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Differential Expression of Two Different *EeSTM* Genes of Leafy Spurge (*Euphorbia esula*) and Root-directed Expression from *EeSTM* Promoter in Leafy Spurge and *Arabidopsis*

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Abstract

SHOOTMERISTEMLESS (*STM*) encodes a member of the class I *KNOX* homeodomain protein family that is required for meristem development and maintenance. Leafy spurge is a model perennial weed that produces adventitious meristems on its roots and hypocotyl. These buds are capable of displaying para-, endo- and ecodormancy. We have cloned two different full length cDNAs of *STM* (*EeSTM1* and *EeSTM4*) from leafy spurge that displays different tissue specific expression patterns. *EeSTM1* gene appears to be expressed only in young root and hypocotyl tissue. *EeSTM4* is co-expressed with *EeSTM1*, but it is also expressed highly in the shoot apical meristem and in mature roots. An *EeSTM* promoter was able to drive *GUS* expression in the roots, hypocotyl, and shoot apical meristem of *Arabidopsis*, whereas the *AtSTM* promoter only produced *GUS* expression in the shoot apical meristem. Expression analysis indicates that *EeSTM* expression is detectable in dormant adventitious buds but is up-regulated within three days following defoliation, a treatment that initiates bud regrowth. Seasonal changes in expression of *EeSTM* correlated to dormancy status in underground leafy spurge buds were observed.

Keywords: *STM*; Leafy spurge; Promoter; Gene Expression; *GUS*; Bud Dormancy; *Arabidopsis*

Introduction

Many plants propagate through the development and regulated growth of adventitious shoot buds. Additionally, many plants maintain a pool of viable but dormant shoot buds as a means to survive periods of growth inhibiting environmental conditions. In the perennial weed leafy spurge, such buds initiate and develop on the underground roots and/or crown region and are essentially fully formed shoot meristems. Recent studies on model organisms such as *Arabidopsis* (*Arabidopsis thaliana* Hyne) have provided a greater understanding of the genes required for proper shoot meristem initiation and development. One of the genes required for shoot development is *SHOOTMERISTEMLESS* (*STM*). *STM*, recognized as a developmentally important gene, was first cloned from maize [1] and then later from cauliflower (*Brassica oleracea* var. *botrytis*) and *Arabidopsis* [2]. *STM* is a member of the Class I *KNOX* homeobox gene family. *KNOX* encoded transcription factors are important in regulating meristem development and maintenance [3-5]. *STM* in particular is required for maintaining a supply of non-differentiated cells in the central zone of the shoot apical meristem (SAM).

STM is primarily expressed in the central zone of the SAM in *Arabidopsis*. *STM* also has a very important role in maintaining meristematic identity in cells of the peripheral zone that are not recruited to form organ primordia. *ROUGH SHEATH2* (*ZmRS2*) in maize and *ASYMMETRIC LEAVES1* (*AS1*) negatively regulate *KNOX* (*KNOTTED1*-like homeobox) genes such as *STM* in *Arabidopsis* [6-8]. *RS2* and *AS1* encode MYB-domain proteins that down-regulate *KNOX* genes in developing leaves. *ASYMMETRIC LEAVES2* (*AS2*), a *LATERAL ORGAN BOUNDARIES* (*LOB*) protein, along with *AS1* repress *KNOX* gene expression to promote leaf differentiation [9]. *CUP-SHAPED COTYLEDON* (*CUC*) initially induces *AtSTM* expression in the developing SAM [10]. There is evidence of both chromatin modification and transcription factor binding sites playing a role in

regulation of *STM* in *Arabidopsis* and other species [11,12].

STM has been extensively studied in annual plant species such as *Arabidopsis* and likely orthologues have been identified in many plant species. However, very little is known about how *STM* is expressed in perennials that produce adventitious buds capable of various states of dormancy. It is unknown if *STM* is expressed in dormant buds, or if it is differentially regulated between growth and dormancy transitions. We have chosen to study *STM* expression in leafy spurge (*Euphorbia esula* L.). Leafy spurge is an herbaceous perennial weed, native to the central Europe, but which has become invasive in the Northern Great Plains of the US and Canada [13]. Genetically, leafy spurge is an auto-allo hexaploid [14].

Leafy spurge is a model perennial that is ideal for studying bud dormancy [15]. Leafy spurge produces large numbers of easily identifiable adventitious buds along the lateral roots (often referred to in the literature as root buds) and on the underground stem (referred to as crown buds) [16]. Leafy spurge root and crown buds display all three types of dormancy (para-, endo- and ecodormancy) [17,18]. After formation in early summer, root and crown buds enter a state of paradormancy. This paradormant state is maintained throughout the growing season by auxin and sugar signals produced in the growing

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shoot [19,20]. Root and crown bud growth is reinitiated by separation of buds from the growing shoot. In the fall, root and crown buds of leafy spurge enter an endodormant state and will not grow even if plants are returned to growth-conducive conditions [18,20]. Prolonged periods of cold temperatures release the endodormant state [18]. The buds remain in an ecodormant state and will not develop into growing shoots until growth-conducive conditions return in the spring [18].

We previously isolated and characterized several genomic and cDNA clones of *STM* from the leafy spurge [21]. A comparison to other class I *KNOX* genes indicates that these *EeSTM* genes represented orthologues of *KN2* from poplar and likely were related to *ARBORKNOX1* of poplar and *STM* of *Arabidopsis*. 5' RACE indicated that the transcription initiation site is close to the start of translation and is conserved between *Arabidopsis* and leafy spurge. Putative *cis*-acting elements were identified in an *EeSTM* promoter, including several long elements that were conserved between leafy spurge and poplar that were independently identified as part of a larger conserved region in other species [12]. Another identified putative *cis*-acting element resembled a tuber-specific sucrose-responsive element which was hypothesized to play a role in the expression of *EeSTM* in root tissue [21].

We report here the differential expression of *EeSTM* during various dormancy transitions, and tissue specific expression of two different *EeSTM* genes (*EeSTM1* and *EeSTM4*). Additionally, reporter constructs indicate that *EeSTM* can drive tissue specific expression of a reporter gene in a heterologous system (*Arabidopsis*). We also test the activity of the tuber-specific sucrose-responsive element in driving *STM* expression outside the SAM in *Arabidopsis*. The results should prove beneficial in identifying molecular targets for regulating the development and growth of underground adventitious buds in leafy spurge and could result in better control strategies for leafy spurge and other perennial weeds with similar growth habits.

Materials and Methods

Plant material

Plants of *Euphorbia esula* used for the experiments were grown in cones (5 by 20 cm) with Sunshine mix (SUN GRO Horticulture, WA, USA) under greenhouse growth conditions as single stems under 16 h of natural and artificial lighting. All experiments were replicated at least twice for confirmation of results.

In order to study differential expression of *EeSTM* after paradormancy release, leafy spurge plants growing in the green house were defoliated by excising the aerial portions at the soil surface. Adventitious buds from 21-28 plants were then harvested, pooled, and stored in liquid nitrogen at various times following excision (0, 2, 4, 8, 16, 24, 48, 72 h). All the buds were harvested at the same time of the day to avoid artifacts caused by circadian effects. Real-Time PCR was done to quantify *EeSTM* expression (see below). Fold differences are shown relative to the 0 h time point. The experiment was repeated twice (two independent time course collections done several months apart).

To follow seasonal changes in *EeSTM* expression, adventitious root buds were harvested from leafy spurge from August 2001 through February 2005. Total RNA was extracted from these buds and cDNA synthesized. Semi-quantitative RT-PCR was done on seasonal cDNAs from 2001-02, 02-03 and 03-04. Serial dilutions of *EeSTM* containing plasmid was amplified in parallel as a concentration standard, and resulting gel was blotted and hybridized to ³²P labeled *EeSTM* probe. Label hybridizing to each band was quantified using a Packard Instant Imager. Real-Time PCR was performed on 2004-05 samples. The data

obtained was converted to fold differences in expression for each year using August cDNA as the calibrator.

For tissue specificity studies, leafy spurge seeds were germinated on moist filter paper in Petri dishes. The Petri dishes containing leafy spurge seeds were kept in an incubator at 37°C. The hypocotyl and radicle tissues were collected 8-10 d after emergence and elongation from the seed. Mature root tissue was collected from 4 month old leafy spurge plants. Adventitious buds were removed from the roots before harvest. Leafy spurge leaves were collected from middle 1/3rd of the stalk. Meristems were collected by excising the tip of the leafy spurge plant and removing most of the immature leaves from the shoot tip. All the leafy spurge tissues (hypocotyl, radicle, old root, leaf and meristem) were frozen in liquid nitrogen and stored at -80°C.

Real-Time PCR

Expression analysis of *EeSTM* was characterized by Real-Time PCR. For all analyses except the time course following paradormancy release, TaqMan chemistry was used in a 7300 Real-Time PCR system. A two-step RT-PCR assay was used for relative quantitation of *EeSTM* cDNA synthesized from time-course and seasonal RNAs. Total RNA was extracted from underground adventitious buds of leafy spurge at various times using the pine tree extraction method [22]. cDNA was synthesized from samples and trace amounts of ³²P-dCTP were included to help assess the quality and quantity of the resulting cDNA via gel electrophoresis, blotting, and quantification of the incorporated label. All samples were amplified in triplicate using ATG ATT GCT TTT GGA GAC AAC A (5') and AGT CTG CAT TAT GAT GAT GAT G (3') primer sequences. The FAM dye-labeled TaqMan probe sequence used for this study was TGG AGG AGG AGG AGG AGG AG. T_m for primers and probe was 60°C and 70°C. Various microarray experiments suggest that a large number of endogenous genes are differentially regulated during dormancy transitions, thus we chose to use an exogenous spike control (total Human cDNA) to normalize quantification of the target cDNA. Multiplex PCR was performed in each reaction tube containing 0.02 µg of total leafy spurge cDNA and 0.01 µg of total human cDNA with primers and probes corresponding to *EeSTM* and human *GAPDH*. A comparative CT (Threshold cycle) method was used in which the *EeSTM* cDNA was normalized to an exogenous (spiked control) reference (Human *GAPDH*). Serial dilutions (10 fold) of the *EeSTM* cDNA clone were included as external quantification controls. A negative control without *EeSTM* cDNA and a *GAPDH* control were also included in this expression study. All reactions were run in triplicate. For the paradormancy release time course, equal amounts of cDNA were used in each reaction and reactions were run in triplicate. SYBR green and the endogenous ROX reference dye were used to determine relative CT values. Numerous genes with expression patterns confirmed by northern hybridization (*HISTONE H3* being only one such gene shown) were run concurrently to ensure appropriate amplification and quantification of each sample.

Allelic discrimination assay

An allelic discrimination assay was conducted on cDNA synthesized from various leafy spurge tissues. All samples (hypocotyl, radicle, old root, leaf, and meristem) were amplified in triplicate using GCACCTACTGGTGGTTCTTCTTCT (5') and GCAGTGTGTTGAGTTTGAGTTTGTGT (3') primer sequences. Two probes were used, one specific to *EeSTM1* (VIC labeled – TGATCGTCAATAATCAT) allele and the other specific to *EeSTM4* allele (FAM labeled – ATCGTCAAGAATCAT). A standard 96-well reaction plate was used for loading samples and for the Pre-read,

Amplification, and Post-read steps of the assay. Both negative and positive controls were included. Analysis was done using the SDS software (v 1.4.1).

Transgene construction

Primers sequences CCGCTCTAGAAGTACTCTCG (5') and CTCTCCTCAACAAATCCTAC (3') were used to amplify 1,918 base pairs (bp) of promoter sequence (including 5' UTR) from the *EeSTM* genomic clone (Genbank accession #EF636204). Likewise, primers sequences ACGAATCACTGTCCTTAACC (5') and CTTCTCTTCTCTCACTAG (3') were used to amplify up to 1,934 bp of the *STM* promoter sequence (including 5' UTR) from genomic DNA of *Arabidopsis*. For functional analysis of the putative sucrose-responsive element - *STK* (*STOREKEEPER*) binding site, primers were developed to replace the *STK* binding site with a sequence containing a *Bam*-HI restriction site. Two primer sets, CCGCTCTAGAAGTACTCTCG (5'), ATGGATCCAGTGGTCTCCCG (3') and GTGGATCCCTTTGTGGCTAT (5'), CTCTCCTCAACAAATCCTAC (3') were used to amplify two fragments from the *EeSTM* promoter sequences immediately flanking the *STK* element. The PCR amplified promoter fragments were digested with *Bam*HI and ligated to form a single contiguous promoter without the sucrose element. The amplified DNAs were gel purified and ligated into pBI121, replacing the cauliflower mosaic virus 35S promoter (CaMV 35S). Constructs were confirmed by PCR and used to transform *Arabidopsis*.

Transformation of *Arabidopsis*

Seeds of the wild-type *Arabidopsis* (Columbia) were sown in green house pots and grown until they begin flowering and ready for transformation. These flowering *Arabidopsis* plants were transformed by *Agrobacterium tumefaciens* (strain GV3101) harboring the binary vector pBI 121 containing *EeSTM* promoter constructs using the floral dip method [23]. Putative transgenic seeds (T_1) from 7-10 independent lines for each construct were collected and re-screened for kanamycin resistance (Kan^r). Several plants each from at least three lines from each construct that produced Kan^r seeds (T_2) were transplanted in potting mix and allowed to mature. Transformation of T_2 plants was confirmed by PCR using a GUS primer (5' TTCATGACG ACCAAAGCCAGTAAAGT 3') and one of the 5' *STM* promoter primers from leafy spurge or *Arabidopsis*. The amplified DNA band was confirmed for each construct by sequencing. Transgenic plants were allowed to grow and produce seeds (T_3). 20-40 T_3 seedlings each from at least three lines for each construct were GUS stained to characterize the activity of the leafy spurge and *Arabidopsis* *STM* promoters.

Results

EeSTM expression following release from paradormancy

Two full length cDNA (EF636205, EF636206) of *EeSTM* were obtained (*EeSTM1* and *EeSTM4*) [21]. Since both clones were similar, the initial analysis of expression was done using a primer and probe set designed to detect both transcripts. *AtSTM* is required for development and maintenance of the shoot apical meristem in *Arabidopsis* [3]. However, it is not clear if *EeSTM* was expressed in dormant meristems or if growth induction had any impact on *EeSTM* expression. We investigated *EeSTM* expression in paradormant and growing buds of leafy spurge using Real-Time PCR with primers that would amplify any known *EeSTM* genes (Figure 1). *EeSTM* is initially down-regulated shortly after release of buds from paradormancy by defoliation and then within 4 h, *EeSTM* expression steadily increases above initial levels for 2-3 d. Interestingly, increased *EeSTM* expression clearly precedes

Figure 1: Real-Time PCR analysis of *EeSTM* and *HISTONE H3* expression in underground buds of leafy spurge. Crown buds were collected at various times following release of buds from paradormancy. Real-Time PCR analyses from two independently isolated time course series are shown. Expression pattern was relative to non-growing crown buds (time 0).

Figure 2: Seasonal expression of *EeSTM*. *EeSTM* expression was examined in crown buds collected monthly over 4 years. August cDNA was used as a calibrator for relative quantification of *EeSTM*. Error bars represent standard error. The average temperature during the week prior to bud harvest is noted below each sample point.

the induction of *HISTONE H3* which was up-regulated only after 16 h following growth induction.

Seasonal expression of *EeSTM*

Leafy spurge buds can be maintained in a paradormant, endodormant or ecodormant state, and the transition from paradormancy to growth resulted in a change in *EeSTM* levels. Thus, it was of interest to determine if *EeSTM* expression was differentially regulated during the seasonal transition from paradormancy to endo- or ecodormancy. *EeSTM* expression was examined in buds collected monthly over several different years. Although there was a significant trend for *EeSTM* expression to drop in Dec. and Jan. and then increased in late winter, *EeSTM* expression fluctuated substantially between monthly samples depending on the year, particularly during Oct. and Nov. when buds were endodormant (Figure 2).

Tissue specific expression of *EeSTM*

The presence of possible root-specific *cis*-acting elements in the promoter of *EeSTM*, and the ability of leafy spurge to develop adventitious buds on its root system suggested the possibility that *EeSTM* could be expressed in root tissue. To determine which tissues *EeSTM* was expressed in leafy spurge, Real-Time PCR was used to analyze RNA from leaf, meristem, young hypocotyl, young root, and old root tissue (devoid of visible adventitious buds) (Figure 3). Surprisingly, these experiments indicated that maximum expression of *EeSTM* was

Figure 3: Relative tissue specific expression of *EeSTM* from three independent samples as quantified by Real-Time PCR. Error bars standard error from three different samples for each tissue. Tissues from young hypocotyl (YH), young root (YR), shoot meristem (M), leaves (L), and mature root (MR), were used for this study.

Figure 4: Real-Time Allelic Discrimination Assay of *EeSTM1* and *EeSTM4* alleles. Each dot represents a well in the 96-well reaction plate of Real-Time assay. Tissues containing only *EeSTM4* (diamonds) are aligned more towards the upper left corner of the plot, whereas control samples containing only *EeSTM1* (circles) are aligned more towards the lower right corner of the plot. Tissues containing both alleles (triangles) are approximately midway between the above two groupings. Negative controls (squares) grouped in the bottom left as expected. Data were plotted by using the absolute fluorescence of each reporter dye probe.

in old roots followed by young roots, young hypocotyl, meristem, in that order, but not in the leaf tissue as would be expected for an *STM* orthologue. Expression of *EeSTM* in old and young roots and hypocotyl of leafy spurge seedlings is consistent with the ability of leafy spurge to form adventitious meristems in these tissues.

Differential tissue specific expression of *EeSTM1* and *EeSTM4*

The differences in the coding sequences of *EeSTM1* and *EeSTM4* suggested they might be homeologous or paralogous genes rather than alleles of the same gene. Thus, it was possible that these genes might have different expression profiles. To test this hypothesis, an allelic discrimination assay was conducted to detect variation in expression patterns of *EeSTM1* and *EeSTM4* in various tissues of leafy spurge (Figure 4). *EeSTM4* expression was detected in all tissues tested, however surprisingly *EeSTM1* was only called as “present” in the young root and hypocotyl.

Heterologous activity of the *EeSTM* promoter in *Arabidopsis*

Screening of a leafy spurge genomic library only identified a single clone with defined promoter sequence [21]. The coding sequence from this clone was most similar, but not identical, to *EeSTM4*. The presence of conserved sequences in this *EeSTM* promoter suggested that it might be able to accurately direct expression of a reporter gene in a heterologous system such as *Arabidopsis* [21]. To test this hypothesis, the 2000 bp fragment of the *EeSTM* genomic clone including the 5'UTR sequence was used to drive *GUS* expression in transgenic *Arabidopsis*. The results indicated that this *EeSTM* promoter could direct *GUS* expression in the SAM of *Arabidopsis* (Figure 5). Interestingly, *GUS* expression was also observed in the hypocotyl and root pericycle of the transgenic plants carrying the *EeSTM:GUS* construct, whereas the construct containing a similar region of the *AtSTM* promoter only drove *GUS* expression in the meristem (Figure 5). This observation is consistent with expression of *EeSTM* in the root and hypocotyl observed in leafy spurge, and supports the hypothesis that elements within this *EeSTM* promoter might be needed for development of underground adventitious shoots in leafy spurge.

Root and hypocotyl expression is not dependent on *STK* element

Initial characterization of the *EeSTM* promoter identified a putative root-specific sucrose-responsive element (*STOREKEEPER* binding site- *STK*) that was unique to leafy spurge [21]. Consequently we hypothesized that this element might be involved in regulating expression of *EeSTM* in root tissue. We therefore deleted this element in our reporter gene and analyzed *GUS* expression in transgenic *Arabidopsis*. The results clearly indicated that *GUS* was still expressed in roots and hypocotyls of transgenic *Arabidopsis* (Figure 5). Thus, it appeared that deletion of this element had no effect on expression from the *EeSTM* promoter.

Figure 5: *GUS* expression in transgenic *Arabidopsis* on whole plant (A), close up of meristem region (B), or of hypocotyl-root boundary (C). Representative *GUS*-stained plants transformed with a construct in which the *AtSTM* promoter and 5'UTR are driving *GUS* expression (*AtSTM*), *EeSTM* promoter and 5'UTR driving *GUS* (*EeSTM*), or the *EeSTM* promoter and 5'UTR in which the *STK* binding site was replaced with a *Bam*HI restriction site (*EeSTMΔSTK*).

Discussion

Characterization of the *EeSTM* genes

The minor differences observed between cDNA and genomic clones of *EeSTM* suggest there may be several alleles of *EeSTM* in leafy spurge [21]. This was expected since leafy spurge is an auto-allo hexaploid [14]. Given that poplar has two *STM* orthologues and that poplar is in the same order as leafy spurge, there could be as many as 12 or more different copies (alleles, paralogues, and homeologues) of *STM* in leafy spurge. *EeSTM* genes form a separate clade with orthologous genes from other perennials such as poplar (*Populus alba*), and snapdragon (*Antirrhinum majus*). The separate clade also includes *AtSTM* from *Arabidopsis* but excludes *KNAT1* from *Arabidopsis* and the *STM-like* genes from monocots [21]. These results suggest that our *EeSTM* genes represent orthologues or paralogues of *AtSTM* rather than a different member of the Class I *KNOX* gene family.

Regulation of *EeSTM* in leafy spurge and *Arabidopsis*

The observation that the *EeSTM* promoter can direct expression in the roots of leafy spurge and *Arabidopsis* indicates that some conserved element directs root specific expression. Thus, the presence of a putative *STK* binding site in the promoter of the *EeSTM* gene was intriguing. *STOREKEEPER (STK)* is a conserved DNA-binding protein shown to recognize the 10 bp motif (GCTAAACAAT) in potato and regulate the expression of patatin [24] in potato tubers. It is interesting to note that this 10 bp motif is not found in the promoter of *STM* gene in *Arabidopsis* or poplar. *AtSTM* is not normally expressed in the roots of *Arabidopsis*, and poplar plants do not normally maintain a population of preformed adventitious shoot buds on their root system, although poplar can and does form adventitious shoots from its roots following damage to the root system or other shoot inducing treatments. Leafy spurge however forms viable shoot meristems on both the lateral roots and hypocotyl as early as 8 days following germination [16]. Thus it was hypothesized that this *STK* binding site might be important for bud formation on underground organs of leafy spurge. However, the results of these experiments (Figure 5) suggest that the putative *STK* binding site is not required for *EeSTM* expression in roots of leafy spurge. It will be interesting to determine what other elements are required for root specific expression of *EeSTM* genes. There are several related spurges that only form buds on hypocotyls or that do not form underground buds at all [25]. A comparison between *STM* gene regulations between these related species could identify elements required for root specific expression of *STM*.

In *Arabidopsis*, it appears that *AtSTM* is regulated by mechanisms involving chromatin remodeling [11], however there is also evidence that Myb-type transcription factors such as *AS1* and *CUC* also regulate *STM* expression [8,10]. Although some critical biochemical components of this chromatin remodeling are well characterized, there is insufficient understanding of the targeting and action of such proteins and protein complexes to allow specific manipulation of chromatin regulated genes. Consequently, coordinated cross-species comparisons of regulatory mechanisms could shed much needed light on such processes. Since the *EeSTM* promoter can accurately direct meristem specific expression in a heterologous system such as *Arabidopsis*, and since there appear to be both conserved and species specific elements within the *EeSTM* promoter, chimeric constructs can be produced with elements from leafy spurge and *Arabidopsis* to dissect the sequences responsible for regulating this critical developmental gene.

Tissue-specific expression of *EeSTM* and its alleles in leafy spurge

The Real-Time expression of *EeSTM* in hypocotyl, old root, young root (radicle), and meristem and not in the leaf tissue is consistent with the *EeSTM* promoter driven *GUS* expression in *Arabidopsis*. We detected expression of both *EeSTM1* and *EeSTM4* genes in the juvenile tissues (radicle and hypocotyl) but only *EeSTM4* in older tissues (meristem, and old root). This suggests that *EeSTM1* is responding to developmental specific signals that either turn this gene on in juvenile tissue or turn it off in older tissue. Other perennials such as poplar also have paralogous copies of *STM*. It would be interesting to determine if there is also tissue specific regulation of paralogous in these other species. It was also surprising to note that there was relatively higher expression of *EeSTM* in the root and hypocotyl tissues than in the meristem. The reason for this is unclear, and needs further investigation. However, the fact that a relatively limited number of cells in the SAM express *STM* in *Arabidopsis* and that our meristem samples contained a number of immature leaves and a small portion of the stem could account for the relatively lower concentration of *STM* mRNA in these samples. In situ hybridizations with *EeSTM* specific probes and antibodies on root and meristem sections will be needed to determine if indeed *EeSTM* was more highly expressed on a cell by cell basis between the various root cell types and cells in the central zone of the meristem, and to determine if the *EeSTM* gene expression is indicative of *EeSTM* protein accumulation.

EeSTM expression in relation to growth induction

There is differential regulation of other genes responsive to cell cycle in growing versus non-growing tissues (dormant buds) of leafy spurge [19,26]. Genes like *HISTONE H3*, *TUBULIN*, and *CYCLIN D3* are up-regulated in underground buds after induction of growth by defoliation [19]. The Real-Time PCR results indicate that induction of *EeSTM* follows a roughly similar pattern of expression observed for *CYCLIN D* or *HISTONE H3* but that the subsequent induction of *EeSTM* expression occurs earlier than the rise of *HISTONE H3* or *CYCLIN D3* expression (Figure 1) [26]. *AtSTM* up-regulates the cytokinin biosynthesis gene *isopentenyl transferase 7 (AtIPT7)* and activates cytokinin response factor, resulting in increased synthesis of cytokinin and up-regulation of cytokinin-responsive *CYCLIN-D* [27]. *AtSTM* and *CYCLIN D3* are both up-regulated in *abnormal meristem mutant 1 (amp 1)*, indicating a link between growth and development [28]. Thus, our observations are consistent with studies that suggest *AtSTM* expression may impact cell division processes and *CYCLIN* expression. Our results also suggest that induction of developmental responsive signaling systems precede induction of growth *per se*. Additionally, the up-regulation of *EeSTM* following induction of active growth suggests that *EeSTM* may be needed to sustain shoot growth and development in adventitious buds following re-initiation of growth.

The initial drop in *EeSTM* expression is also intriguing. The leafy spurge plants could be responding to wounding stress following decapitation. Down-regulation of genes for a brief period might be one of the ways to conserve energy under stress and to readjust physiology before putting forth new shoots. Elevated levels of jasmonic acid and oxophytodienoic acid produced during wounding stress [29] have an antagonistic effect on the endogenous cytokinin levels [30]. *AtSTM* is positively regulated by cytokinins [28]. A decreased cytokinin level could therefore down-regulate *EeSTM* immediately after decapitation.

Expression of *EeSTM* during dormancy transitions

The relative down regulation of *EeSTM* in early winter as the buds transition into ecodormancy, and subsequent up-regulation as the buds move out of ecodormancy in early spring is consistent with similar expression patterns observed for various cell cycle genes such as *HISTONE H3* and *CYCLIN D3* [26]. Also, there was substantial variation in *EeSTM* expression during Oct. and Nov. when the buds are in endodormancy. This variation did not appear to correlate either with temperatures near the time of harvest or the depth of the dormant state of the plants as noted in parallel experiments (data not shown). This indicates that *EeSTM* expression during endodormancy may be regulated by other unknown factors.

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