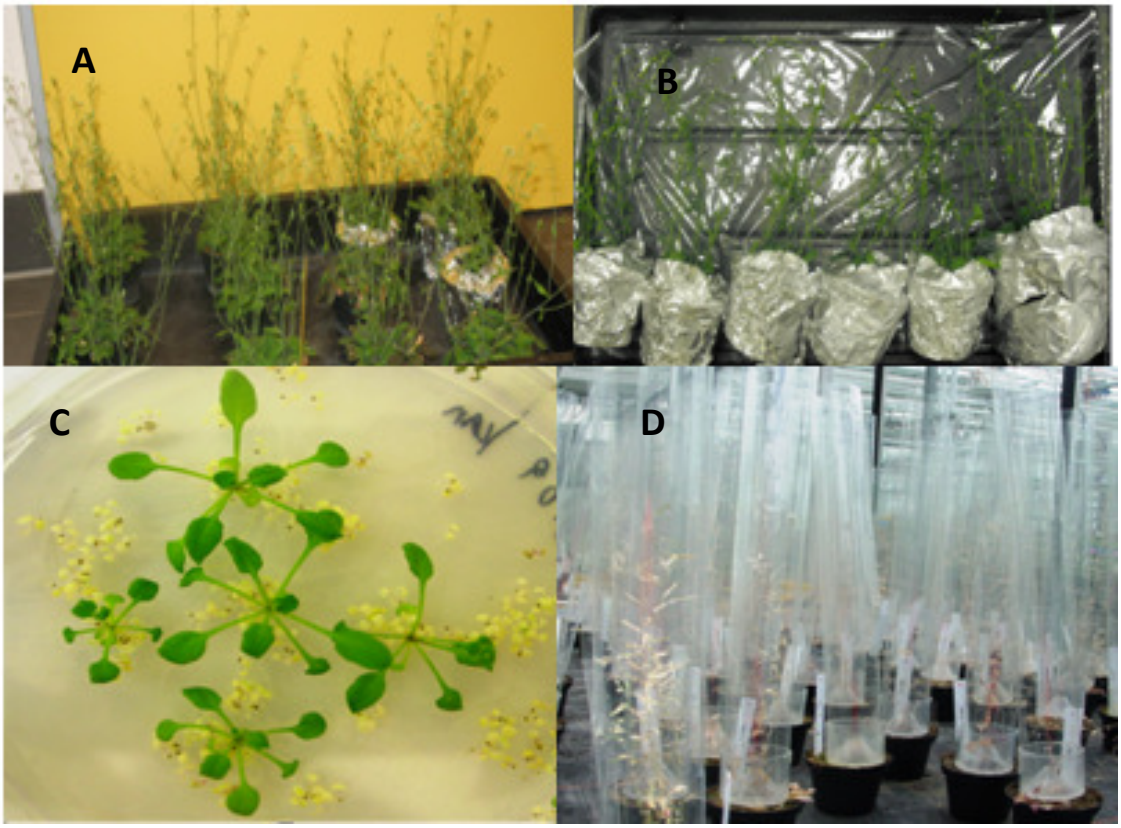

Figure 2. Steps towards harvesting T_1 seeds; healthy wild type grown until flower initiation (A), transformed plants covered overnight with plastic to maintain 100% moisture (B), screening T_0 seeds on 50 µg ml^{-1} kanamycin (C), vigorous and deep green transformants were transferred to pots and self pollinated in the greenhouse until they set T_1 seeds (D).

phosphorus (P) (Figure 3A and B). Similarly, T_1 and wild type were grown on the surface of MS media with and without P (data not shown). In both cases, regardless of the phosphorus deficiency symptoms, there was no reporter gene expression for T_1 plants at the time of staining (Figure 3A and B). There was also no clear difference in terms of root morphology between knockout and wild type grown with phosphorus and without phosphorus. This indicates that Phosphorus does not contribute for gene expression.

On the other hand, there was reporter gene expression when T_1 plants were grown on MS media containing

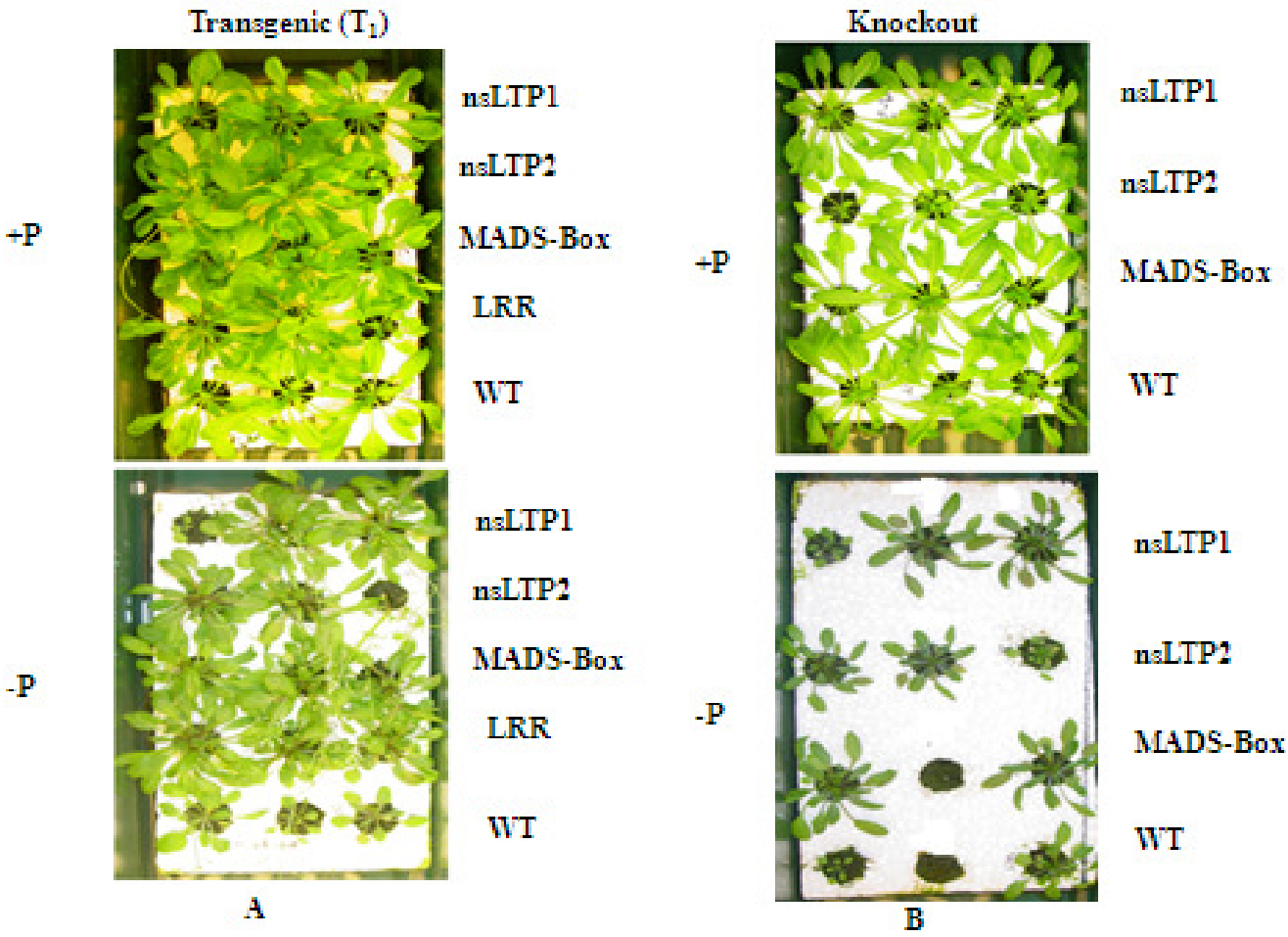

Figure 3. *Arabidopsis thaliana* grown hydroponically: wild type, T_1 (A) and knockout plants for nsLTP1, nsLTP2 and MADS-Box genes (B) grown with and without phosphorus.

kanamycin and fully inside solid media. Despite the differences in the types of construct, there was reporter gene expression when T_1 plants were grown with and without phosphorus. However, there were differences in reporter gene expression patterns among the constructs (Figures 4 and 5).

The reporter gene expression pattern in nsLTP1 was strong at the root tips and was distributed throughout the root system (Figure 4C, D, K and L). Whereas, the expressions were at the primary and secondary root tips extending back to meristematic, elongation and maturation zones in MADS-Box and LRR genes (Figure 4E, F, M, and N; Figure 4G, H, O and P), respectively.

Reporter gene expression was also observed in all T_1 plants grown on MS media containing kanamycin. The expressions were intense and it was all over the root systems in the cases of nsLTP1 and nsLTP2 constructs (Figure 5A and B). There was strong staining at the root tips of primary and secondary roots in MADS-Box, (Figure 5D). Whereas, the reporter gene expression in the case of the LRR construct was very slight (Figure 5E). This indicated that these genes were highly involved in the root system to overcome the metabolic burden that is created probably due to the high expression of selectable marker to degrade the kanamycin. This is inline with research result that states marker gene likely incur a substantial metabolic burden in cases where the marker protein constitutes as much as 10% of total soluble cellular protein (Maliga, 2002). It has also been mentioned that there exist unintended effects of the selectable marker gene or its regulatory elements and genetic elements at the site of insertion (Brian et al., 2008).

Reporter gene expression was not observed in any of the stained wild type plant grown with and without phosphorus under different growth conditions (Figure 3A and B; Figure 4A, B, I and J; Figure 5A). This showed that there was no reporter gene (Auid) background in the wild plant to interfere with reporter gene that was fused with the promoter as stated by Jefferson et al. (1987).

The root specific reporter gene expression in all the constructs indicated that these promoters have role in

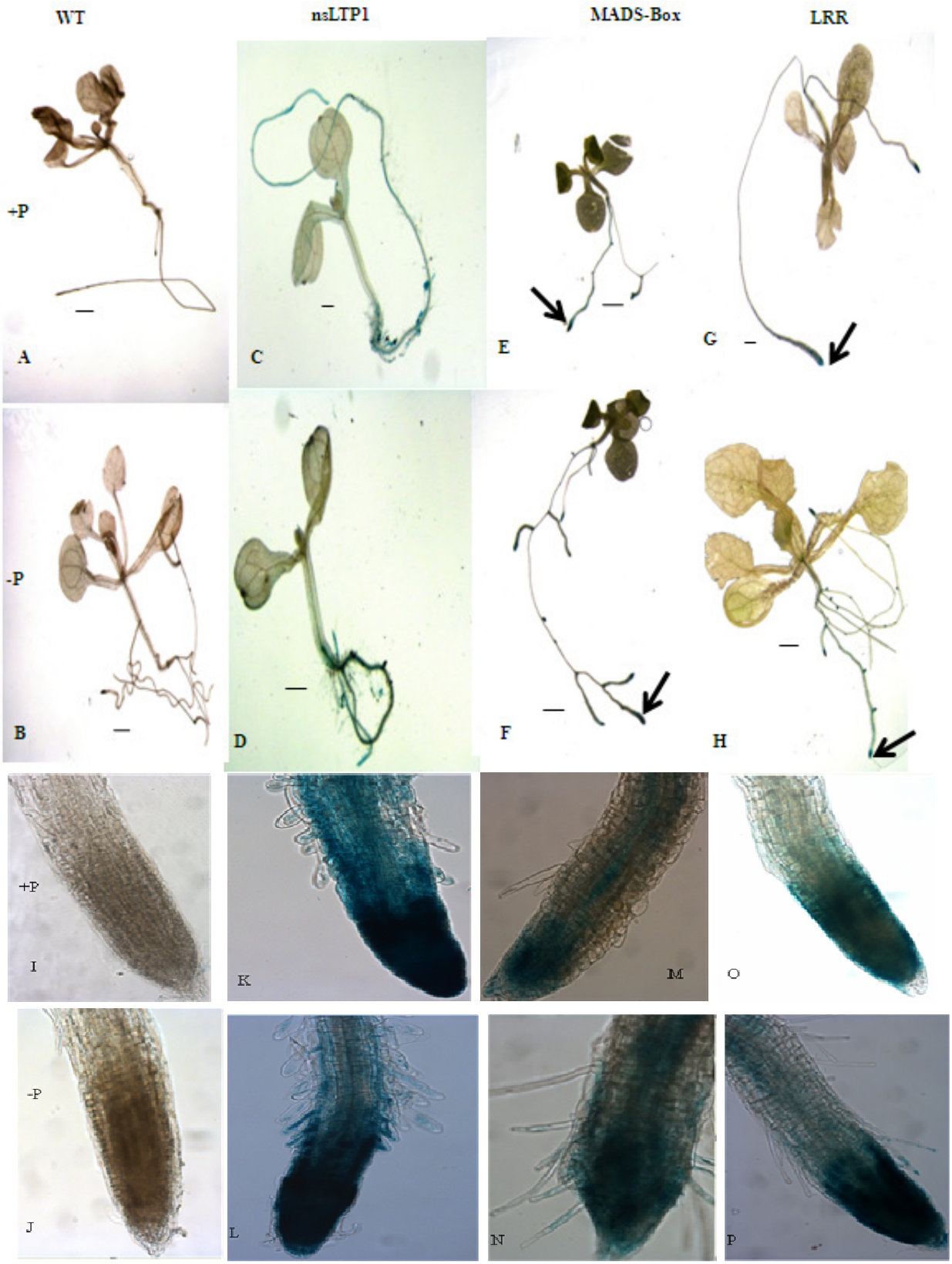

Figure 4. GUS staining results of wild type (A, B, I, J) and T_1 plants for nsLTP1 (C, D, K, L), MADS-Box (E, F, M, N) and LRR (G, H, O, P) fully grown inside solid media with P and without P. Bar = 1 mm and arrows indicate the GUS expression region (A to H). Magnification = 200× (I-P), AxioSkop2 mounted with Axiocam MRC5 (Germany). NsLTP2 was not GUS stained because T_1 seeds were not ready during this experiment.

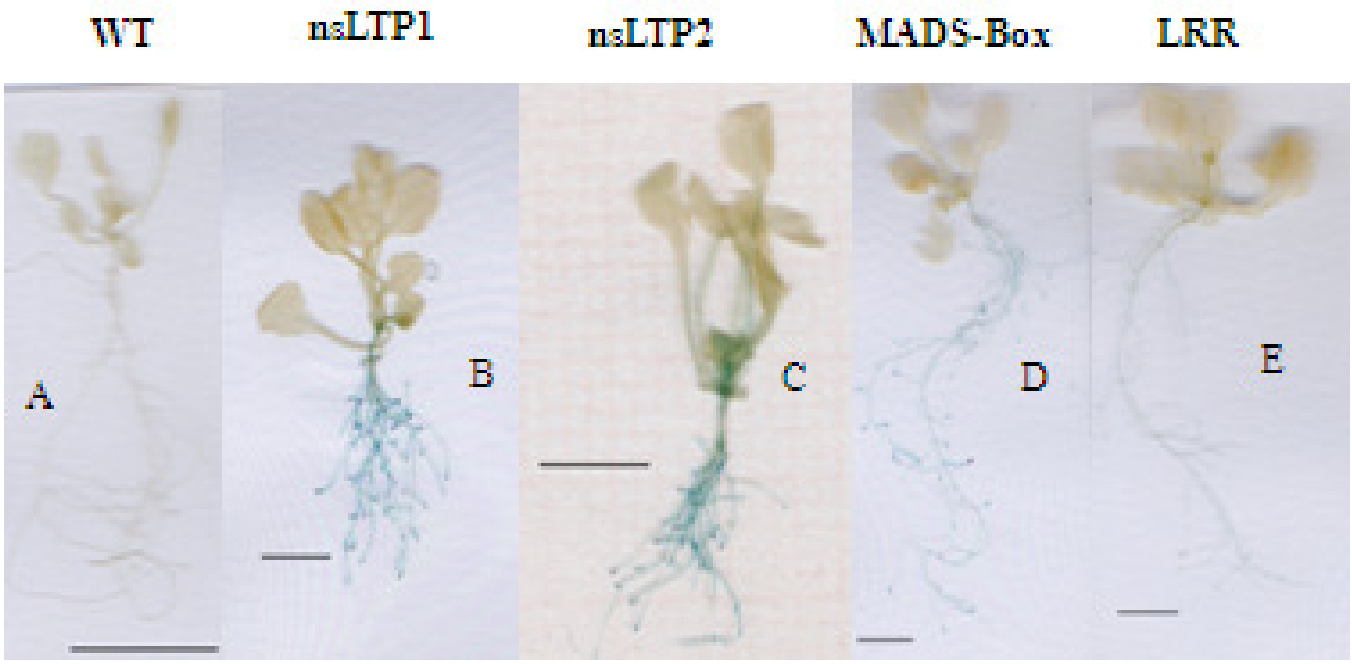

Figure 5. GUS staining result of wild type (A) grown on 1% MS media and T_1 plant for nsLTP1 (B), nsLTP2 (C), MADS-Box (D) and LRR (E) grown on MS medium containing 50 µg/ml kanamycin for three weeks (bar = 5 mm).

root development. The difference in the patterns of gene expression may suggest that these genes function at different parts of the root. For instance, the gene expression was at the primary and secondary root tips extending back to meristematic, elongation and maturation zones as in the cases of MADS-Box and LRR or in the whole root system as it was observed in non-specific lipid transport proteins, nsLTP1 and nsLTP2. The study investigated that the promoters under study were regulated promoters. Because, if promoter is activated under specific condition, then the promoter activates the reporter gene, which is under the control of that promoter to be expressed (Jefferson et al., 1987). This is also consistent with the report of Werner (1999) in that the regulated promoter was the one whose transcription is subjected to the control of various extracellular and intracellular signals.

This indicates that there are other important stress factors that could induce the gene expression other than phosphorus, when T_1 plants are fully grown inside the solid media and on the MS media containing Kanamycin. Previous experiments using suppression subtractive hybridization (SSH) method, however, showed that some genes were differentially regulated in the roots of *Brassica carinata* at different phosphorus conditions (Bremer and Schenk, 2009). The observed discrepancy between the previous experiment and the current result could possibly be due to the difference in the plant species and variation in age of the plants. Aksamit et al. (2005) suggested that phosphorus starvation needs optimization with regard to age of the plant and duration of phosphorus starvation. However, the result from this study indicates that P stress was not responsible for promoter induction of these genes at least in *A. thaliana* and signifies the importance of other stress factors for the gene expression.

The possible explanations for promoter induction and reporter gene expression could be mechanical impediment and oxygen deficiency to the roots during root development fully inside the solid media. And, catabolism of kanamycin may also trigger the gene expression when T_1 plants grow on selectable marker containing MS media. This is because; these abiotic factors are not problems when T_1 plants were grown on the surface of the solid media as well as hydroponically. This is in line with the report of Kilian et al. (2007) who proposed by AtGenExpress software that abiotic stresses such as oxidative and wounding stresses were found to be responsible for high gene expression value of nsLTP2, MADS-Box and LRR genes in the roots of *A. thaliana.* Nevertheless, the genes under study belong to family protein and studies indicate that some genes from the family involve in root development. For example, XAL1 (AGL12) belongs to MADS-Box family protein and regulates root meristem cell proliferation (Tapia-López et al., 2008), LRX1 encodes chimeric LRR and specifically expressed in root hairs (Baumberger et al., 2001) of *A. thaliana*.

Analyses by computer program PLACE helps to confirm the existence of several putative cis-elements on the promoters region (Prestridge, 1991; Higo et al., 1999), which are important for gene transcription. According to

these analyses, nsLTP1 was found to contain W-box (defense) in *Arabidopsis* (Xu et al., 2006), Cyc6 and Cpx1 (oxidative stress) in chlamydomonas (Quinn et al., 2000), and RAV1 (a novel DNA-binding protein) in higher plants (Kagaya et al., 1999). NsLTP2 was reported to contain anaerobically (ANAERO1CONSENSUS) induced genes in different plant species (Mohanty et al., 2005), W-box (defense) activation by transcription factors in *Arabidopsis* (Eulgemt et al., 1999). Likewise, MADS-Box was found to hold WEB (wound responsible) (Palm et al., 1990) in potato, and WRKY (transcription factor) (Zhang et al., 2004) in plants. LRR has been shown to consist of anaerobically (ANAERO1CONSENSUS) induced genes in different plant species (Mohanty et al., 2005). Thus, computer programs PLACE and AtGenExpress analysis strengthen oxygen deficiency, mechanical impedance and selectable marker that are prevalent when T_1 plants were grown fully inside the solid media and on MS media containing kanamycin. The strong reporter gene expression of nsLTP1, nsLTP2 and MADS-Box in the root system when grown on MS media with kanamycin may shows that these genes are highly regulated in the root as adaptation mechanism against metabolic burden. Therefore, these abiotic stresses were responsible for the promoter induction and reporter gene expression than phosphorus stress.

Conclusion

In this study, the promoter fragments analyzed do not show responsiveness to the phosphorus stress. This signifies the importance of undertaking further studies in order to analyze the effect of phosphorus stress on root specific expression of the studied genes. According to this study, abiotic stresses such as mechanical impedance, oxygen deficiency and Kanamycin are reasons for the gene expression. Thus, investigation made on the aforementioned genes have shown the involvement of these genes in plant adaptation and root development under mechanical impedance and oxygen deficiency. Further, quantifying the reporter gene activity in the T_1 plants by real time PCR helps to explore the real function of these genes. Hence, it is possible to make use of these genes as an adaptation mechanism to grow plants on rigid soils and under water logging condition where oxygen is limiting.

ACKNOWLEDGEMENTS

The authors are thankful to the German Academic Exchange service (DAAD) for financing this research and Professor Dr. M.K. Schenk for providing his lab. Thanks to the Ethiopian Institute of Agricultural Research (EIAR) for the study leave during the study period. The authors do appreciate Nottingham University for the provision of knockout seeds.

REFERENCES

Aksamit A, Korobczak A, Skala J, Lukaszewicz M, Szopa J (2005). The 14-3-3 Gene expression specificity in response to stress is promoter-dependent. Plant Cell Physiol., 46(10): 1635-1645.

Baumberger N, Ringli C, Keller B (2001). The chimeric leucine-rich repeat /extensin cell wall protein LRX1 is required for root hair morphogenesis in *Arabidopsis thaliana*. Genes Dev., 15: 1128–1139.

Bechtold N, Ellis J, Pelletier G (1993). In planta Agrobacterium-mediated gene transfer by infiltration of adult *Arabidopsis thaliana* plants. Academic Science Paris. Life Sci., 316: 1194-1199.

Bent AF (2000). Arabidopsis in planta transformation. Uses, mechanisms and prospects for transformation of other species. Plant Physiol., 124: 1540-1547.

Bremer M, Schenk MK (2009). The Expression of P-Responsive Genes is related to Root Hair Growth. The Proceedings of the International Plant Nutrition Colloquium XVI, Department of Plant Sciences, UC Davis.

Brian M, Ashraf A, Yuzuki M, MacDonald P (2008). Selectable marker genes and unintended changes to the plant transcriptome. Plant Biotechnol. J., 7, 211–218.

Buchanan B, Gruissen W, Jones R (2000). Biochemistry and Molecular Biology of plants. American Society of Plant Physiologists, Maryland. pp. 4-1139.

Desfeux C, Clough SJ, Bent AF (2000). Female reproductive tissues are the primary target of *Agrobacterium*-mediated transformation by the *Arabidopsis* floraldip method. Plant Physiol., 123: 895–904.

Doebley J, Lukens L (1998). Transcriptional regulators and the evolution of plant form. Plant Cell, 10, 1075-1082.

Dolferus R (1991). Isolation of DNA and RNA from Arabidopsis thaliana. In: Negruku J. and Ghark-Chherti GB (eds.). Bio-methods: A laboratory Guide for Cellular and Molecular Plant Biology. pp.133-152.

Eulgemt, Rushton PJ, Schmelzer E, Hahlbrock K, Somssich IE (1999). Early nuclear events in plant defence signalling: Rapid gene activation by WRKY transcription factors. EMBO J., 18: 4689-4699.

Forsthoefel NR, Cutler K, Port MD, Yamamoto T, Vernon DM (2005). A novel class of plant intracellular leucine-rich repeat proteins. Plant Cell Physiol., 46: 913–922.

Galune, Breiman A (1997). Transgenic Plants. Imperial College Press, London. pp. 1-122.

Gateway cloning technology; Instruction manual (2005). Available: http//www.lifethecnology.com/gateway. Accessed: on 10.06.2009.

Higo K, Ugawa Y, Iwamoto M, Korenaga T (1999). Plant cis-acting regulatory DNA elements (PLACE) database. Nucleic Acids Res., 27(1): 297-300.

Höfgen R, Willmizer L (1988). Storage of competent cells for *Agrobacterium* transformation. Nucleic Acids Res., 16:9877.

Jefferson RA, Kavanaga TA, Bevan MW (1987). GUS Fusion: β-glucuronidase as a sensitive and versatile gene fusion marker in higher plants. EMBO J., 6: 3901-3907.

Kagaya Y, Ohmiya K, Hattori T (1999). RAV1, a novel DNA-binding protein, binds to bipartite recognition sequence through two distinct DNA-binding domains uniquely found in higher plants. Nucleic Acids Res., 27: 470-478.

Magnani E, Bartling L, Hake S (2006). From Gateway to MultiSite Gateway in one recombination event. BMC Mol. Biol., 7:46.

Maliga P (2002). Engineering the plastid genome of higher plants. Curr. Opin. Plant Biol. 5, 164–172.

Mohanty B, Krishnan SP, Swarup S, Bajic VB (2005). Detection and preliminary analysis of motifs in promoters of anaerobically induced genes of different plant species. Ann. Bot., 96: 669-681.

Nieuwland J, Feron R, Huisman BAH, Fasolino A, Hilbers C, Derksen WJ, Mariani C (2005). Lipid Transfer Proteins Enhance Cell Wall Extension in Tobacco. Plant Cell, 17(7), 2009–2019.

Palm CJ, Costa MA, An G, Ryan CA (1990). Wound-inducible nuclear protein binds DNA fragments that regulate a proteinase inhibitor II gene from potato. Proc. Nat. Acad. Sci. USA., 87(2): 603-607.

Prestridge DS (1991). SIGNAL SCAN: a computer program that scans DNA sequences for eukaryotic transcriptional elements. Comput. Appl. Biosci., 2: 203-206.

Quinn JM, Barraco P, Eriksson M, Merchant S (2000). Coordinate copper- and oxygen-responsive Cyc6 and Cpx1 expression in

Chlamydomonas is mediated by the same element. J. Biol. Chem., 275: 6080-6089.
Rozen S, Skaletsky HJ (2000). Primer3 on the WWW for general users and for biologist programmers. In: Krawetz, S. and Misener, S. (eds) Bioinformatics Methods and Protocols: Methods in Molecular Biology. Humana Press, Totowa, NJ. pp. 365-386.
Schmid M, Davison TS, Henz SR, Pape UJ, Demar M, Vingron M, Scholkopf B, Weigel D, Lohmann JU (2005). A gene expression map of Arabidopsis thaliana development. Nat. Gent. 37, 501-506.
Schwechheimer C, Bevan M (1998). The regulation of transcription factor activity in plants. Trends Plant Sci. 3: 378-383.
Smale ST, Kadonaga JT (2003). The RNA polymerase II core promoter. Annu Rev Biochem. 72, 449-479.
Stanislava G (2007). Barley Grain Non-specific Lipid-Transfer Proteins (ns-LTPs) in Beer Production and Quality. J. Inst. Brew., 113(3): 310–324.
Sterk P, Booij H, Schellekens GA, Kammen A, Vries SC (1991). Cellspecific expression of the carrot EP2 lipid transfer protein gene. Plant Cell. 3(9): 907-921.
Tapia-López R, García-Ponce B, Dubrovsky JG, Garay-Arroyo A, Pérezruíz RV, Kim SH, Acevedo F, Pelaz S, Alvarez-Buylla ER (2008). A GAMOUS-related MADS-box gene, XAL1 (AGL12), regulates root meristem cell proliferation and flowering transition in Arabidopsis. Plant Physiol., 146: 1182-1192.
Werner T (1999). Models for prediction and recognition of eukaryotic promoters. Mam. Genome, 10: 168-175.
Xu X, Chen C, Fan B, Chen Z (2006). Physical and functional interactions between pathogen-induced *Arabidopsis* WRKY18, WRKY40, and WRKY60 transcription factors. Plant Cell, 18: 1310-1326.
Zhang ZL, Xie Z, Zou X, Casaretto J, Ho TH, Shen QJ (2004). A rice WRKY gene encodes a transcriptional repressor of the gibberellin signaling pathway in aleurone cells. Plant Physiol., 134(4): 1500-1513.