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Activity of AtDrm1 Promoters in Transgenic Arabidopsis thaliana

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Abstract

The focus of this work involves the molecular regulation of dormancy in plants. This includes the analysis of genes that are activated when cells stop growing. Dormancy is the temporary arrest of cell growth as well as a group of changes that aid the plant in surviving adverse conditions. There are hormones involved in this process and their significance helps to demonstrate that dormancy is an active physiological process that requires altered levels of gene expression. Dormant plants have been found to contain unique patterns of gene expression, as well as protein synthesis. Additionally, dormant plants have unique processes such as metabolic activity, just as growing plants do. Gene promoters are regions upstream of the coding region where RNA polymerase binds to initiate transcription. In this experiment, two promoters of *Arabidopsis thaliana* dormancy genes were isolated and fused with reporter genes in order to study their expression in plants. These observations were compared with that of a Northern blot, which involved probing *Arabidopsis thaliana* RNA with specific sequences from the 3' UTR of each gene's cDNA. In most cases, similarity was observed between mRNA accumulation in the Northern blots and expression of the reporter gene protein *in planta*. As a consequence of a deletion analysis on one of the promoters, significant differences in expression were observed with relation to the size of the promoter.
Introduction

In order for a plant to be successfully reproductive, it must undergo several physiological alterations to maintain itself and its progeny. Plant physiology is a complex system of chemical and physical interactions. One of these interactions involves a process known as dormancy. Dormancy is the temporary arrest of cell growth as well as a group of changes that aid the plant in surviving adverse conditions. This is assisted by the triggering mechanism associated with abscisic acid. Abscisic acid (ABA) is a messenger commonly referred to as a stress hormone because of its tendency to promote changes in plants that are experiencing adverse conditions. Such stresses may include too high or low of temperature as well as variations in salt or nutrient levels. In the presence of these stresses, the concentration of abscisic acid may increase several fold. Such high levels can trigger certain responses in the plant such as the closing of the stomata to help retain water. In addition to ABA, there are other plant hormones that are involved in dormancy. These include auxin, cytokinin, and gibberellins, which have been shown to aid in reverting from dormancy and triggering the plant to begin growth and cell division. Although all of these hormones can be involved in dormancy, they are not the actual cause or inhibitor of it. They are messengers. These hormones are of vital significance to the process of dormancy and help demonstrate that dormancy is an active physiological process that requires altered patterns of gene expression. Furthermore, the process of dormancy is dependent on the site of the response. Roots, buds, seeds, and other organs respond differently to these hormones implying the complexity of the physiological processes of dormancy.
Dormant plants have been found to contain unique patterns of gene expression, as well as protein synthesis. Furthermore, metabolic activity is a component of dormancy, which implies that these plants have many unique processes, just as growing plants do. Research on pea buds has revealed dormancy genes in enough abundance to create a dormant bud library. Two cDNAs, PsDRM1 and PsDRM2 were isolated via screening. PsDRM1 mRNA was found to be abundant in non-growing buds as well as other non-growing plant organs. In experiments by Stafstrom and coworkers (Stafstrom et al. 1998), levels PsDRM1 and PsDRM2 mRNAs accumulated to higher levels in dormant axillary buds than did RPL27, a growth associated mRNA. Expression of PsDRM1 and PsDRM2 in a measured axillary bud is greatest just before its terminal bud is decapitated. Decapitation allows growth in the axillary buds due to an allocation of auxin, which is usually most concentrated in the terminal bud. This experiment shows that when buds are dormant, they express DRM genes and when growing, express RPL27. In addition, the degree to which the DRM1 was down-regulated after decapitation was significant. Further Northern blot analysis revealed that expression of PsDRM1 was apparent in non-growing stems and roots. Conversely, PsDRM2 was present in many growing tissues and was thus deemed an inadequate dormancy marker. Further significance in PsDRM1 was observed when its expression was measured in terms of days after decapitation. The growth-dormancy cycle in pea axillary buds was observed, and levels of these genes displayed a unique pattern during that time. After decapitation, it was significantly down-regulated only to be turned back on after two days. After five days, it was turned back off. This complimentary expression further indicated that it is a good marker for bud dormancy.
Studies of PsDRM1 should provide insight into dormancy since it is a good dormancy marker. The amino acid sequence of PsDRM1 is 75% identical to a strawberry clone (Poovaiah and Reddy 1990). This clone is an example of an auxin-repressed gene, which implies that PsDRM1 may be regulated in the same way. Studies with this strawberry clone, called λSAR5, involved analysis of the repression by auxin by inducing the plant into a dormant or non-growing state by deaachening the fruit, which reduces auxin production that controls recepticle growth. This fruit was then either exposed or not exposed to auxin. The RNA from these specimens was collected and probed with λSAR5. A five fold higher level of λSAR5 mRNA was apparent in the fruit that was not exposed to auxin, implying that auxin may repress the transcription of λSAR5 mRNA. Ultimately, the study showed a positive correlation between the decline of mRNA in the auxin repressed clone and fruit growth in strawberries.

In addition to the PsDRM1 gene, two additional dormancy associated genes have been isolated from Pisum sativum. These are all pea-associated and may be orthologous to proteins found in Arabidopsis thaliana (Stafstrom et al. 1999). Termed AtDRM1-1, and later renamed AtDrm1a, it was sequenced and is available under the accession number AF053746. This cDNA contains 745bp of combined ORF and untranslated 3' and 5' regions. A 366bp ORF encodes a 122 amino acid peptide. AtDrm1a shows 66% amino acid similarity to PsDRM1. Northern analysis was able to show that the corresponding mRNA levels of young leaves was near absent suggesting that AtDrm1a is repressed in growing organs. In addition, another gene on chromosome II of Arabidopsis thaliana was identified in Genebank, which encodes a protein of 108 amino acids. It is 72% similar to AtDrm1a (Stafstrom, Krueger, and Stoudt 1998). A third gene has been
identified in the Arabidopsis genome from a genomic clone (AAD25779), which encodes a relatively larger protein of 166 amino acids. Blastp analysis shows numerous related genes in other plant species. It is unknown whether any of the other DRM1-like genes are regulated by auxin.

Gene promoters are regions upstream of the coding region where RNA polymerase binds to initiate transcription. Transcription factors are proteins that associate with the promoter to enable the binding of RNA polymerase. These proteins are called transcription factors. The complex that forms between the transcription factors and the promoter is what the RNA polymerase recognizes and subsequently binds to. There are usually common characteristics to promoters such as a -35/-10 or -24/-12 structure as well as a TATA and Shine Delgarno sequences. Additional transcription factors must associate with the RNA polymerase before it can proceed with transcription. Promoters have cis-acting elements such as activators and repressors. Activators allow optimal transcription to occur, leading to a larger amount of RNA produced, whereas repressors down-regulate transcription and are thus a form of negative regulation. Various transcription factors are responsible for binding to cis-elements, which may lead to differences in eventual RNA production.

The emphasis of this work involves the molecular regulation of dormancy in plants. In this study, the expression of two genes, AtDrmla and AtDrm1b was quantified in two ways. First, a segment of 3’ UTR from each gene’s cDNA was selected, amplified and used as a probe in Northern blots. And second, the two complete promoters were cloned into Agrobacterium Ti plasmids containing reporter genes. These promoter-marker gene fusions were transformed into plants to create transgenics. Study
of expression with regards to developmental stages as well as organ differentiation could be observed directly, since the reporter genes would be expressed in the same cells, tissues, and developmental stages as the original AtDrm1 genes. The activity of these promoters was further studied by creating a series of deletions in the promoter sequence of AtDrm1a and studying them in the same manner, *in planta*. Doing so could give insight to any of the regions that may contain cis-acting elements contributing to enhanced or reduced expression.
Materials and Methods

Northern Blot procedures. RNA samples were collected from different organs of wild type Arabidopsis plants by Michelle Devitt. These samples of RNA were run on a formaldehyde gel (57.7 mL of water, 1.2 g of agarose, 8 mL of MOPS buffer, and 14.3 mL of 37% formaldehyde). Two combs divided the gel so that two duplicate sets of the different samples of RNA could be loaded. All conditions were kept RNAase free. Samples of RNA were added to formamide, 10X MOPS buffer, formaldehyde, and loading buffer. The gel was blotted onto a nylon membrane by capillary transfer and then fixed onto the membrane by a UV cross-linker machine. The gel was cut to divide the duplicate sets. Conditions of prehybridization and hybridization solutions and washes were identical to those of the Southern blots (below). Two different probes were made to label the two separate but identical blots mentioned above. These two unique sequences of DNA were created by making forward and reverse primers for the 3' UTR region AtD1a cDNA as well as the 3' UTR region of AtD1b cDNA.

AtDrm1a:
Forward-CTTCCGCCACCTTACTTTG
Reverse-CAACCATCATCAAAATCCAAC

AtDrm1b:
Forward-CCGAGAGGTAGAGTGAC
Reverse-CAAGACACTGAAGACGCATC

These segments were amplified in a PCR reaction and were used to generate radioactive probes using the Decaprime II kit by Ambion. The blot was exposed to Biomax film for six days.
Isolation of promoters. The two promoters were handled differently. Promoter AtDrmla, 2.5 Kb of an upstream sequence of a genomic clone isolated in Stafstrom’s lab, was amplified by PCR into four different fragments: Fragment1(2600bp), F2(1400), F3(950), F4(400).

F1 forward-GCTAAGCTTATCTAGATTTGTGTCAC
F2 forward-CGAAAGCTTCGTGGAAACCTTTTGTG
F3 forward-TGCAAGCTTCCATGCTTTTGAC
F4 forward-GCTAAGGTTATCTAGATTTTGTTCAC

Reverse-GACCATGGTAGATCTTTCAATGATTTTGTAGACAG

Promoter AtDrmlb was amplified in whole using PCR primers as well.

Forward-CACGGATCCTTTCACGGATATAGAGAATCTAGG
Reverse-AGAGAAAGTAGTGACAAGTG

Each respective promoter fragment was run on an agarose gel from where it was purified by ethanol precipitation or GeneClean.

Cloning techniques. Promoter fragments of AtDrmla were ligated into pCAMBIA 1381, which contains the β-glucuronidase (GUS) reporter gene (Jennifer Nunn started this work and was successful in creating clones of the different fragments of AtDrmla into pCAMBIA 1381). Promoter construct AtDlb was ligated into pCAMBIA 1391, which also contains GUS. The five constructs were transformed into E. coli strain JM109, selected for by blue-white screening, and plasmid purified. The purified plasmid clones of AtDrmla, containing their respective inserts, were digested with NcoI and HindIII. The AtDlb clone was digested with Bam H1 and NcoI. These restriction digests were performed as a primary test to show that the colonies selected were actually
transformants. After proper transformation had been confirmed, colonies that contained the proper insert were selected and all five constructs were transformed into Agrobacterium strain GV3101. This was done with the aid of electroporation in place of heat shock. The cultures were plated on Kanamycin selection plates and grown for two days at 30°C. The constructs were then ready to be transformed into Arabidopsis thaliana.

**Plant Material.** Arabidopsis thaliana seeds were sown in trays containing Metromix, covered with bridal veil, and grown in a Conviron growth chamber. Temperature was adjusted to a 16/8 hour light cycle with temperatures set at 20°C and 15°C degrees, respectively. Plants were allowed to grow until they contained 8 to 10 rosette leaves about 1.5 inches long and had begun to flower.

**Transformation techniques.** The five promoter constructs were transformed into Arabidopsis by a floral dip process (Clough and Bent 1999). A large liquid culture of LB was grown with appropriate antibiotic until mid-log phase. The Agrobacterium cells of each culture was spun down and resuspended in a 5% sucrose solution. Silwet L-77 was added to a concentration of 500uL/L. The plants were dipped for 2-3 seconds with mild agitation or until the plant was covered with a film of liquid coating. The plants were placed under a dome for a day and then allowed to grow.

**Analysis of transgenic plants.** The seeds from the dipped plants were collected after two months. They were rinsed in a 50% bleach and 50% TritonX solution at .02% concentration. They were agitated for 5-7 minutes and added to 1% phytoagar containing 50mg/mL of hygromycin. After hygromycin resistant strains were selected off the plate, the plantlets were sown in trays containing Metromix and allowed to grow until
approximately six inches. Tissue was collected by razor blade. Samples of young and older leaves were collected from each plant and frozen in liquid nitrogen. DNA was extracted by Edward’s PCR DNA miniprep. Tissue was ground and combined with Edward’s extraction buffer. The samples were microfuged and the supernatant was combined with isopropanol. The samples were microfuged again, supernatant decanted, and the pellet resuspended in 100 microliters of TE.

**Southern blot of hypothetical transgenic plants.** Specific primers were designed to recognize the promoter regions of interest.

Forward-TGGTCTTCTGAGACTGTATC

Reverse primer-CGCGATCCAGACTGAATGCC

These primers were used to excise all promoters since each plant was independently dipped and these primers would recognize the vector just outside the sites where each promoter fragment was inserted. The regions recognized were the CaMV35S promoter and the gusA (N358Q) gene. PCR reactions were performed using the extracted DNA and the specified primers. PCR products were then analyzed by Southern blot. The PCR products were run on a 1.35% agarose gel along with a standard. The gel was blotted onto a nylon membrane overnight by capillary transfer. The blots were prehybridized in 25 mL of 50% formamide, 5X Denhardt’s reagent, 0.5% SDS, 5X SSPE, and 100ug/mL of salmon sperm DNA for 1.5 hours at 42C. The blot was hybridized, overnight, with a P32 labeled probe. Blots were washed in stringent conditions and exposed on Biomax film for one day.

**GUS staining to verify transgenic plants.** Leaf tissue from young and old leaves was collected and tested for protein expression. NaHPO4 (5.8mL), NaH2PO4 (4.8mL), and
90 mL of water was mixed to make 50mM sodium phosphate buffer. This sodium phosphate buffer (19.80 mL at pH 7) and 0.20 mL of X-GLUC (substrate) was combined and 500 uL of the solution was added to each leaf sample (three from each construct). The samples were incubated overnight. The next day, the solution was removed by aspiration and replaced with 95% ethanol to remove the chlorophyll and to better quantify expression. Four washes with alcohol were done over a two-hour period. Samples were stored in 70% ethanol at 4°C.

**Gus staining at different developmental stages.** Seeds were harvested from plants known to be transgenic. Some were grown on phytoagar plates that contained hygromycin to identify resistant plants. Others were allowed to grow in 4" pots. Tissue was collected from the plants once a week for four weeks. All organs that were present at each stage of growth were collected at each time interval. GUS staining (as described above) was performed on these tissues and both the time of collection as well as the location and intensity of expression was noted.
Results

**Northern Blot.** Northern blot analysis revealed similar patterns of steady state levels of mRNA accumulation between AtDrmla and AtDrmlb (Figure 1). RNA accumulation patterns showed variable but distinct expression in flowerbuds, open flowers, siliques, and stems. RNA accumulation in eight day leaves was not detected and accumulation in six week leaves and roots was high.

**Transgenic plant constructs.** The schematic of the promoter regions was as follows:

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2600bp------------------1400bp-----------------------9506p-----------------------400bp--------------

Forward primer1   Forward primer2   Forward primer3   Forward primer4
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2a. AtDrmla in pCAMBIA 1391 containing GUS

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2500bp

Forward primer
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2b. AtDrmlb in pCAMBIA 1381 containing GUS

**Figure 2**

After being transformed into *Arabidopsis thaliana*, seeds were harvested and selected on hygromycin and putative transformed plants were selected, their tissue was collected and DNA extracted. Those plants whose chromosomes had successfully integrated the left and right T borders of the *Agrobacterium* Ti vector, which would contain the insert, would be transformants.

**Southern Blot.** Southern blots used to confirm whether the plants sampled were transgenics contained positive results. Primers were created to recognize the region on the plasmid adjacent to the insertion sites and were used in a PCR reaction with the respective plant DNA specific to each transformant. These were run on gels and southern
probed with promoter-specific probes. In the case of AtDrm1a, the probe was made of fragment 1, which would recognize all primers, although any of the fragments would have been just as effective. Corresponding plant seeds were collected and sown in trays. Progeny, T2, was representative of a partially hemizygous generation with 1/4 homozygous transgenic, 1/4 wild type, and 1/2 hemizygous. Optimal testing parameters will require homozygous transgenics.

**GUS expression at different developmental stages.** Overall, there were few noticeable differences in expression corresponding to different developmental stages. There is some evidence that expression in roots is minimal early in development and progresses with the plant’s age. Paradoxically, there is some expression in 5day leaves although northern results of 8-day leaves show no measurable expression. Collectively, the amount of expression was consistent throughout the different stage of growth.

**GUS expression in different organs.** The most abundant expression of the proteins was in flower buds and leaves. This is consistent with the Northern analysis of mRNA production. There was significant expression in and around the nodes, which were not specifically tested, in the northern analysis. Expression in the stems was the lowest and the most variable, in some cases being significant and other cases variable.

**Analysis of AtDrm1a constructs.** There was a similar trend observed several times during collection and staining concerning the intensity of expression in each promoter fragment construct. The strongest intensity of expression was seen in the fragment 4 construct (Figure 6). Intensity increased as the promoter got smaller (F1=<F2;<F=<3F4) (Figure 7). Whether or not this trend is representative is yet to be tested.
Comparison between $AtDrm1a$ and $AtDrm1b$. Similar trends were observed between $AtDrm1a$ and $AtDrm1b$ (Figures 3 and 8). Both had fairly low expression, the most intense being concentrated in the leaves and flower buds. $AtDrm1b$ contained very little expression in its stems.
Discussion

Data that has been observed is preliminary, especially concerning the prevalence of AtDrm1 like genes in young tissues. Little mRNA was observed via Northern blot analysis of 8-day leaves. However, GUS staining did reveal some translation of protein in young leaves, although not in extremely young leaves (less than 5 days old). Theory may give hint to the possibility that there would be little production of AtDrm1 in young leaves and fruits since these may be organs that contain high levels of auxin, a hormone that hypothetically represses this gene. If auxin represses these genes, then organs with high levels of auxin activity may have low levels of DRM1 expression. Consideration must be taken when observing these constructs since their expression may be different assuming they do or do not contain the site where auxin represses transcription of RNA. Nevertheless, all other data seems to be consistent between mRNA production detected in the Northern and protein observations through GUS substrate binding. Quality of expression appears to be consistent with exception to roots, though the quantity of expression observed in AtDrm1a deletions seems to correlate to the size of the promoter fragment. Further analysis will be done to try and map any activator or repressor sequences that may be causing this varied expression. Deletions will be made in AtDrm1b to determine whether it has similar patterns of GUS activity. RNA of nodes may also be collected, with some difficulty, and subject to the same type of Northern blot that the other wild type RNA was subject to. One would expect to see considerably high levels of mRNA in these parts of the plant since GUS expression is high there. Further analysis may also be done with another marker gene, green fluorescent protein (GFP),
fused to the exact same promoter fragments. This requires a special type of fluorescence microscope that was unavailable during this testing.

Another pertinent analysis already in progress involves the comparison of mRNA patterns to protein patterns. Since mRNA undergoes modification on its way to being translated, there is a possibility that these patterns may not be exactly the same. This will be done using AtDrm1 specific antibodies, which will be used as a probe in a Western blot analysis to quantify accumulation of AtDrm1 protein in wild type Arabidopsis thaliana plants.

Ideal testing will be done on homozygous plants. As of now, the transgenic plants are in a hemizygous state, which requires tests of several plants at a time to ensure transgenics are included in the analysis. Plants expressing the marker gene will be selected and their seeds will give way to a homozygous generation.

Tests to be done in the future concerning the transgenic plants may involve down-regulating the amount of mRNA production and thus the amount of protein expressed by generating anti-sense mRNA. This may bind with the sense mRNA, which will inhibit it from being translated. A dominant-negative mutation can be made by over-expressing a mutated protein with a 35S promoter. This would allow substrate to bind to the mutated protein, and reduce the amount available for the unmutated protein to bind to. This type of mutation may lead to loss of function. Mutations that may lead to gain of function is termed can be achieved through reverse genetics. Here, you utilize a cloned gene and alter the amount and location of its expression. You can turn it on where it normally would not be by attaching it to a constitutive 35S promoter. On the gene level, insertional mutagenesis can be used to create knockouts in genes of the plant and then
study their phenotypes. A fusion of AtDrm1 promoter, AtDrm1 gene, as well as a marker
gene can be made. These constructs can be mutated as well. These mutations will help
further characterize the activity of these promoters and help characterize the gene by
defining changes in phenotype due to altered expression of AtDrm1.

Further tests will be done by modifying the environment in which the transgenics
grow. By doing so, it will become apparent if any growth conditions suppress or enhance
the production of AtDrm1. Such tests can involve altering temperature, light exposure,
and, most significantly in this case, hormone exposure. Specifically, tests can be done
altering auxin concentration in growth medium or by applying the hormone directly to the
leaves or other organs. Such variation in auxin levels may give insight to the hypothesis
that AtDrm1 is an auxin-repressed gene. If high levels of AtDrm1 expression are on
growth plates with little or no auxin, and depleted expression is seen when there is an
abundance of auxin there is implication that auxin may be repressing the activity of the
promoters. This can be done for both promoters, as well as with all deletions. By doing
so, we may be able to map where the auxin may be repressing expression and if the maps
are identical or similar between the two different promoters.
References


Stafstrom J. (in press) Regulation of Growth and Dormancy in Pea Axillary Buds. Dormancy in Plants: 331-346

FIGURES
Top:

Northern blot showing increased amounts of AtDrm1 mRNA accumulation in 6 week leaves and buds as well as diminished accumulation of AtDrm1 mRNA in 8 day leaves.

Bottom:

Formaldehyde gel with duplicate sets of Arabidopsis thaliana RNA samples from different plant organs (as ordered above).
Figure 3
Entire AtDrm1a promoter at 8 days old. This tissue shows little GUS expression.
Figure 4

Fragment 2 of AtDrm1a promoter (1400bp) at 8 days. This construct shows slightly increased levels of GUS expression and is comparable to F1.
Figure 5

Fragment 3 of AtDrm1a promoter (950bp) at 8 days. This construct shows increased levels of GUS expression.
Figure 6

Fragment 4 of AtDrmla promoter (400bp) at 8 days. This construct shows high levels of GUS expression.
Figure 7

Fragments 4, 3, 2, and 1 of the AtDrm1a promoter at 28 days. This comparative picture shows the variation in expression depending on promoter size.
Figure 8

Entire AtDrm1b promoter at 8 days. Expression is similar to that of AtDrm1a F1.