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EXPRESSION PROFILING OF THE SPERMIDINE SYNTHASE3 (SPDS3) AND SPERMINE SYNTHASE (SPMS) GENES DURING THE LIFE OF ARABIDOPSIS

BY

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B.S. University of New Hampshire, 2004

THESIS

Submitted to the University of New Hampshire
in
Partial Fulfillment of the Requirements for the Degree of

Master of Science

In

Plant Biology

December, 2006
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Associate Professor of Plant Biology and Genetics

Date

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DEDICATION

I would like to dedicate this thesis to my parents who never let me give up on my goals; to my three brothers who provided an entertaining childhood; and to my best friend and the love of my life, Amanda.
ACKNOWLEDGMENTS

I would like to thank all the people who contributed to the completion of this work. First, I express my deepest gratitude to my advisor, Dr. Subhash C. Minocha, for his guidance throughout this research, which started with his willingness to take me on as an undergraduate. I would also like to thank Dr. Estelle Hrabak for her countless hours of technical assistance and support. I would also like to thank Dr. Rakesh Minocha who continuously provides insight and technical support for our lab and only offers encouraging criticism. I thank the entire Department of Plant Biology for their outstanding academic support. I must also note the work of several undergraduates, Scott Sanders, Evan Jordan, and Vivian Wan, who provided assistance in one way or another. I must thank Todd Bezold who was brave enough to provide my initial training and guide me to my first research project. I am also indebted to Jeff Mitchell and Dr. Andrew Page who provided an equal amount of technical support. Of course, I offer my sincere thanks to the other Minocha lab members who provided an entertaining and supportive research atmosphere throughout my years: Sridev “The Dude” Mohapatra, Michelle Serapiglia, Katherine Tiberghien, Rajtilak Majumdar, Smita Singh, and Dr. Sridevi Ganapathi. Finally, to all my friends and family who supported me through the years.
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ABSTRACT

EXPRESSION PROFILING OF THE SPERMINIDINE SYNTHASE3 (SPDS3) AND SPERMINE SYNTHASE (SPMS) GENES DURING THE LIFE OF ARABIDOPSIS

by

Charles Rice

University of New Hampshire, December 2006

Polyamines are low molecular weight nitrogenous compounds found in all living organisms. These omnipresent molecules have been extensively studied in plant systems and have been implicated in a number of physiological responses including growth, development, and stress response. The proposed role of polyamines in vital processes such as cell division and differentiation has sparked enthusiasm in further investigation of this relatively small biosynthetic pathway. Very little is known about the regulation of polyamine biosynthetic genes, an aspect that can elucidate further functions of these ubiquitous compounds. In the following study, the expression profiles of two of these genes, spermidine synthase3 (SPDS3) and spermine synthase (SPMS), were analyzed in the model plant, Arabidopsis thaliana. The promoter::GUS fusion technique was used to provide a detailed expression profile of both genes during the entire life cycle of A. thaliana. Three constructs were designed for each gene containing different segments of the putative promoter region, the entire 5'UTR, and in some cases, a portion of the open reading frame. Each construct was individually transformed into A. thaliana and transformed plants were assayed for GUS activity in every organ, during various time points of development. Overall, the expression of SPDS3 was found to be high in young developing tissues with continued, but weaker, expression in the vascular tissue of mature plants. A similar expression profile was observed for SPMS, however, expression was observed in meristematic and elongating regions of tissue. In addition to the developmental profile, the changes in expression were observed during various abiotic stress conditions. Overall, both SPDS3 and SPMS appear to be induced in response to drought and 100 mM salt stress. There appears to be a slight increase in expression during chilling stress, but expression soon decreased over a 24 h period. SPDS3 was also induced during wounding.
INTRODUCTION

Polyamines

Polyamines are low molecular weight nitrogenous compounds found in all living cells. The three common polyamines are putrescine (Put), spermidine (Spd), and spermine (Spm). Other polyamines termed ‘uncommon’, include norspermidine (caldine), norspermine (thermine), pentamine, and hexamine. The uncommon polyamines are only found in specific plant families and microbes (Kuehn and Rodriguez-Garay, 1990) and will not be discussed here. In nature, polyamines exist either in a free form or conjugated with phenolic acids and bound to various macromolecules (Martin-Tanguay, 1997; Bagni and Tassoni, 2001). In plant cells, polyamine titers range from a few μmol/g of fresh weight to several mmol/g fresh weight. At cellular pH, polyamines are cationic, which partly explains their association with anionic cellular macromolecules such as DNA, RNA, phospholipids, or certain proteins (Bachrach et al., 1983; Cohen, 1998). At the molecular level, polyamines have been shown to stabilize nucleic acids, promote the accuracy of mRNA translation, and aid in the activation of specific enzymes (Cohen, 1998). In addition to these functions, they have been implicated in other developmental and growth processes within plants such as cell division, flower bud development, embryogenesis, ripening of fruit, leaf senescence, differentiation of tissues and organs (Bagni et al., 1993; Minocha and Minocha, 1995; Walden et al., 1997; Kakkar and Sawhney, 2002), and response to environmental stresses including drought, salinity and soil pH (Minocha et al., 2003; Bouchereau et al.,
1999). In addition, polyamines can serve as precursors for secondary metabolites such as nicotine and tropane and, when conjugated with phenolic acids, they produce defense-related compounds for plants (Smith et al., 1979; Flores et al., 1989; Hashimoto and Yamada, 1994; Martin-Tanguay, 1997).

There are five key enzymes in the pathway that are involved in the biosynthesis of polyamines (Figure 1); these enzymes are ornithine decarboxylase (ODC; EC 4.1.1.17), arginine decarboxylase (ADC; EC 4.1.1.19), S-adenosylmethionine decarboxylase (SAMDC; EC 4.1.1.50), spermidine synthase (SPDS; EC 2.5.1.16), and spermine synthase (SPMS; EC 2.5.1.22). The diamine putrescine is synthesized either from arginine or from ornithine by ADC and ODC, respectively. In most animals, putrescine is produced exclusively through ODC, while plants use both ADC and ODC with their presence being tissue specific and developmentally regulated. Despite the occasional reports of ODC activity, to the contrary, it appears that *Arabidopsis* does not contain the ODC enzyme or *ODC* gene (Hanfrey et al., 2001). Thus, *Arabidopsis* exclusively utilizes the ADC pathway to produce putrescine. The only other organism known to lack *ODC* is *Trypanosoma cruzi* (Carrillo et al., 1999). From putrescine, the triamine spermidine is formed by the addition of an aminopropyl group and the tetramine spermine is, in turn, formed by the addition of another aminopropyl group to spermidine. These additions are catalyzed by the enzymes SPDS and SPMS with the aminopropyl group being derived from decarboxylated SAM which is the product of SAM decarboxylation by SAMDC.

Numerous studies have been performed on the essentiality of polyamines in both prokaryotes and eukaryotes. Some studies involve single gene mutants for key biosynthetic enzymes in the polyamine pathway. In both *Escherichia coli* and
*Saccharomyces cerevisiae*, mutants fail to grow without medium supplemented with specific polyamines (Tabor and Tabor, 1984; Hanzawa et al., 2002; Panicot et al., 2002). Other mutants lacking the SPDS or SPMS gene have numerous morphogenic problems such as the inability to sporulate and errors in RNA/DNA synthesis. Mammalian cells have also demonstrated a requirement for polyamines for normal growth in tissue culture (Pegg, 1986). Despite the obvious importance of polyamines in growth and development, the precise mechanism of their action remains unclear. Although this remains a topic of debate, several ideas have been postulated due to their ability to interact with anionic macromolecules. Aside from protecting nucleic acids from various modifications (Rajalakshmi et al., 1978; Feurstein and Marton, 1989), polyamines have been shown to play a role in the synthesis of DNA and RNA (Nishiguchi et al., 1986; Jacob and Stetler, 1989), as well as in translation (Barbiroli et al., 1989).

![Polyamine biosynthetic pathway](image)

Figure 1. Polyamine biosynthetic pathway (adapted from Minocha and Minocha, 1995).

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Polyamine Biosynthetic Genes in *Arabidopsis*

In the year 2000, *Arabidopsis thaliana* became the first plant to have its entire genome sequenced (Arabidopsis Genome Initiative, 2000). Due to its small genome size (125 Mb) and small amount of repetitive DNA, *Arabidopsis* has become a model system for a number of genetic and genomic studies. *Arabidopsis* has also become a model plant for numerous biochemical and physiological studies and is currently the focus of a study aimed at analyzing the function of all ~25,000 genes in its genome (of which ~12,000 are unique in *Arabidopsis*). In this study, we have chosen *Arabidopsis* as a model organism for studying the polyamine biosynthetic pathway.

There are a number of biosynthetic enzymes involved in the polyamine pathway, although ADC, ODC, SAMDC, SPDS, and SPMS are considered to be the most important due to their location in the pathway and ultimate function of producing the most common polyamines (putrescine, spermidine, and spermine). It has been shown that *Arabidopsis* has multiple copies of the genes coding for these enzymes (Urano et al., 2003). Table 1 summarizes the known sequences of the polyamine biosynthetic genes identified from the NCBI (National Center for Biotechnology Information; www.ncbi.nlm.nih.gov) and TAIR (The Arabidopsis Information Resource; www.arabidopsis.org) databases. Whereas the genomic sequences for these genes have been identified, the function and expression patterns remain ambiguous. In my study, the expression profiles of two of these genes (*SPDS3* and *SPMS*) were investigated during the entire life of *Arabidopsis thaliana*.

As previously mentioned, *Arabidopsis* apparently lacks the *ODC* gene despite the occasional detection of decarboxylation of ornithine *in vitro* (Hanfrey et al., 2001).
Therefore, *Arabidopsis* relies solely on the ADC pathway for production of putrescine. The *Arabidopsis* genome contains two paralogs of the *ADC* gene (Table 1), denoted *ADC1* (accession # NM_127204) and *ADC2* (accession # NM_202955, NM_119637). It has been postulated that the *ADC* gene was duplicated at the origin of the Brassicaceae family (Galloway et al., 1998). In *Arabidopsis*, the *ADC1* gene is located on chromosome 2 (At2g16500), while the *ADC2* gene is located on chromosome 4 (At4g34710). The nucleotide coding sequences of these genes share a high degree of homology (78% identity). The *ADC* gene appears to be regulated at both the transcriptional and post-translational levels. Watson and Malmberg (1996) depicted the oat (*Avena sativa*) ADC as a proenzyme synthesized as a 66 kDa preprotein that is cleaved into 42 kDa N terminal and a 24 kDa C-terminal domain polypeptides that are joined by a disulfide bridge. The cleavage activity was found to involve another enzyme separate from ADC itself. The *Arabidopsis* ADC protein has been suggested to be

<table>
<thead>
<tr>
<th>Gene</th>
<th>Chromosome Location</th>
<th>E.C.</th>
<th>Accession Number</th>
</tr>
</thead>
<tbody>
<tr>
<td>Arginine Decarboxylase 1</td>
<td>At2g16500</td>
<td>4.1.1.19</td>
<td>NM_127204</td>
</tr>
<tr>
<td>Arginine Decarboxylase 2</td>
<td>At4g34710</td>
<td>4.1.1.19</td>
<td>NM_202955, NM_119637</td>
</tr>
<tr>
<td>Spermidine Synthase 1</td>
<td>At1g23820</td>
<td>2.5.1.16</td>
<td>NM_102230, NM_202171</td>
</tr>
<tr>
<td>Spermidine Synthase 2</td>
<td>At1g70310</td>
<td>2.5.1.16</td>
<td>NM_105699</td>
</tr>
<tr>
<td>Spermidine Synthase 3</td>
<td>At5g53120</td>
<td>2.5.1.16</td>
<td>NM_124691, NM_180848, NM_180847</td>
</tr>
<tr>
<td>Spermine Synthase</td>
<td>At5g19530</td>
<td>2.5.1.22</td>
<td>NM_121958</td>
</tr>
<tr>
<td>S-adenosylmethionine decarboxylase 1</td>
<td>At3g02470</td>
<td>4.1.1.50</td>
<td>NM_111114, NM_001035545</td>
</tr>
<tr>
<td>S-adenosylmethionine decarboxylase 2</td>
<td>At5g15950</td>
<td>4.1.1.50</td>
<td>NM_121600, NM_001036811</td>
</tr>
</tbody>
</table>
similar to the ODC protein which is a head-to-tail homodimer with two active sites acting in trans across the interface of the dimer (Hanfrey et al., 2001).

Physiological roles of ADC have been suggested in both developmental and stress responses in plants. An increase in ADC expression has been shown to occur in the early stages of fruit development in a number of species (Egea-Cortines and Mizrahi, 1991), including peas (Pisum sativum L.; Perez-Amador and Carbonell, 1995) and tomato (Lycopersicon esculentum L.; Rastogi et al., 1993). Watson et al. (1998) studied adcl/adc2 double mutants in Arabidopsis which reduced ADC activity to 20% of wild type. The resulting phenotypes displayed kinked and clustered roots and narrow leaves, sepals, and petals. Further indication of ADC’s role in development came from the work of Urano et al. (2005) who studied defects in both paralogs using mutants. In mutants defective in the ADC1 gene, HPLC analysis revealed no changes in polyamine levels of vegetative tissues. However, mutants defective in the ADC2 gene displayed a 70% reduction of putrescine and a 35% reduction of spermidine, suggesting that ADC2 plays an important role in putrescine production of Arabidopsis. Yet, no phenotypic changes were observed for either mutant, further suggesting that ADC1 is capable of compensating for the loss of ADC2. To further test this hypothesis, the authors attempted to create double mutants (ADC1−ADC2−) lacking all ADC transcripts and thus eliminating putrescine. All seeds with the double mutant genotype failed to germinate indicating that at least one copy of ADC is required for seed development. RT-PCR analysis of wild type plants found a strong increase in ADC2 expression during late seed development and a slight increase in ADC1 expression during the same stage. This further suggests that the ADC genes, and thus polyamines, are essential during seed development.
Seed development is characterized by two phases, the embryo growth phase and the seed maturation phase. Polyamines have been shown to play an important role during embryogenesis, as the inhibition of ADC in carrot cells via DFMA (DL-α-Difluoromethylarginine) prevented somatic embryogenesis (Robie and Minocha, 1989). Also the over-expression of a mouse ODC in carrot cells yielded a high degree of somatic embryogenesis (Bastola and Minocha, 1995). With the use of promoter-reporter gene analysis, Hummel et al. (2004) reported ADC1 activity in *Arabidopsis* primarily in developing roots, whereas ADC2 expression was predominant during seed germination and seedling development. Yet, a previous study detected ADC1 expression in all tissues and ADC2 activity in the siliques and cauline leaves only (Watson and Malmberg, 1996). These findings were similar in the Urano et al. (2003) study using RT-PCR to detect transcript levels of the ADC paralogs in *Arabidopsis*. ADC1 mRNA was detected equally in all organs during development except in mature siliques. ADC2 transcripts were detected predominately in the flowers, buds, immature siliques and rosette leaves, indicating that ADC1 is constitutive, whereas ADC2 is an organ-specific gene. Using the promoter::GUS technique, ADC2 expression was found to be weak and sporadic throughout the development of *Arabidopsis*, with particular localization in the trichomes of rosette and cauline leaves (Mitchell 2004). It appears through a number of studies that ADC plays an extremely important role in the growth and development of a plant starting at the embryo stage. The role of ADC in stress is discussed later.

As with ADC, there are multiple copies of SAMDC in *Arabidopsis*. Through genome analysis, four paralogs of SAMDC have been identified (Table 1), of which two have been shown to code for SAMDC (Franceschetti et al., 2001). SAMDC1 (accession #
NM_111114, NM_001035545) is located on chromosome 3 (At3g02470) and \textit{SAMDC2} (accession # NM_121600, NM_001036811) is located on chromosome 5 (At5g15950). The two paralogues share 49% nucleotide sequence identity and 81% amino acid sequence identity. The \textit{SAMDC} mRNAs contain two highly conserved upstream open reading frames (uORFs) in their 5’ untranslated region. The two uORFs are characterized as a tiny uORF, 3-4 codons in size, and a small uORF coding for approximately 50 amino acid residues (Franceschetti et al., 2001). The tiny uORF, which is highly conserved among species, is not translated. However, the small uORF (conserved among monocots, dicots, and gymnosperms) is translated and may play an important role in the repression of translation of \textit{SAMDC} due to altering polyamine levels, as shown in both humans (Ruan et al., 1996) and \textit{Arabidopsis} (Hanfrey et al., 2002). The uORFs may explain why the putative \textit{SAMDC3} (accession # NM_113454; At3g25570) and \textit{SAMDC4} (accession # NM_121898; At5g18930) genes are not functional as there appear to be defects in the uORF suggesting a possible reason for the lack of expressed sequence tags for each gene (Franceschetti et al., 2001). It should also be mentioned that the two \textit{ADC} paralogs, as well as the \textit{SAMDC1} and \textit{SAMDC2} genes, do not have introns in the coding region, but do contain some introns in the 5’UTR which is unique to other polyamine genes.

It has been repeatedly mentioned that the ethylene pathway is in direct competition with the polyamine pathway for the substrate of SAMDC, S-adenosylmethionine (reviewed by Moffatt and Weretilnyk, 2001). The activity of SAMDC has been shown to be inversely proportional to ethylene production. Apelbaum et al. (1985) observed the decrease in SAMDC activity which enhanced ethylene

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production, whereas Roustan et al. (1992) reported the inhibition of ethylene synthesis with an increase of SAMDC activity. Therefore, it is presumed that the polyamine pathway plays a role in development in order to counteract the senescent effects of ethylene by utilizing the SAM substrate. This assumption is strengthened by the expression patterns observed for SAMDC in multiple plant species. SAMDC transcripts have been observed in all vegetative tissues of pea plants (Pisum sativum L.; Marco and Carrasco, 2002) and potato plants (Solanum tuberosum L.; Taylor et al., 1992). Mad-Arif et al. (1994) also observed high SAMDC expression in young, developing vegetative and reproductive tissues when compared to mature tissues. Similar findings were observed in transformed cultures of Datura stramonium (Michael et al., 1996), tobacco seedlings (Nicotiana tabacum; Scaramagli et al., 1999), and carnation petals (Dianthus caryophyllus L.; Lee et al., 1997a). RT-PCR analysis of SAMDC1 transcripts in Arabidopsis displayed a ubiquitous profile in all organs through development, whereas SAMDC2 mRNA was detected in flowers, buds, immature siliques and cauline leaves (Urano et al., 2003). This was further supported through promoter::GUS fusion analysis, in which SAMDC1 expression was strong and ubiquitous in all organs throughout the life cycle of Arabidopsis (Mitchell 2004). These patterns would suggest that SAMDC1 is a constitutively-expressed gene, whereas SAMDC2 is organ-specific.

Arabidopsis has three SPDS paralogues (Table 1) of which two (SPDS1 and SPDS2) have been shown to code for active SPDS enzymes (Hanzawa et al., 2002; Urano et al., 2003; Imai et al., 2004a). The first two genes are located on chromosome 1: SPDS1 (accession # NM_102230; At1g23820) and SPDS2 (accession # NM_105699; At1g70310). The third putative gene, SPDS3 (accession # NM_124691), is located on
chromosome 5 (At5g53120). These spermidine synthases represent three of the four amino- propyl transferases found in Arabidopsis (the other being SPMS). The nucleotide sequences of this family of genes share approximately 83% identity between SPDS1 and SPDS2, 65% between SPDS1 and SPDS3, and 62% between SPDS2 and SPD3 (Table 2). The intron-exon structures of the genes also appear to be highly conserved (Hanzawa et al., 2002; Panicot et al., 2002) despite the presence of a large intron, 603 bp, in the 5’ untranslated region of SPDS3. The molecular mass of the SPDS proteins in Arabidopsis is 36.6, 37.1, and 39.2 kDa for SPDS1, SPDS2, and SPDS3, respectively (Hanzawa et al., 2002). Whereas other plant species, such as Datura stramonium, Hyoscyamus niger, and pea (Pisum sativum L.), have fairly similar protein sizes as Arabidopsis, there is some variation of structure including a 74 kDa soybean SPDS and a 43 kDa maize SPDS (Panicot et al., 2002). Panicot et al. (2002) further reported the formation of heterodimers between Arabidopsis SPD1/SPDS2 and SPDS2/SPDS3 in vivo.

Table 2. Nucleotide sequence identities between the Arabidopsis coding regions of the SPDS1, SPDS2, SPDS3, and SPMS genes

<table>
<thead>
<tr>
<th></th>
<th>SPDS1</th>
<th>SPDS2</th>
<th>SPDS3</th>
<th>SPMS</th>
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<tr>
<td>SPDS1</td>
<td>-</td>
<td>83%</td>
<td>65%</td>
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<td>SPDS2</td>
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<td>SPMS</td>
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SPDS has recently been characterized with studies indicating the importance of spermidine for survival (Hanzawa et al., 2002; Panicot et al., 2002; Imai et al., 2004a; Ikeguchi et al., 2006). Imai et al. (2004a) created Arabidopsis double mutants of the SPDS1 and SPDS2 genes. The resulting phenotype was found to be embryo lethal,
indicating that spermidine is essential for embryo development and is produced largely by SPDS1 and SPDS2. Hanzawa et al. (2002) and Panicot et al. (2002) showed the requirement of spermidine using yeast mutants lacking SPDS activity. cDNAs for Arabidopsis SPDS1, SPDS2, and SPDS3 were cloned into yeast expression vectors and subsequently transformed into mutant yeast cells lacking SPDS activity (spe3 mutants). Using these mutants, Panicot et al. (2002) reported normal growth in cells expressing AtSPDS1 and AtSPDS2 indicating that the two Arabidopsis SPDS genes can complement the native yeast SPDS deficiency. However, transformants containing AtSPDS3 displayed growth arrest, indicating that AtSPDS3 does not produce a functional SPDS enzyme or the Arabidopsis SPDS3 requires certain conditions for optimal activity not met by the yeast cells. Further investigation of SPDS3 involved the use of the spe4 mutants lacking SPMS activity. Mutant cells transformed with Arabidopsis SPDS3 complemented the lack of SPMS, indicating that SPDS3 could be a functional enzyme showing spermine synthase activity. Similar results were seen in the Hanzawa et al. (2002) study using Y480 mutant cells with SPDS deficiencies. In cells complemented with Arabidopsis SPDS1 and SPDS2, there were large amounts of spermidine detected, however there was a small amount of spermidine accumulated in cells with SPDS3.

Using E. coli cells, feeding experiments with radiolabeled spermidine indicated that SPDS3 does indeed convert spermidine into spermine. The authors suggest that SPDS3 may have both spermidine and spermine synthase activities. Due to the results of the aforementioned studies, many have named the putative SPDS3 as SPMS, indicating that Arabidopsis possesses two SPMS genes (the other being ACL5). However, both NCBI
and TAIR have retained the *SPDS3* annotation despite the characterized SPMS activity. Therefore, we have also maintained the annotation of *SPDS3* in this study.

Hanzawa et al. (2002) examined the transcript levels of each *SPDS* gene using RNA gel blot hybridization. The authors were able to detect all three SPDS transcripts in whole seedlings, leaves, stem internodes, roots, inflorescences, and siliques. *SPD1* and *SPDS2* transcripts were predominant in root tissue and *SPDS3* expression was predominant in stem internodes, flower buds, and roots. Similar findings were reported by Urano et al. (2003) who detected *SPDS* transcripts in *Arabidopsis* organs via RT-PCR. *SPDS1* and *SPDS3* were constitutively expressed in all organs, whereas *SPDS2* transcripts were not found in mature siliques and upper stems. Zhang et al. (2003) reported *SPDS* expression patterns in apple (*Malus sylvesteris*) finding three homologs, of which two were actively expressed. The mRNA levels for each *SPDS* were higher in young leaves than in mature leaves and shoots. Bagga et al. (1997) also found high *SPDS* activity in alfalfa meristematic shoot tips and floral buds when compared to older, non-proliferating tissue. These expression patterns indicate that SPDS activity appears to be associated with actively-growing tissues.

Currently, only one *SPMS* gene, annotated as the *ACAULIS5* (*ACL5*) gene (accession # NM_121958), has been identified on chromosome 5 of *Arabidopsis* (*At5g19530; Table 1*). However, as previously mentioned SPDS3 has been shown to have both SPDS and SPMS activity, yet has maintained the *SPDS3* annotation in the genomic databases. Being an aminopropyltransferase, the *ACL5* gene shares a fairly high homology with the *Arabidopsis SPDS* genes, sharing close to 50% nucleotide sequence
identity amongst the three SPDS homologues (Table 2). The protein mass is also very similar, estimated at 38.5 kDa (Hanzawa et al., 2002).

ACL5 was first identified in Arabidopsis by Hanzawa et al. (1997) as a gene required for internodal growth and the maintenance of proliferating activity of inflorescence meristems. ACL5 mutants displayed a severe defect that restricted cell elongation, specifically in apical meristems. This gene was later characterized as encoding a spermine synthase (Hanzawa et al., 2000, 2002). RNA gel blot analysis revealed that the ACL5 transcripts accumulated in the stem internodes, flower buds, and root tissue, with much lower levels in vegetative tissue. RT-PCR analysis of transcript levels revealed expression in immature siliques, cauline leaves, and roots (Urano et al., 2003). This correlates with the mutant phenotype observations depicting the defects in reproductive tissues. However, in a later study by Imai et al. (2004b), SPMS was shown not to be essential for survival of the plant. In this study, mutants for ACL5 and the proposed functional SPMS gene, SPDS3, were created. There were no obvious phenotypic changes in mutants lacking the SPDS3 gene. Double mutants for acl5-l/spds3-l displayed no distinguishable difference from the acl5-l mutants which had the characteristic reduced stem growth. This indicated that either spermine is not essential for the survival of Arabidopsis or the roles of spermine can be compensated by spermidine and/or putrescine (Imai et al., 2004b). This study revealed further evidence that SPDS3 encodes spermine synthase activity. HPLC analysis of the spds3-l mutants revealed a dramatic decrease in free and conjugated spermine levels when compared to wild type plants, down to 5.8% and 3.4% respectively. However, there was no significant change in spermine levels in the acl5-l mutants. This indicated that SPDS3
plays a major role in spermine biosynthesis. The authors speculated that the heterodimerization between SPDS3 and SPDS2 (reported by Panicot et al., 2002) may favor SPDS3 as a SPMS taking into account that spermidine is a precursor to spermine. Yet, this raises the question of why ACL5 is solely involved in stem elongation and maintenance of meristematic activity? There are a number of speculations, however, it is apparent that these two genes need to be further characterized in order to better understand their functional roles. The published literature has given a great deal of insight (reviewed by Ikeguchi et al., 2006), yet no clear expression pattern has been reported at the cellular level, which made these two genes good candidates for my study.

The Role of Polyamines in Plant Stress

As mentioned previously, polyamines not only play a role in growth and development, but also play a major role in response to both abiotic and biotic stress. In plants, a number of nitrogenous compounds accumulate in response to environmental stress; including amino acids, amides, and polyamines (Bohnert and Jensen, 1996; Holmberg and Bulow, 1998; Minocha et al., 1997, 2000, 2003; Baur et al., 2004). Polyamines have been implicated in molecular signaling events in plant-pathogen interactions and responses to microbial symbionts which are important in plant nutrition (Bouchereau 1999). Polyamines can also serve as precursors for several classes of alkaloids, such as nicotine and tropane (Hashimoto and Yamada 1994; Flores et al., 1989; Smith et al. 1979), which may play important roles in plant defense during biotic stress. During abiotic stress, polyamines have been implicated as osmoprotectants due to their
cationic state and ability to interact with various anionic molecules such as DNA, RNA, certain proteins, and components of the cell membrane and wall.

Numerous attempts have been made to correlate polyamines and their effects on stress. One of the earliest studies was performed by Richards and Coleman (1952) who discovered a dramatic increase in putrescine levels during increased potassium titers in barley. Since then, putrescine accumulation has been shown to occur under various stresses such as water deprivation, high external osmolarity, high external concentration of ammonium or hydrogen ions, deficiency or excess of other monovalent cations, SO$_2$ fumigation, atmospheric pollutants, low temperature (chilling), as well as the aforementioned pathogenic responses (reviewed by Galston and Sawhney, 1990; Bouchereau et al., 1999). Further studies have shown direct correlations of polyamines and stress tolerance using polyamine biosynthetic enzyme inhibitors (Lee et al., 1997b; He et al., 2002). In these studies, stress-tolerant plant species were subjected to a polyamine enzyme inhibitor and placed in its “adapted stress environment”. The inability to produce the particular polyamines drastically decreased stress tolerance and, thus, the survival rate. However, if the exogenous polyamines were supplied to the same plants, stress tolerance was restored. Subsequent studies show that polyamine levels during various stress conditions can increase 2-3 fold when compared to non-stressed plants (Kasukabe et al., 2004). Aside from enzyme inhibitors, polyamines and stress response have been extensively studied using transgenic plants (Capell et al., 2004; Kasukabe et al., 2004). These transgenic plants can be designed to over-express a biosynthetic enzyme, which often results in the increased levels of a particular polyamine. This
method has been particularly helpful in studying polyamine roles in drought and salinity stress and will be discussed later.

The best characterized abiotic stress treatments in plants are salt and drought. Much work has been focused on understanding the molecular and biochemical response to these significant environmental factors. Plant cells undergo a variety of changes during both salinity and drought stress. Both stresses initially involve the lowering of the extracellular water potential. Plant cells often respond to such cellular dehydration by producing cystolic, low molecular weight, organic compounds and accumulating ions in the vacuole (Leshem and Kuiper, 1996). This process, called osmoregulation, aids in lowering the cellular water potential in order to restore turgor pressure. During such a stress, the formation of reactive oxygen species (ROS) is a common event within the cytoplasm. These molecules are very reactive and often inhibit enzyme functions and oxidize lipids in membranes. During long term salt stress, ion toxicity and energy imbalances greatly affect the cell, as sodium chloride has been shown to cause extensive damage to membrane integrity (Greenway and Munns, 1980) and interfere with multiple biochemical reactions such as respiration, photosynthesis, and protein and nucleic acid metabolism (Kakkar and Rai, 1997).

Salinity and drought stress have also been the focus of studies in relation to polyamines. Early salt treatment experiments demonstrated a trend of increased putrescine levels in plants. Strogonov (1964) observed putrescine accumulation in salt stressed leaves of Gossypium herbaceum with subsequent results in Vicia faba (Strogonov et al., 1972). Shevyakova (1981) observed an increase in putrescine in peas (Pisum sativum L ) and Vicia faba with the addition of 50 mM NaCl. Basu et al. (1988)
reported increased polyamine levels in salt-treated rice (*Oryza sativa* L.) seedlings with similar findings by Krishnamurthy and Bhagwat (1989). Prakesh and Prathapasenan (1988) observed a 56% increase in growth in salt-treated rice seedlings when exogenous putrescine was applied. Another interesting study compared the use of the ADC and ODC pathways during salt stress (Chattopadhay et al., 1997). Salt-sensitive and salt-tolerant lines of rice (*Oryza sativa* L.) were treated with increasing 150 mM salt increments over a 72 h period. Initially, there was a sharp increase in ADC for both lines, but the salt-sensitive plants displayed lower ADC levels beyond 6 h. The salt-tolerant line continued to show high ADC levels, but also showed high ODC levels. This supports other studies which suggest that, in some plant species, the ADC pathway is active during both normal and stress conditions, whereas the ODC pathway is functioning only during stress (Aziz et al., 1998). Kasukabe et al. (2004) performed a study using transgenic *Arabidopsis* transformed with the figleaf gourd (*Cucurbita ficifolia*) SPDS gene under the control of a 35S promoter. This construct showed over-expression of the SPDS protein, leading to an accumulation of spermidine. The transgenic lines were shown to have a significant increase in SPDS activity and spermidine content in the leaves which led to the enhanced tolerance to chilling, freezing, hyperosmosis, paraquat toxicity (oxidative stress), drought, and salinity. In addition, a cDNA microarray analysis revealed that there were a number of genes transcribed in the transgenic lines during stress that were not expressed in the wild type plants. These genes included stress-responsive transcription factors such as DREB and the protective protein rd29A. This suggests that polyamines may play a role as a signaling molecule in stress response. This correlates with similar studies which found polyamines promoting gene expression and
increasing the DNA-binding activity of other transcription factors (Gupta et al., 1998; Childs et al., 2003). This further supports the notion that the cationic state of polyamines enables the interaction with anionic macromolecules, such as DNA. Kasukabe et al. (2004) also showed a direct effect of increased polyamine levels during salinity and drought stress. The transgenic and wild type plants were subjected to a separate 15 day drought period and 75 mM NaCl stress. In both stress conditions, the transgenic plants were far more apt to tolerate the treatment than the wild type and displayed enhanced survival.

However, not all species have been found to accumulate polyamines during salinity stress, as a decrease in overall polyamine levels was observed in some halophytes (Priebe and Jager, 1978). This suggests that the role of polyamines in salinity stress may be species-specific. It has been suggested that the osmotic contribution of polyamines may not be as significant as some other compatible solutes, such as proline and glycine betaine (Kakkar and Rai, 1993). However, there is evidence that low levels of exogenously supplied putrescine stimulate the accumulation of proline, suggesting that the two are connected by a precursor-product relationship (Bouchereau et al., 1999). In addition, polyamines, in particular spermidine and spermine, play a significant role in the overall protective response during salt stress (to be discussed further).

The Kasukabe et al. (2004) study not only showed a direct role of polyamines during salinity stress, but also during drought stress. The role of polyamines during osmotic stress appears much more universal than during salt stress. Studies involving plants and drought have provided a clearer function for polyamines during this particular stress. Flores and Galston (1984) reported high levels of putrescine and ADC activity
during sorbitol-induced and conventional drought in detached oat (*Avena sativa*) leaves. Another study found that transgenic rice (*Oryza sativa* L.) over-expressing various polyamine biosynthetic enzymes conferred a dramatic tolerance to drought (Capell et al., 2004). In this study, a variety of transgenic plants were produced, over-expressing either rice ADC, *Datura stramonium* ADC (under control of a strong maize promoter), or rice SAMDC (which related to spermidine and spermine over-production). The transgenic lines increased cellular putrescine levels and were able to tolerate drought stress far better than the wild type plants. The non-transformed plants exhibited a characteristic curling of the leaves which was not observed in the transgenics. The authors proposed that the ability of plants to tolerate abiotic stresses relies heavily on the production of spermidine and spermine. The sudden burst of putrescine during abiotic stress may play a much greater role than just being a simple precursor to the higher polyamines; however, over-accumulation of putrescine in normal, unstressed plants has resulted in cellular toxicity. Transgenic tobacco with inducible over-expression of oat ADC (thus increased putrescine levels) showed decreased vegetative growth upon induction only (Masgrau et al., 1997) and over-expression of *Arabidopsis ADC2* caused dwarfism and late-flowering (Alcazar et al., 2005). Putrescine was also reported to cause depolarization of membranes and increased potassium leakage (Tiburcio et al., 1990). In some plants, the application of exogenous putrescine leads to the loss of turgor and causes necrotic spots (Flores et al., 1991). The physiological response to increased putrescine has lead to chlorophyll loss and accelerated senescence (Capell et al., 1993), which is attributed to the depolarization of membranes. Generally, it appears that the best equipped stress-tolerant plants are those that can efficiently convert elevated levels of putrescine to the higher polyamines,
spermidine and spermine. Capell et al. (2004) presented a unified model which suggests that putrescine levels must reach a particular threshold in order to be efficiently converted. As levels of putrescine rise in transgenic and wild type lines during drought, the wild-type putrescine levels can not reach this threshold before senescence sets in. The transgenic line, however, can reach this threshold and convert the diamine into spermidine and subsequently spermine, which increases the tolerance to stress.

Many studies support this model. Oat leaf segments during a 72 h osmotic stress were allowed to accumulate putrescine and supplied with exogenous spermine (Capell et al., 1993). The segments with applied spermine were able to retain chlorophyll and phenotypically appeared normal. It has been suggested that spermidine and spermine interact with membranes by inhibiting transbilayer movement of phospholipids (Bratton, 1994) or by stabilizing molecular complexes of thylakoid membranes (Popovic et al., 1979, Besford et al., 1993). Similar studies in cereals and various dicots also note that spermidine and spermine reduce chlorophyll breakdown (Kushad and Dumbroff, 1991). The exogenous treatment of plants with spermidine and/or spermine have been shown to reduce the harmful effects of tobacco mosaic virus (Yamakawa et al., 1998), paraquat toxicity (Kuerpa et al., 1998), chilling (Shen et al., 2000), and osmotic stress (Besford et al., 1993).

Urano et al. (2003) provided a characterization of all the key polyamine biosynthetic genes in Arabidopsis (Table 1) during various abiotic stress conditions. The expression profiles of these genes during NaCl, dehydration, and abscisic acid were separated into three categories: stress-inducible, which includes ADC2, SAMDC2, and SPDS3; constitutive, which includes ADC1, SAMDC1, SPDS1, and SPDS2; and stress-
repressible, including *SPMS*. The induction of *Arabidopsis ADC2* was also reported during osmotic stress (Soyka and Heyer, 1999) and abscisic acid treatments (Perez-Amador et al., 2002). Microarray analysis demonstrated similar induction of rice (*Oryza sativa*) *SAMDC2* during salt stress (Kawasaki et al., 2001). These profiles further support the role of polyamines during abiotic stress responses.

The overall mechanism of action of polyamines during abiotic stress is still a matter of debate; however, numerous studies correlate the mode of action during stress based on their mechanism during normal cellular conditions. Roberts et al. (1986) demonstrated that polyamines make membrane surfaces rigid, which, again, relates to their cationic nature. This can correlate to the ability of polyamines to retard membrane deterioration during stress, thus preventing the deleterious effect of NaCl and ROS and aiding in the prevention of ion leakage and chlorophyll loss. ROS are commonly produced during many abiotic and biotic stresses. These oxygen species often react with membranes causing lipid peroxidation and damage to other cellular components, which greatly enhances cell injury during stress. Polyamines have long been known to have ROS scavenging capabilities (Bachrach 1983; Roberts et al., 1986; Bors et al., 1989; Ha et al., 1998). The amino groups of polyamines have been shown to have a high affinity for ROSs (Kushad and Dumbroff, 1991) showing that the scavenging ability is related to the amino groups, which would suggest that the triamine spermidine and the tetramine spermine would be more effective in scavenging. This was further supported by the Kasukabe et al. (2004) study in which an increase in SPDS was shown to protect against paraquat toxicity, a herbicide that functions similar to oxidative stress leading to the formation of superoxide and hydrogen peroxide. In addition to the overaccumulation of
putrescine enhancing senescence, production of the hormone ethylene during abiotic stress has been shown to have the same effect. Wang et al. (1990) describe how water stress in drought sensitive plants triggers production of ethylene, which leads to widespread senescence of the plant. The same phenomenon was observed during increased salt levels in salt-sensitive plants (Morgan and Drew, 1997). Spermidine and spermine have been shown to reduce ethylene synthesis by inhibition of both ACC synthase and conversion of ACC into ethylene (Davies et al., 1991). Consequently, the ethylene and spermidine/spermine pathways both compete for the same substrate, SAM, which is produced by SAMDC (Figure 1). This competition limits the amount of substrate available for production of ethylene. Thus, spermidine and spermine provide a widespread anti-senescent effect during abiotic stress.

Polyamines have also been implicated in many other stress related functions. Along with ROS scavenging, membrane protection, and ethylene reduction, polyamines could sequester NH$_3$ which is highly toxic to cells, maintain cellular pH and anionic/cationic balances, and stabilize anionic macromolecules via electrostatic binding (Bouchereau et al., 1999; Minocha et al., 2000). Because of this evidence, the desire to learn more about the mechanism and function of polyamines during stress is greater than ever. Polyamines are of interest to those studying plant stress response, parasitic diseases, and genetic manipulation. Their omnipresence and involvement in so many vital cellular functions makes them crucial for the field of plant stress physiology.
Studying Gene Expression

One of the fastest growing fields in biology today is functional genomics. As the number of genome projects grows, the race to determine the function of each DNA sequence becomes increasingly significant. Interest has shifted in genomics from identifying the DNA sequence to understanding the functional role of the various genes contained in the genome. Early functional genomics utilized the powerful technique of studying mutants. In this approach, a cell or organism, which lacks a gene of interest or expresses an altered form of it, is observed for altered phenotypes or cellular disruptions. This method has become much improved with the advent of insertional mutagenesis, in which a fragment of exogenous DNA is randomly inserted in a nucleotide sequence to disrupt a gene. However, this method is limited to organisms with rapid life cycles and genomes which are compliant to genetic manipulations. In addition, this method often results in a lengthy screening process. Also, the study of genes is limited as knockouts of certain genes may be lethal to the organism. Despite such disadvantages, this method is still a significant contributor to this field for a number of organisms. Since the rise of biotechnology and gene cloning, even more powerful and precise methods have appeared. The most-widely utilized techniques include microarrays, northern blots, and quantitative Real Time PCR. Whereas each method differs in its goal and approach, they collectively measure transcript levels which are a direct measure of gene expression. As with every technique, each has its own advantages and disadvantages.

Microarrays are one of the latest breakthroughs allowing the simultaneous analysis of genome-wide expression (Brazma and Vilo, 2000; Lockhart and Winzeler, 2002). A microarray is a glass slide containing single-stranded DNA molecules on fixed
locations. Each fragment or “spot” is a probe representing a single, specific gene. The mRNA is isolated from a sample and used to make complementary DNA (cDNA) via reverse transcription. The cDNA is labeled with a fluorescent tag and hybridized to the fixed complementary single-stranded DNA. The array is then washed to remove any cDNA that is not tightly bound. The expression pattern is elucidated by either the different levels of fluorescence or the different colors of fluorescence (Russell 2002). The positions to which the labeled DNA is bound is usually monitored by an automated scanning-laser. Microarrays allow the monitoring of thousands of genes at once, which enables the identification of genes which are induced or repressed during various cellular events, whether developmental or environmental. However, there are drawbacks to this method. Because RNA preparations are required, tissue localization is very difficult. For example, in the case of plants, the observation of gene expression can be done in whole organs such as leaves and stems, however, particular structures such as vascular bundles or xylem and phloem cells can not be studied. Also, specificity is a problem when studying members of a gene family with a high degree of homology. Probes are often not specific enough to differentiate between these gene family members.

Another technique which is highly suitable for single gene analysis is northern blots. This is a standard technique which can show the presence or absence of a transcript. This method is performed by first isolating total RNA or mRNA which is separated by size using agarose gel electrophoresis. The RNA is then transferred to a membrane and hybridized with a specific probe. The sensitivity is somewhat low compared to other techniques when trying to quantify the transcript level by comparing the strength of the hybridization signal with an internal control (Russell 2002).
provides information about the presence of a transcript and estimated levels. However, there are a number of disadvantages with this technique. As with microarrays, the lack of specificity with the probes makes it difficult to successfully differentiate between members of a gene family with a high degree of homology. This technique also requires a large amount of total RNA which can become time-consuming and decrease sensitivity when working with genes that have low levels of expression. In addition, due to RNA preparations, analysis at the tissue or cellular level is not possible.

A more sensitive tool for analyzing transcript levels is reverse transcription PCR (RT-PCR). RT-PCR has become one of the most common methods for characterizing gene expression patterns. There are two types of RT-PCR; qualitative and semiquantitative. For both methods, the procedure begins by isolating either total RNA or polyadenylated RNA from a particular sample. The mRNA of the gene of interest is used as the template in a reverse transcription reaction to produce a cDNA molecule. Primers for this reaction are often gene specific which increases the specificity of the reaction. The cDNA then becomes the template for a standard PCR reaction. Qualitative RT-PCR succeeds in showing the presence or absence of a transcript with a high degree of sensitivity and specificity. Semiquantitative RT-PCR often requires a modification of the PCR, called Real Time PCR, in which two separate primer sets are utilized. The primers include a set for the gene of interest and one set for a control gene that is presumed to be constitutively expressed. The reaction also contains fluorescent tags which can be used to monitor the levels of each gene during the reaction. This method provides an indirect method for quantifying the amount of transcript, and thus gene expression, in a particular sample (Freeman et al., 1999; Bustin, 2002; Page and Minocha...
Both methods are capable of high-throughput with a high degree of specificity enabling one to distinguish between members of a gene family. Whereas RT-PCR increases the sensitivity and specificity when compared to northern blots and microarrays, this method still requires cumbersome RNA preparations, thus anatomical localization is limited.

In order to examine gene expression at the tissue and cellular level, there are several techniques which can be used. One such technique is *in situ* hybridization. As the name may suggest, this method localizes and detects specific mRNA sequences in preserved tissue samples or cell preparations by hybridizing a specific anti-sense DNA or RNA probe to the mRNA of interest (Franco et al., 2001; Page and Minocha 2004; Pineau et al., 2006). Identification (and partial quantification) of gene expression using this technique offers a high degree of specificity (depending on the probes used) in the cellular context. However, sensitivity is limited to the abundance of transcript present in a particular group of cells, as low levels may be difficult to detect a signal. The mRNA may also be masked by proteins or protected within a cellular structure, once again decreasing the probe signal. Because of its complexity, this method is difficult to perform and is prone to multiple errors for quantification. However, regardless of its disadvantages, *in situ* hybridization has been frequently used for detecting gene expression at the cellular level (Franco et al., 2001; Pineau et al., 2006).

Alternatively, an equally powerful technique is the use of reporter genes. The premise behind this approach involves the use of reporter genes controlled by the native promoter region of a particular gene of interest which enables one to examine where and when a gene is expressed. The objective behind this approach is to fuse or replace the
coding region of a gene of interest with a reporter gene whose expression can be visually identified within an organism. The promoter::reporter gene fusion is then transformed into the organism and the expression of the reporter gene should mimic the expression pattern of the gene of interest and can be visually identified at the cellular level. The promoter::reporter gene fusion is a useful technique for analyzing the expression patterns of inducible or constitutive genes, which may be regulated in a cell/tissue-specific manner. The assumption behind this approach is that the 5' flanking region of a gene (promoter) contains the regulatory elements responsible for driving expression in response to developmental and/or environmental signals. There are two types of promoter::reporter gene fusions that can be designed: a transcriptional fusion in which the promoter region is fused to a reporter gene and a translational fusion which includes the promoter region, the 5'UTR, and all or part of the gene of interest’s open reading frame (ORF) fused to the reporter gene (Hanfrey et al., 2002). Because the promoter region is poorly defined, it is customary to include approximately 1500-2000 bp upstream of the transcription start site to ensure all regulatory sequences are included (Guilfoyle, 1997). There are two primary advantages to this system: 1) the reporter gene system provides a high degree of specificity in utilizing the native promoter region. This also eliminates problems when studying gene family members with a high degree of homology; 2) expression of a gene can be studied at the cellular level in all tissues, at any developmental time point without cumbersome RNA preparations. However, there are some disadvantages to this technique. The reporter gene construct may fail to contain certain regulatory sequences not found in the promoter region, but rather are included in the exon of the gene or elsewhere in the genome. Secondly, post-transcriptional
regulation may be affected due to differences in the reporter gene and native gene transcripts. Overall, the reporter gene system provides a high degree of specificity, sensitivity, and allows tissue localization which is not offered by the aforementioned techniques (Table 3).

<table>
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<th>Method</th>
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<th>Localization</th>
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<td>Northern Blot</td>
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<td>Low</td>
<td>Low</td>
</tr>
<tr>
<td>RT-PCR</td>
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<td>Low</td>
</tr>
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<td>Microarray</td>
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<tr>
<td><em>In situ</em> hybridization</td>
<td>Medium</td>
<td>High</td>
<td>High</td>
</tr>
<tr>
<td>Promoter:Reporter Fusion</td>
<td>High</td>
<td>High</td>
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</table>

Table 3. Comparison of the various methods for studying gene expression in plant systems.

There are a number of reporter genes that have been used in the study of gene expression, including *lacZ*, β-galactosidase, galactokinase, luciferase, green fluorescent protein (*GFP*), and β-glucuronidase (*GUS*) (Naylor 1999). In plant studies, the reporter genes of choice have been *GFP* and *GUS*. Both genes provide unique advantages in that they are both relatively easy to visually detect and quantify, both have been extensively characterized in a number of studies, and a number of vectors have been designed to allow easy insertion of a sequence to produce transcriptional or translational fusions. Yet, each gene has its own advantages and disadvantages. The *GUS* (*uidA*) gene originating from *E. coli* codes for the β-glucuronidase enzyme (Jefferson et al., 1987), which can be qualitatively assayed using the colorimetric substrate 5-bromo-4-chloro-3-indoyl-β-D-glucuronide (X-gluc). The *GUS* enzyme can also be quantitatively assayed using the fluorogenic substrate 4-methylumbelliferyl-β-D-glucuronide (4MUG) (Martin et
al., 1992). The GUS system has been extensively used due to its relative ease of use, cost efficiency of assay substrates, and the lack of need for special equipment. The GUS gene has also been shown to be easily detectable when under the control of a weak promoter (Mantis and Tague, 2000) and because it is an enzymatic reaction, the signal is amplified producing a direct detection of activity. The enzymatic product is also very stable enabling samples to be preserved for a long period of time without losing detection. However, this also offers a drawback as the GUS product has been shown to leach into surrounding tissues providing false identification of GUS activity. The stability also prevents the accurate measurement of changing transcript levels. Perhaps the most significant disadvantage with this system is its destructive properties to cells, which prevents analysis of live tissues.

The GFP gene, isolated from the jellyfish Aequorea victoria, was first demonstrated to be an effective reporter gene in bacteria and Caenorhabditis elegans by Chalfie et al. (1994). One of the principal advantages to GFP over GUS is that the protein is autofluorescent, and therefore does not require any substrates or cofactors for detection. The protein is a useful reporter when excited at a particular wavelength, producing a characteristic fluorescent green color. The most significant advantage to this system is its ability to non-destructively monitor gene expression and can also be used to study sub-cellular localization. However, aside from the obvious need of expensive microscopy and photography equipment, it is difficult to distinguish from background fluorescence in plant systems often caused by chlorophyll. Such inhibitions were seen by Mantis and Tague (2000) when comparing the GFP and GUS systems under the control of a weak promoter (AtZFPI) in Arabidopsis. The relatively weak gene activity was
difficult to detect using GFP, yet the GUS system was sensitive enough to detect the low levels of activity. This shows that the GUS system may provide slightly more sensitivity in the study of gene expression.

Despite the aforementioned drawbacks, the reporter gene system has, and continues to be, a proven method for studying gene expression. This technique offers sensitivity, specificity, and anatomical localization not offered by the other systems mentioned previously. The number and scope of studies utilizing this technique further supports the importance and fundamental acceptance of this method which enables the study of simultaneous tissue-specific, developmental-specific, and cell-specific gene expression patterns. However, perhaps the best characterized studies are those that utilize multiple techniques in the study of gene expression.
Objectives of Study

Numerous studies have shown the importance of polyamines in plants during growth, development, and in response to stress. These studies have shown a correlation of either an increase in polyamine levels or the presence and activity of the biosynthetic genes. However, there is a lack of information regarding the regulation of the expression of the key polyamine biosynthetic genes. Studies showing the expression profile of these genes, which provide the developmental timing and tissue localization, are of great importance to understanding the overall function of polyamines. The present study is aimed at analyzing two key genes in the polyamine biosynthetic pathway, SPDS3 and SPMS, during the entire life cycle of Arabidopsis thaliana using the promoter::gus fusion technique. The objectives of this study are:

1. To examine the expression pattern of SPMS and SPDS3 during the entire life of Arabidopsis
2. To examine the effects of various abiotic stresses on the expression patterns of SPMS and SPDS3
3. To clone the promoters of SPDS1 and SPDS2
CHAPTER 1

MATERIALS AND METHODS

Bacterial Culture

*Escherichia coli* and *Agrobacterium tumefaciens* cultures were grown in Luria broth medium (10 g/L Bacto Tryptone, 5 g/L Bacto Yeast Extract, and 10 g/L NaCl) (Maniatis et al., 1982). For solid medium, 1.3% Bacto agar was added. *E. coli* cultures inoculated in broth or on solid agar plates were incubated at 37° C overnight (18 h). *A. tumefaciens* cultures were incubated at 28° C from 18 to 48 h. Liquid cultures were incubated on a shaker at 250 rpm.

Plasmid DNA Isolation

Plasmid DNA was isolated from *E. coli* cultures using a modified alkaline lysis protocol from the Wizard Plus Miniprep DNA Purification System (Promega, Madison, WI). Typically, 3 ml cultures were pelleted at 10,000 g for 30 s. Sequentially, the cells were treated with 200 µl of Resuspension Solution (50 mM Tris-HCl pH 7.5, 10 mM EDTA, 100 mg/L RNase A), Lysis Solution (0.2 M NaOH, 1% SDS), and Neutralization Solution (1.32 M potassium acetate, pH 4.8). The mixture was then centrifuged at 14,000 g for 10 min at 4° C. The supernatant was recovered and an equal volume of cold isopropanol added. The DNA was precipitated at -20° C for 30 min. DNA was pelleted at 14,000 g for 15 min at 4° C, washed with 70% ethanol, and centrifuged at 14,000 g. The supernatant was removed and the pellet dried in a vacuum centrifuge. The DNA pellet was resuspended in 20-50 µl sterile distilled water.
Genomic DNA Isolation

Genomic DNA was isolated from *Arabidopsis thaliana* plants using a modified protocol from Murray and Thompson (1980). Approximately 100-300 mg of plant tissue was ground directly in 500 µl of CTAB buffer (2% Hexadecyltrimethylammonium bromide (w/v), 1.4 M NaCl, 20 mM EDTA, 100 mM Tris-HCl pH 8.0, and 0.2% (w/v) β-mercaptoethanol added just before use) preheated to 60°C in 1.5 microfuge tubes. The ground tissue was incubated at 60°C for 30 min with gentle agitation. An equal volume of chloroform:isoamyl alcohol (24:1) was added, homogenized through inversion, and centrifuged at 14,000 g for 5 min. The upper aqueous layer was removed and mixed with an equal volume of cold isopropanol. The tubes were incubated at -20°C for 15-30 min. The precipitated DNA was pelleted through centrifugation at 14,000 g at 4°C for 15 min. The pellet was washed with 70% ethanol/10 mM ammonium acetate and recentrifuged for 5 min at 14,000 g. The pellet was dried in a vacuum centrifuge and resuspended in 20-50 µl of TE buffer (10mM Tris pH 8.0, 1 mM EDTA).

Polymerase Chain Reaction (PCR)

PCR was performed using either Ready-To-Go PCR Beads (Amersham Pharmacia Biotech, Piscataway, NJ), Taq PCR Master Mix (USB Corp.), or New England Biolab Taq Polymerase. All reactions were performed in 25 µl volumes using approximately 100-150 ng genomic DNA template or 50-100 pg of plasmid DNA. Primers were supplied at 5-10 pmol/reaction. Buffer and dNTP's were supplied in the PCR beads and Taq Master Mix, however reactions using the NEB Taq required the addition of a final concentration of 1X buffer and 1 µM of each dNTP. Reactions were

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run in a PTC 100 Programmable Thermocycler (MJ Research, Waltham, MA) with a heated lid.

**Restriction Enzyme Digests**

Restriction enzymes were purchased from either New England Biolabs (Ipswich, MA) or Promega (Madison, WI). Digests were performed to analyze plasmid DNA or prepare DNA for ligations. Each reaction contained a final concentration of 1x buffer, 1x bovine serum albumin (if required), approximately 2 units/µg DNA of restriction enzyme, 150-200 ng of template DNA (for analysis), and brought to volume with sterile distilled water. Reactions were incubated for 2 h to overnight at the manufacturer's specified temperature. Enzymes were typically inactivated by a 20 min, 65° C incubation.

**Agarose Gel Electrophoresis**

Agarose gel electrophoresis was performed for analyzing or separating DNA. Typically, gels were composed of 1% Seakem GTG or LE agarose (Cybrex) dissolved in 1x TAE buffer (40 mM Tris-acetate, 1 mM EDTA). Samples were mixed with 6x loading buffer containing EDTA with at least one sample being a DNA size standard. Gels were electrophoresed at 5 V/cm for approximately 1 h. Gels were stained with 0.5 µg/ml ethidium bromide for 10 min followed by destaining in distilled water for 10 min. Gels were visualized on an UV trans-illuminator and digitally photographed using Nucleotech Gel Expert version 3.5 software and the Nucleotech gel-documentation.
Figure 2. DNA size standards used on agarose gels. New England Biolabs DNA ladders used in this study include the A) 2-Log DNA Ladder, B) 1 Kb DNA Ladder, and the C) Low Molecular Weight DNA Ladder.

system (Nucleotech, San Mateo, CA). Gels were analyzed with either the NEB 2-Log DNA Ladder, 1 kb DNA Ladder, or Low Molecular Weight DNA Ladder (Figure 2).

**DNA Sequence and Sequence Analysis**

Typical 20 µl reactions included 8 µl of premix, 66-132 ng DNA, 5 pmol primer, and were brought to volume with sterile water. Reactions were cycled 20-30 times followed by an ethanol precipitation. The purified sequencing reactions were sent to the UNH Hubbard Genome Center for load only sequence analysis. DNA sequences were analyzed and aligned using the BioEdit Sequence Alignment Editor (Hall, 1999).
Ligations

Ligations were performed using New England Biolabs (Beverly, MA) T4 DNA Ligase. Reactions were performed using either a 1:1 or 1:3 vector:insert ratio. Reactions were incubated at 16°C overnight. Reactions were purified by bringing the reaction volume to 100 μl with water and adding 10 μg of muscle glycogen. DNA was precipitated in 0.3 M sodium acetate, pH 5.6, and 76% ethanol. Reactions were incubated at -80°C for 15 min, followed by centrifugation at top speed and washing the pellet with 70% ethanol. Pellets were dried in a Speed-Vac and resuspended in 10 μl water.

Electrocompetent Bacterial Cells

For bacterial transformation, *Escherichia coli* (TOP10) and *Agrobacterium tumefaciens* (GV3101) cells were made competent for electroporation. For preparation of the cells, a 3 ml culture of LB was inoculated with the desired bacterial strain and incubated overnight as described previously. The entire 3 ml culture was used to inoculate 400 ml of fresh LB medium which was also incubated overnight with vigorous shaking. The cultures were grown to an OD$_{600}$ of 0.5-0.7 monitored with a spectrophotometer. The culture was chilled on ice and centrifuged at 4000 g for 10 min at 4°C in prechilled, sterile 250 ml centrifuge bottles. After centrifugation, the supernatant was discarded and the pellet resuspended in 200 ml sterile, ice-cold water. Next, cells were pelleted at 4000 g for 10 min at 4°C and resuspended in 100 ml sterile, ice cold water. The suspension was once again centrifuged at 4000 g for 10 min at 4°C and the pellet was resuspended in 10 ml sterile, ice cold 10% glycerol. The cells were
combined in the same tube and once again centrifuged at 4000 g for 10 min at 4° C. The pellet was resuspended in 2 ml sterile, ice cold 10% glycerol, aliquoted into 50 μl volumes, and frozen immediately in dry ice. The cells were stored at -80° C.

Electroporation

Electroporations were performed using TOP10 *E. coli* or GV3101 *A. tumefaciens* electrocompetent cells in 50 μl aliquots. A 1-2 μl aliquot of a purified ligation product or plasmid DNA was added to the electrocompetent cells which were thawed on ice. The cell-DNA mixture was transferred to a prechilled cuvette with a 1 mm gap. The cells were electroporated at 1800 V in an Eppendorf model 2510 electroporator and incubated in fresh LB for 1 h at 37° C for *E. coli* and room temperature for *A. tumefaciens*. Cells were plated on LB medium supplemented with the appropriate antibiotic and incubated overnight. Resulting colonies were screened for the presence of the desired insert via plasmid isolation and restriction digest or PCR.

Glycerol Stocks

Colonies containing the desired plasmid were used to inoculate a 3 ml culture of LB and incubated overnight at 37°C. Glycerol stocks were prepared by mixing 85% culture with 15% sterile glycerol in cryo-vials. Stocks were incubated on dry ice for 15 minutes and cryo-preserved at -80°C. Remaining LB was used for plasmid isolation and screened for desired insert via PCR or restriction enzyme digest.
Construction of pCAM-SPDS3 and pCAM-SPMS Constructs

*SPDS3* and *SPMS* promoter:GUS fusions were constructed previously by Todd Bezold and are described here briefly. Three separate constructs for each gene were designed, each containing various regions of the putative promoter and open reading frame (Figure 3). The constructs were named *SPDS3-A, SPDS3-B, SPDS3-C,* and *SPMS-A, SPMS-B, SPMS-C.* These constructs were named earlier in the notebooks according to the primers used for cloning: *SPDS3 F1-R2B (SPDS3-A), SPDS3 F2-R1B (SPDS3-B), SPDS3 F1-5UTR (SPDS3-C), and SPMS F2-R1B (SPMS-A), SPMS F3-R1B (SPMS-B) SPMS F1-5UTR (SPMS-C).* In this study, the promoter region was defined as 800-1500 bp upstream of the transcription start site, truncation with a flanking gene, or the presence of repetitive sequences. The *SPDS3-A* construct contains a ~1.8 Kb fragment which includes 935 bp upstream of the transcription start site (putative promoter), the entire 875 bp 5'UTR, and 57 bp of the *SPDS3* ORF. The *SPDS3-B* construct has only 214 bp of the putative promoter, the 875 bp 5'UTR, and 170 bp of the ORF. The *SPDS3-C* construct was designed to have 935 bp of the promoter and the 875 bp 5'UTR, but no part of the ORF.

The *SPMS* constructs were designed in a similar manner as the *SPDS3* constructs. *SPMS-A* contains 1416 bp upstream of the transcription start site, the entire 90 bp 5'UTR, and 61 bp of the *SPMS* ORF. *SPMS-B* contains 939 bp of the promoter, the 90 bp 5'UTR, and 61 bp of the ORF. The *SPMS-C* construct was designed to contain 1416 bp of the putative promoter and the 90 bp 5'UTR. The various promoter regions were PCR amplified from genomic *Arabidopsis* DNA using PCR cloning primers listed in Table 4. The resulting PCR products were cloned into the pCR2.1-TOPO TA cloning
vector (Invitrogen, Carlsbad, CA; Figure 4). The inserts were sequenced using the M13 and T7 primers (Table 4). The \textit{SPDS3-A}, \textit{SPDS3-B}, \textit{SPMS-A}, and \textit{SPMS-B} PCR products were removed from TOPO using the 5' flanking \textit{EcoRI} restriction site and the 3' \textit{BamHI} site incorporated by the \textit{SPDS3} R1B, R2B and \textit{SPMS} R1B cloning primers. The inserts were gel purified using the QIAquick Gel Extraction Kit (Qiagen, Valencia, CA). The \textit{SPDS3-C} and \textit{SPMS-C} promoter fragments were removed with the flanking TOPO \textit{EcoRI} sites. The fragments were also gel purified. The purified fragments were ligated into the appropriately digested pCAMBIA 2381 (www.cambia.org) (Figure 5) vector where they were fused with the \textit{GUS} gene. The \textit{SPDS3-A} and \textit{SPDS3-B} fragments were ligated into pCAMBIA x C and the \textit{SPMS-A} and \textit{SPMS-B} fragments ligated into pCAMBIA x A. Both \textit{SPDS3-C} and \textit{SPMS-C} were ligated into the pCAMBIA GUS+ATG vector. The promoter::GUS fusion was sequenced using sequencing and cloning primers (Table 4) to ensure all protein fusions were in frame.
Figure 3. Diagram of A) *SPMS* and B) *SPDS3* constructs used in this study. Various regions of the promoter region and ORF of these two genes were PCR amplified and fused to the GUS reporter gene. All constructs contain the entire 5' UTR as defined by the TAIR database (www.arabidopsis.org). Diagram not drawn to scale.
Figure 4. pCR2.1-TOPO TA cloning vector used for ligating PCR products.

Figure 5. Maps of pCAMBIA vectors used for creating promoter::GUS fusions. The A) pCAMBIA x A and B) pCAMBIA x C have a GUS gene lacking the ATG start site. C) pCAMBIA GUS+ATG has a GUS gene possessing its own ATG start site.
Table 4. Primers used in this study. Primers were used for cloning of the promoter region from *Arabidopsis* genomic DNA, sequencing of cloned regions in either the TOPO or pCAMBIA plasmid, and RT-PCR analysis of native and transgene transcripts.

<table>
<thead>
<tr>
<th>Primer Name</th>
<th>Function</th>
<th>Primer Sequence</th>
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<tbody>
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<td>SPDS1 F2</td>
<td>Cloning/Seq</td>
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Transformation and PCR Screening of *Agrobacterium tumefaciens*

Electrocompetent GV3101 *A. tumefaciens* cells were transformed with the pCAM-SPDS3 or pCAM-SPMS plasmids as described earlier. The resulting colonies were screened for the presence of the pCAMBIA plasmid by PCR using the appropriate primer combinations. To extract the DNA, a small sample of the colony was removed with a sterile toothpick and resuspended in 50 μl of water. The cells were boiled at 100°C for 10 min. The boiled suspension was centrifuged at 10,000 g for 30 sec to pellet the debris. The resulting supernatant was used as the PCR template.

**Transient Expression of the pCAM-SPDS3 and pCAM-SPMS Plasmids**

The pCAM-SPDS3 and pCAM-SPMS plasmids’ functionality were confirmed using transient assays by biolistic bombardment of 3-4 day old poplar (*Populus nigra x maximowiczii*) cell cultures (Bhatnagar et al., 2001). The biolistics protocol was slightly modified from Walter et al. (1998). Poplar cells were aliquoted (1-2 ml) onto medium (Murashige and Skoog basal salts, 1X Gamborg’s B-5 vitamins, 2% sucrose, 0.2 M sorbitol, 0.8 % type A agar, adjusted to pH 5.7 with NaOH; Gamborg et al., 1968) overlaid with 60 mm #1 filter papers. Approximately 2 μg DNA was coated onto 1.5-3 μm Aldrich gold particles (Aldrich, Milwaukee, WI) in the presence of 1 M CaCl₂ and 16.7 mM spermidine. Biolistics was performed using a BIO-RAD PDS 1000/He gene gun (BIO-RAD, Hercules, CA) with 1350 psi rupture disks. Following a 36-48 h incubation, poplar cells were stained for GUS activity (Martin et al., 1992) using GUS stain with X-gluc (1 mM 5-bromo-4-chloro-3-indoyl-β-D-glucuronide, 1mM potassium ferricyanide, 1mM potassium ferrocyanide, 100 mM sodium phosphate buffer pH 7.0,
5 mM EDTA, 0.1% Triton X-100, 20% methanol. The cells were incubated overnight at 37°C and the total number of cells showing GUS activity was counted as blue spots.

**Growth of Arabidopsis Plants in Soil**

*Arabidopsis thaliana* ecotype Columbia plants were grown in 3 parts Scott's 360 Metro-Mix (Scotts Company, Marysville, OH) and 1 part perlite. Moist soil mix was placed in 3 inch pots and arranged in a flat which allowed watering via capillary action. Plants were watered on alternate days with the addition of ¼ strength Miracle-Gro (Scotts Company) synthetic fertilizer every five days. The plants were grown under an 18 h photoperiod at 21°C under 80 μEm²/sec fluorescent lighting. A clear plastic lid was used to maintain humidity for newly sown seeds or transferred seedlings. For each pot, approximately 25 seeds were sown and placed in a flat at 4°C for 48 h for seed stratification. Once placed in light, the plastic lid remained on the flat for 3-5 days, followed by a three day hardening off period in which the lid was slowly opened until it was completely removed. The pots were thinned to approximately 10 well-spaced, healthy plants.

Plantlets surviving antibiotic selection were removed from GM plates using fine tip tweezers and the roots guided into the same soil mixture. Once again, a clear plastic lid was placed over the flat for 7 days and plants were hardened off over a one week period.
Floral Dip Transformation of *Arabidopsis*

*Arabidopsis thaliana* (ecotype Columbia) plants were transformed using a modified floral dip method previously described by Clough and Bent (1998). For each transformation, eight pots of plants were prepared one week prior to dipping by clipping primary bolts to encourage branching and synchrony of budding. Approximately 5-7 days later, plants with many green developing buds and few open flowers were used for dipping.

For preparation of the *A. tumefaciens*, two 3 ml tubes of LB supplemented with 100 µg/ml kanamycin were inoculated with pCAM-SPDS3 or pCAM-SPMS A. *tumefaciens*. The cultures were grown overnight at 28°C with shaking at 250 rpm. The 3 ml cultures were used to inoculate two 500 ml flasks of LB medium supplemented with 100 µg/ml kanamycin. The 500 ml cultures were grown overnight in a 28°C shaker at 250 rpm. The following day, the 500 ml cultures were centrifuged at 5000 g for 10 min. The pelleted cells were resuspended in 600 ml of 5% (w/v) sucrose. The bacterial solution was gently mixed using a magnetic stirrer to prevent settling. Just before dipping, L-77 Silwet (Lehle Seeds, Round Rock, TX) was added at a final concentration of 0.005% (w/v). The plants were dipped in the bacterial solution for approximately 8-10 s with slight agitation, avoiding contact with the soil and basal leaves. Pots were laid on their sides in a flat and covered with a clear plastic lid overnight. The following day, the plants were inverted, rinsed with distilled water, and returned to normal growth conditions. After seven days, the floral dip was repeated and plants produced seed 2-4 weeks later. Each pot was independently harvested for T₁ seeds, removing plant debris.
with a 30 mesh sieve. Seeds were dessicated at room temperature for 5-7 days in 1.5 ml microfuge tubes.

**Sterilization and Plating of *Arabidopsis* Seeds**

Approximately 40 mg of seeds were sterilized using a series of ethanol washes, starting with 1 ml of 70% EtOH and one drop of 10% Triton X-100 (v/v). The seeds were incubated for 5 min with occasional, gentle agitation. The supernatant was removed and replaced with 1 ml 100% EtOH and 1 drop of 10% Triton X-100. The seeds were once again incubated for 5 min with agitation. After the incubation, the supernatant was poured off and a final wash with 1 ml of 100% EtOH was performed for another 5 min. After the final incubation, all supernatant was removed and the seeds were air dried for 24-48 h under a laminar flow hood.

For selection of transformed plants, dry, sterile seeds were plated on germination medium (GM; 4.3 g/L Murashige and Skoog basal salts, 0.5 g/L MES, 1 g/L sucrose, and 0.8% type A agar (w/v)). Medium was adjusted to pH 5.7 with 4 M KOH and autoclaved. For selective media, GM was supplemented with 50 mg/L kanamycin and sterile 1x Gamborg’s B-5 vitamins (Gamborg et al., 1968). Approximately 200 sterile seeds were plated on solid selection medium, wrapped in aluminum foil, and placed at 4°C for 48 h to achieve seed stratification. After cold treatment, plates were placed in 25°C growth chamber with 70-80 μEm²sec⁻¹ fluorescent lights with a 12 h photoperiod for 10-14 days. Surviving plants were transferred to soil and allowed to produce seed. Seeds were subjected to two generations (T₁ and T₂) of selection on kanamycin. Stocks were named according to T₁ seed stock (T₀ pot number used for floral dip), the individual T₁
plant number and T₂ plant number (if applicable). For example, line 1-3-2 was selected from T₁ seed stock #1, T₁ plant #3, and T₂ plant #2. Experiments were performed with T₃ plants.

**PCR Screening of Transformed Arabidopsis**

Transformed *A. thaliana* plants were screened for transgenes by PCR. Genomic DNA was isolated from approximately 50 mg of leaf tissue as described earlier. PCR was performed using 1 µl of DNA preparation in two separate reactions using *NPTII* and *GUS* primers (Table 4) to screen for the kanamycin resistance gene and the *GUS* gene. PCR screening was performed on T₁ and T₂ plants.

**β-Glucuronidase (GUS) Assays**

GUS activity was monitored in transgenic plants using a histochemical GUS stain to provide a qualitative assay. Plant organs were submerged in GUS stain (1 mM 5-bromo-4-chloro-3-indoyl-β-D-glucuronide, 1 mM potassium ferricyanide, 1 mM potassium ferrocyanide, 100 mM sodium phosphate buffer pH 7.0, 5 mM EDTA, 0.1% Triton X-100, 20% methanol) and vacuum infiltrated for 5 min. The samples were incubated for 18 h at 37° C. After incubation, the stain was removed and replaced with 70% ethanol to clear chlorophyll (ethanol changed if needed to completely clear chlorophyll). Stained samples were stored at 4° C until analysis and photography (Martin et al., 1992).
Developmental Expression Analysis of SPDS3 and SPMS

Approximately 200 T3 seeds from 5 transformed lines for each of the three SPDS3 and SPMS constructs were sterilized and plated on GM supplemented with 50 µg/ml kanamycin (wild type plants were also sterilized and plated on GM w/o antibiotic). Plates were placed at 4°C for 48 h for seed stratification. Plates were then placed at 25°C under 80 µE m²sec⁻¹ fluorescent light. Upon germination, 10 seedlings from each of the 5 lines were removed and submerged in GUS stain (providing 50 total samples for each construct). The tissue was vacuum-infiltrated for 5 min and incubated at 37°C overnight. After incubation, the stain was removed and the tissue was decolorized with 70% ethanol to remove all chlorophyll. Samples were collected and assayed from the petri plates at germination and 1, 2, 3, 5, 7, 9, 11, 13, and 15 days post germination (DPG). Upon sowing seeds on plates, seeds were simultaneously sown on soil and cold stratified for 48 h. Plants were grown under an 18 h photoperiod at 21°C under 80 µEm²sec⁻¹ fluorescent lighting. A total of 5 samples were collected from each line (25 total samples for each construct) at 18, 21, 24, 28, 32, 38, and 45 DPG and stained for GUS activity.

Following GUS staining, samples were analyzed for the presence of blue color indicating GUS activity. The localization of blue coloration was recorded in each tissue and organ at various time points throughout development. The counts were tabulated and converted into a percentage of plants showing expression in any particular organ/tissue.

Expression Analysis in Response to Abiotic Stress

Expression analysis of transgenic Arabidopsis plants was performed for induction or suppression of GUS activity. The abiotic stresses included a NaCl treatment, drought,
chilling, and wounding. Treatments were applied to T3 plants at 10, 20, and 35 DPG. A total of 25 samples (5 samples from each of the 5 lines) for each construct were collected and tested for GUS staining as previously described. Wild type plants were used as a control for each experiment which were performed only once.

**NaCl Stress Response**

Seeds sown on petri plates were used at 10 DPG for salt treatments. Approximately 100 seedlings were removed from germination medium and placed in two separate 50 ml flasks each with liquid germination medium containing either 100 mM NaCl or 200 mM NaCl. In addition, approximately 20 ml of 100 mM and 200 mM NaCl solutions were poured directly on the remaining seedlings in the solid GM plates and allowed to soak into the medium. Controls were simultaneously prepared with untreated liquid and solid germination medium. Liquid GM was poured over plated seedlings to control for anoxia. Samples from each of the liquid and solid treatments were collected at 0 (just prior to treatment), 6, 12, 24, and 48 h post treatment.

Seeds sown directly on soil were allowed to reach 20 and 35 DPG. At each time point, a 100 mM and 200 mM NaCl solution was poured directly on the soil until saturation. Once again, 5 samples from each of the 5 lines were collected at 0, 6, 12, 24, and 48 h post treatment and stained for GUS activity. Samples saturated in water were used as a control and collected at the same time points.

**Drought Stress Response**

Seeds sown directly on soil were allowed to reach 20 and 35 DPG. Plants were exposed to a 96 h drought treatment by delaying the normal watering schedule. Plants were watered at 0 h and samples collected at 0, 24, 48, 72, and 96 h after the last normal
watering. Transformants under the normal watering schedule were also collected at these time points and used as a control.

**Chilling Stress Response**

Seeds sown on petri plates containing GM were allowed to reach 10 DPG and placed at 4°C. A total of 25 samples were collected at 0 (just prior to cold treatment), 4, 8, 12, 24, and 48 h and stained. Similarly, seeds sown in soil were allowed to reach 20 and 35 DPG under normal growth conditions, and the plants placed at 4°C. Samples were collected at 0, 4, 8, 12, 24, and 48 h. Transformants kept at 21°C were also collected and stained at the same time.

**Wounding Stress Response**

Plants at 20 and 35 DPG were tested for GUS activity in response to wounding. Various organs of plants, including the primary and secondary rachis, siliques, rosette and cauline leaves, were subjected to small cuts and/or scrapes. Organs were either cut and placed under high humidity in a plastic bag or kept intact on the plant. Wounded and unwounded organs were collected and stained at 0 (immediately upon wounding), 6, 12, and 24 h after injury. Uninjured tissues were also collected at these time points and used as a control.

**RNA Isolation for Preparation of cDNA**

RNA was isolated from both transgenic and wild type *Arabidopsis* plants using a procedure modified from Mason and Schmidt (2002). Tissue was collected and weighed in a 1.5 ml microfuge tube and flash-frozen in liquid nitrogen. The tissue was ground in the tube with a pre-chilled pestle, making sure the tissue did not thaw. A 500 μl aliquot
of 60°C preheated extraction buffer (2% SDS, 1% polyvinylpyrrolidone-10 (PVP-10), 0.2 M Tris pH 8.0, 1.5 M NaCl, 25 mM EDTA pH 8.0, 2% β-mercaptoethanol) was added and vortexed for 30 s. An equal volume of chloroform:isoamyl alcohol (24:1) was added and vortexed and/or inverted for 1 min, followed by centrifugation at 4°C for 5 min at top speed. The upper aqueous layer was removed and transferred to a new tube. This chloroform:isoamyl alcohol extraction was repeated twice more. The final transferred aqueous layer was supplemented with 0.25 volumes of 10 M LiCl and incubated overnight at 4°C. Following incubation, the solution was centrifuged for 20 min at 4°C at top speed and the supernatant discarded. The pellet was washed by adding 1 ml of 80% EtOH, then centrifuged at 4°C for 5 min at top speed. The supernatant was once again discarded and the pellet dried in a vacuum centrifuge. The pellet was resuspended in 30 μl of DEPC-treated water (0.05% (v/v) diethylpyrocarbonate).

**DNase Treatment and Reverse Transcription of Arabidopsis RNA**

*Arabidopsis* RNA was DNase treated to remove all DNA. The DNase treatment was done using RQ1 RNase-Free DNase (Promega, Madison, WI). RNA was first quantified using a spectrophotometer and approximately 3 μg used in a 30 μl DNase reaction containing 3 μl RQ-1 Buffer, and 3 μl RQ-1 DNase enzyme. The reaction was incubated at 37°C for 30 min. Protein was removed by bringing the volume to 100 μl with RNase-free water and adding an equal volume of phenol:chloroform:isoamyl alcohol (25:24:1). The reaction was centrifuged at 4°C for 5 min at top speed. The upper aqueous layer was transferred to a new tube and an equal volume of chloroform:isoamyl alcohol (24:1) added, followed by another 5 min centrifugation at 4°C at top speed. RNA
was precipitated with an equal volume of isopropanol and incubation at -20°C for 20 min, followed by a 15 min centrifugation at 4°C. The supernatant was discarded and the pellet washed with 80% EtOH and centrifuged at 4°C for 5 min. The pellet was dried in a vacuum centrifuge and resuspended in 10 μl RNase-free water.

DNase-treated RNA was reverse transcribed using the cMaster RT System (Eppendorf, Hamburg, Germany). The reverse transcription was performed using two mixes. Using this kit, mix 1 typically contained 2 μl of dNTPs, 0.75 μl oligo dT primer, and 7.25 μl of the DNase-treated RNA. This mix was incubated at 65°C for 5 min and cooled on ice. Mix 2, containing 4 μl of RT buffer, 1 μl of cMaster reverse transcriptase, 0.5 μl RNase inhibitor, and 4.5 μl of RNase-free water, was added to mix 1 and incubated at 42°C for 90 mins. The reaction was further incubated at 85°C for 5 min. The reaction product was used as the cDNA template for RT-PCR screening.

**RT-PCR Primer Design and Screen of SPDS3, SPMS, and GUS Transcripts**

Primers for RT-PCR were designed using the Primer Express v2.0 software (Applied Biosystems, Foster City, CA) from the SPDS1 (Accession # NM_102230), SPDS2 (NM_105699), SPDS3 (NM_124691), SPMS (AF184094) and GUS (pCAMBIA 2381) mRNA sequences. To confirm specificity, primers were screened using specific cDNA clones, which were ordered from a cDNA library stock through the Arabidopsis Biological Resource Center (ABRC). These stocks were prepared from tissue culture grown roots, 7 day old etiolated seedlings, and stems, flowers, siliques, and rosettes from various ages and two light regimes. The cDNA clones of SPDS1 (ABRC stock# 104K18), SPDS2 (220B5), SPDS3 (202J6), and SPMS (147N21) were prepared in the
pZL1 plasmid and cloned in DH10B *E. coli*. The primers were screened against the particular cDNA clone as well as the other clones to confirm specificity.

RT-PCR was performed on cDNA prepared from transgenic 10 DPG whole seedlings, 20 DPG and 35 DPG rosette leaves, 35 DPG roots, and 35 DPG stems. A total of 2 lines for each *SPDS3* and *SPMS* treatment were screened at each time point. RT-PCR was set up as a normal PCR using NEB *Taq* polymerase. Each line was screened for the native *SPDS3* or *SPMS* transcript as well as the *GUS* transcript. Negative controls were prepared using the DNase-treated RNA to ensure no DNA contamination. PCR products were analyzed on a 1.5% gel using sodium borate buffer and electrophoresed at 20 V/cm for approximately 15 min.

**Cloning of *SPDS1* and *SPDS2* Promoter Regions**

Constructs for *SPDS1* and *SPDS2* were amplified for preparation of promoter::GUS fusions. Six separate constructs were designed for *SPDS1* (Figure 6). The putative promoter region of *SPDS1* (accession #'s NM_102230, NM_202171; BAC clone #F508) was PCR amplified from *A. thaliana* (ecotype Columbia) genomic DNA using specific primers (Table 4) designed to amplify various regions of the promoter and, in some constructs, the *SPDS1* open reading frame. The *SPDS1*-A construct was designed using the FI (forward) and RIB (reverse) primers which amplified 1701 bp upstream from the transcription start site (putative promoter region), the entire 123 bp 5′UTR, and 98 bp of the *SPDS1* ORF. The *SPDS1*-B construct was amplified using the F1 (forward) and 5′UTR (reverse) primers which amplified 1701 bp of the putative promoter and the 123 bp 5′UTR. *SPDS1*-C was amplified using the F2 (forward) and
RIB (reverse) primers providing 867 bp of the promoter, the entire 123 bp 5’UTR, and an additional 98 bp of the ORF. The *SPDSI*-D construct was designed with the F2 (forward) and 5’UTR (reverse) primers which amplified 876 bp of the promoter and the 123 bp 5’UTR. The *SPDSI*-E construct was designed with the F3 (forward) and RIB (reverse) primers which amplified 291 bp of the promoter, the entire 123 bp 5’UTR, and 98 bp of the ORF. The final construct, *SPDSI*-F was amplified with the F3 (forward) and 5’UTR (reverse) primers which provided 291 bp of the promoter and the entire 123 bp 5’UTR. The primers were designed to incorporate flanking restriction sites, as the F1, F2, and F3 primers included an *EcoRI* site, the RIB primer incorporated a *BamHI* site, and the 5’UTR primer a *SpeI* site. The PCR products were individually cloned into the pCR2.1 TOPO TA vector (Figure 4) and the plasmids chemically transformed into TOP10 competent *E. coli*. The clones were confirmed with a restriction enzyme analysis and sequenced using the M13, T7 primers, and the cloning primers (Table 4).

Four different fragments of the *SPDS2* promoter (accession # NM_105699; BAC clone # F1707) were designed and amplified from *A. thaliana* genomic DNA (Figure 6). The primer combination for the *SPDS2*-A construct was F1 (forward) and RIB (reverse) primers which provided 987 bp upstream of the transcription start site (putative promoter), the entire 61 bp 5’UTR, and 111 bp of the ORF. The *SPDS2*-B construct was amplified with the forward F1 primer and the 5’UTR (reverse) primer and contained 987 bp of the promoter and the 61 bp 5’UTR. The primers were also designed to incorporate flanking restriction sites as the F1 primer provided a *BamHI* site, the RIB primer incorporated a *SalI* site, and the 5’UTR primer a *SpeI* restriction site. The *SPDS2*-A and *SPDS2*-B PCR products were cloned into the pCR2.1 TOPO TA vector (Figure 4) and
the sequence confirmed using the M13, T7, and cloning primers (Table 4). The SPDS2-C construct was created by digesting the SPDS-A TOPO clone with EcoRI and SalI to produce a 567 bp promoter fragment, the entire 61 bp 5'UTR, and 111 bp of the ORF. The SPDS2-D construct was also designed with the internal EcoRI site by digesting the SPDS2-B clone with EcoRI and SpeI to produce the 567 bp promoter region and the 61 bp 5'UTR.

Figure 6. Diagram of the A) SPDS1 and B) SPDS2 constructs designed in this study. The promoter regions along with the 5'UTR (and ORF if applicable) have been cloned into the TOPO cloning vector and sequenced. Currently the cloned regions have not been fused to GUS, but this diagram represents what the constructs will look like. All constructs contain the entire 5'UTR of the respective gene as described in the TAIR database (www.arabidopsis.org). Diagram not drawn to scale.
CHAPTER 2

RESULTS

Construction of pCAM-SPDS3 and pCAM-SPMS Plasmids

*Arabidopsis* has three paralogues of the *SPDS* gene, currently annotated as *SPDS1*, *SPDS2*, and *SPDS3*. In addition, there is another aminopropyl transferase involved in spermine synthesis, annotated as SPMS. The current study focuses on the expression of the third paralogue of SPDS, *SPDS3*, and the SPMS. The promoter regions of *SPDS1* and *SPDS2* were also cloned into the TOPO cloning vector in order to produce constructs for promoter::GUS fusions in future transformations. Various regions of the promoter for *SPDS3* and *SPMS* were amplified by PCR and fused to the GUS reporter gene in the binary vector pCAMBIA. The resulting plasmids were named pCAM-SPDS3 and pCAM-SPMS. These plasmids were previously constructed by Todd Bezold; therefore, the design and preparation of these plasmids is discussed here briefly.

As shown in Figure 7, three separate constructs were designed for the expression analysis of *SPDS3*. The putative promoter region of *SPDS3* (accession #'s NM_124691, NM_180848, NM_180847; BAC clone # MFH8) was PCR amplified from *A. thaliana* (ecotype Columbia) genomic DNA using primers (Table 4) designed to amplify various regions of the promoter and part of the *SPDS3* open reading frame (Figure 8). The *SPDS3*-A fragment was amplified using the F1 (forward) and R2B (reverse) primers, which amplified an 1867 bp fragment containing 935 bp of the putative promoter (upstream from the transcription start site), the entire 875 bp 5'UTR, and an additional 57 bp of the ORF (Figure 8A). The *SPDS3*-B fragment was amplified using the F2
The various regions of the \textit{SPDS3} promoter, with or without part of the ORF, were PCR amplified from genomic DNA, and cloned into the TOPO TA vector. The PCR products were excised from TOPO, and ligated into the pCAMBIA x C 2381 vector where they were fused with the \textit{GUS} gene to form the pCAM-\textit{SPDS3} plasmids. The \textit{SPDS3-C} PCR product was lacking the \textit{SPDS3} ATG, therefore it was ligated into the pCAMBIA 2381 GUS+ATG vector containing a \textit{GUS} gene with its own ATG site.

Figure 7. Cloning strategy of pCAM-\textit{SPDS3} vectors.
Figure 8. PCR products of SPDS3 promoter regions.
A) SPDS3-A (lanes 1-4) PCR product contains an 1867 bp fragment, the B) SPDS3-B (lanes 5-8) product is 1259 bp, and C) SPDS3-C (lanes 9-12) is 1810 bp. The NEB 2-Log DNA Ladder was used as a marker.

(forward) and R1B (reverse) primers, providing a 1259 bp product which included 214 bp of the putative promoter, the 875 bp 5'UTR, and 170 bp of the ORF (Figure 8B). Both the R1B and R2B reverse primers incorporated a BamHI restriction site at the 3' end of the PCR products. The SPDS3-C construct was amplified using the F1 (forward) and 5'UTR (reverse) primers which provided an 1810 bp fragment including 935 bp of the promoter and the entire 875 bp 5'UTR, but no part of the ORF (Figure 8C). The PCR products were individually cloned into the pCR2.1 TOPO TA vector (Figure 9) and sequenced using the M13 and T7 primers (Table 4) to confirm the presence and orientation of the promoter region. Of the four clones screened for each construct, all were found to contain the insert and be in the correct orientation.

To fuse the SPDS3 promoter region with the GUS gene, the PCR products were excised from the respective TOPO vector using the TOPO EcoRI site and the incorporated BamHI for SPDS3-A and SPDS3-B, and the flanking TOPO EcoRI sites for SPDS3-C. The inserts were separated from the TOPO vector by gel electrophoresis (Figure 10) and the appropriate bands were gel purified. The pCAMBIA 2381 vector was also cut with the same restriction enzymes in order to receive the excised SPDS3...
Various regions of the SPDS3 promoter and ORF were PCR amplified and cloned into the TOPO cloning vector. The resulting ligations yielded the A) SPDS3-A TOPO, B) SPDS3-B TOPO, and C) SPDS3-C TOPO plasmids.

Figure 10. Restriction digests of SPDS3 TOPO plasmids. SPDS3-A (lanes 1-4) and SPDS3-B (lanes 5-8) TOPO plasmids were digested with EcoRI and BamHI to produce ~1.9 and ~1.3 Kb fragments, respectively. SPDS3-C (lanes 9-12) TOPO was digested with EcoRI to produce a ~1.8 Kb fragment. The NEB 2-Log DNA Ladder or 1 Kb DNA Ladder was used as a marker.
promoter region. The SPDS3-A and SPDS3-B promoter regions were ligated into pCambia 2381 x C where it was fused, in-frame with the GUS gene which does not have its own ATG start site. This construct relies on the use of the ATG start site provided with the SPDS3 ORF in expressing the GUS gene. The SPDS3-C PCR product was fused into the pCambia 2381 GUS+ATG vector which contains a GUS gene supplied with its own ATG, as the SPDS3-C involves a direct fusion of the 5'UTR to the GUS gene, lacking the SPDS3 start codon (Figure 11).

Figure 11. Summary of pCAM-SPDS3 maps. The various regions of the SPDS3 promoter were fused to the GUS gene in the binary vector pCambia 2381. The resulting ligations yielded the A) pCAM-SPDS3-A, B) pCAM-SPDS3-B, and C) pCAM-SPDS3-C plasmids.
The three SPMS constructs were designed in a similar manner to SPDS3 (Figure 12). The putative promoter region of SPMS (accession # NM_121958; BAC clone # T20D1) was PCR amplified from A. thaliana genomic DNA (Figure 13) using the primers described in Table 4. The SPMS-A construct was amplified with the F2 (forward) and R1B (reverse) primers which provided a 1567 bp fragment including 1416 bp of the putative promoter, the entire 90 bp 5'UTR, and 61 bp of the SPMS ORF (Figure 13A). The SPMS-B construct was amplified with the F3 and R1B primers to provide a 1090 bp fragment containing 939 bp of the promoter, the 90 bp 5'UTR, and 61 bp of the ORF (Figure 13B). The R1B primer incorporated a BamHI restriction site at the 3' end of the SPMS-A and SPMS-B PCR products. The SPMS-C construct was amplified with the F2 and 5'UTR primers to provide a 1506 bp fragment containing 1416 bp of the promoter region and the 90 bp 5'UTR (Figure 13C). The PCR products were individually cloned into the pCR2.1 TOPO TA vector (Figure 14) and sequenced with the M13 and T7 primers. The DNA sequences once again matched with the published sequences.

The SPMS-A and SPMS-B promoter fragments were excised from the TOPO vector and gel purified using the TOPO EcoRI site and the PCR incorporated BamHI site, whereas SPMS-C was removed from TOPO using the vector's flanking EcoRI sites (Figure 15). The excised fragments were fused to the GUS gene in the pCAMBIA 2381 vector. The SPMS-A and SPMS-B fragments were ligated into the pCAMBIA 2381 x A vector and the SPMS-C fragment ligated into the pCAMBIA 2381 GUS+ATG vector (Figure 16).
Figure 12. Cloning strategy of pCAM-SPMS vectors. The various regions of the SPMS promoter, with or without part of the ORF, were PCR amplified from genomic DNA, and cloned into the TOPO TA vector. The PCR products were further excised from TOPO and ligated into the pCAMBIA x A 2381 vector where they were fused with the GUS gene to form the pCAM-SPMS plasmids. The SPMS-C PCR product was lacking the SPMS ATG, therefore it was ligated into the pCAMBIA GUS+ATG vector containing a GUS gene with its own ATG start site.
Figure 13. PCR products of SPMS promoter regions. A) SPMS-A (Lanes 1-4) PCR products are 1567 bp, B) SPMS-B (Lanes 5-8) 1110 bp, and C) SPMS-C (Lanes 9-12) 1506 bp. The NEB 2-Log DNA Ladder was used as a marker.

Figure 14. Summary of SPMS TOPO maps. Various regions of the SPMS promoter and ORF were PCR amplified and cloned into the TOPO vector. The resulting ligations yielded the A) SPMS-A TOPO, B) SPMS-B TOPO, and C) SPMS-C TOPO plasmids.

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Figure 15. Restriction digests of SPMS TOPO plasmids.
A) SPMS-A (lanes 1-4) and B) SPMS-B (lanes 5-8) TOPO clones were digested with EcoRI and BamHI to produce ~1.6 and ~1.1 Kb fragments, respectively. C) SPMS-C (lanes 9-12) TOPO was digested with EcoRI to produce a ~1.5 Kb fragment. The NEB 2-Log DNA Ladder or 1 Kb DNA Ladder were used as markers.

Figure 16. Summary of pCAM-SPMS maps.
The various regions of the SPMS promoter and were fused to the GUS gene in the binary vector pCAMBIA 2381. The resulting ligations yielded the A) pCAM-SPMS-A, B) pCAM-SPMS-B, and C) pCAM-SPMS-C plasmids.

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The final SPDS3 and SPMS constructs were transformed into TOP10 electrocompetent E. coli and the selected colonies tested for the insert (Figure 17). Of the 9 colonies screened for SPDS3-A and SPMS-A, 8 were confirmed positive. A total of 8 colonies for SPDS3-B, SPDS3-C, SPMS-B, and SPMS-C were screened. For SPDS3-B, all 8 colonies were confirmed positive, 5 for SPDS3-C, 6 for SPMS-B, and 7 for SPMS-C. The pCAM plasmids were sequenced to check integrity of GUS fusions (Figures 18-23). Two colonies for each construct were sequenced and both found to provide in-frame GUS fusions in the correct orientation. The final plasmids were named according to the primer combination used for cloning: SPDS3 F1-R2B (SPDS3-A), F2-R1B (SPDS3-B), F1-5UTR (SPDS3-C), and SPMS F2-R1B (SPMS-A), F3-R1B (SPMS-B), and F2-5UTR (SPMS-C). The positive bacteria were stored as glycerol stocks at -80°C.

![Figure 17. Restriction enzyme analysis of pCAM-SPDS3 and pCAM-SPMS ligations.](image)

A) pCAM-SPDS3-A (lanes 1-9) and pCAM-SPDS3-B (lanes 10-17) were digested with EcoRI and BamHI to produce ~1.9 and 1.2 Kb fragments, respectively. pCAM-SPDS3-C (lanes 18-25) was digested with EcoRI to produce an ~1.8 Kb fragment. B) pCAM-SPMS-A (Lanes 1-9) and pCAM-SPMS-B (Lanes 10-17) were also digested with EcoRI and BamHI to produce ~1.6 and 1.1 Kb fragments, respectively. pCAM-SPMS-C (lanes 18-25) was digested with EcoRI to produce an ~1.5 Kb fragment. Gels were analyzed with the NEB 1 Kb DNA Ladder.
Figure 18. Alignment of pCAM-SPDS3-A sequencing with the SPDS3-A genomic sequence used for cloning (NM_124691) and the pCAMBIA 2381 x C (pCAMxC) sequence. The entire length of the insert was sequenced using the cloning primers and sequencing primers from Table 4. The black underline indicates part of the TOPO sequence carried through restriction enzyme digests. The blue line indicates the location of the 875 bp 5'UTR. The purple line is the 57 bp SPDS3 ORF. The black box denotes the ATG start site provided by the SPDS3 ORF which is in-frame with the first codon of the GUS gene highlighted by the red box.
**Figure 19.** Alignment of pCAM-SPDS3-B sequencing with the SPDS3-B genomic sequence used for cloning (NM_124691) and the pCAMBIA 2381 x C (pCAMxC) sequence. The entire length of the insert was sequenced using the cloning primers and sequencing primers from Table 4. The black underline indicates part of the TOPO sequence carried through restriction enzyme digests. The blue line indicates the location of the 875 bp 5'UTR. The purple line is the 170 bp SPDS3 ORF. The black box denotes the ATG start site provided by the SPDS3 ORF which is in-frame with the first codon of the GUS gene highlighted by the red box.
Figure 20. Alignment of pCAM-SPDS3-C sequencing with the SPDS3-C genomic sequence used for cloning (NM_124691) and the pCAMBIA 2381 GUS+ATG (pCAM GUS+) sequence. The entire length of the insert was sequenced using the cloning primers and sequencing primers from Table 4. The black underlines indicate part of the TOPO sequence carried through restriction enzyme digests. The blue line indicates the location of the 875 bp 5'UTR. The black box highlights the ATG start site of the GUS gene provided by the pCAMBIA vector.
Figure 21. Alignment of pCAM-SPMS-A sequencing with the SPMS-A genomic sequence used for cloning (NM_121958) and the pCAMBIA 2381 x A (pCAMxA) sequence. The entire length of the insert was sequenced using the cloning primers and sequencing primers from Table 4. The black underline indicates part of the TOPO sequence carried through restriction enzyme digests. The blue line indicates the location of the 90 bp 5'UTR. The purple line is the 61 bp SPMS ORF. The black box denotes the ATG start site provided by the SPDS3 ORF which is in-frame with the first codon of the GUS gene highlighted by the red box.
Figure 22. Alignment of pCAM-SPMS-B sequencing with the SPMS-B genomic sequence used for cloning (NM_121958) and the pCAMBIA 2381 x A (pCAMxA) sequence. The entire length of the insert was sequenced using the cloning primers and sequencing primers from Table 4. The black underline indicates part of the TOPO sequence carried through restriction enzyme digests. The blue line indicates the location of the 90 bp 5'UTR. The purple line is the 61 bp SPMS ORF. The black box denotes the ATG start site provided by the SPMS ORF which is in-frame with the first codon of the GUS gene highlighted by the red box.
Figure 23. Alignment of pCAM-SPMS-C sequencing with the SPMS-C genomic sequence used for cloning (NM_121958) and the pCAMBIA 2381 GUS+ATG (pCAM GUS+) sequence. The entire length of the insert was sequenced using the cloning primers and sequencing primers from Table 4. The black underlines indicate part of the location of the 90 bp 5'UTR. The black box highlights the ATG start site of the GUS TOPO sequence carried through restriction enzyme digests. The blue line indicates the GUS+ sequence. The entire length of the insert was sequenced using the cloning primers and sequencing primers from Table 4. The black underlines indicate part of the location of the 90 bp 5'UTR. The black box highlights the ATG start site of the GUS TOPO sequence carried through restriction enzyme digests. The blue line indicates the GUS+ sequence.
Transient Expression of the pCAM-SPDS3 and pCAM-SPMS Plasmids

The functionality of the pCAM-SPDS3 and pCAM-SPMS plasmids was tested using transient expression in poplar (Populus nigra x maximowiczii) cells by biolistic bombardment. Approximately 48 h after bombardment, the cells were stained for GUS activity and the number of blue cells was counted, representing the number of cells expressing the GUS gene. A pCAMBIA 2381 vector containing the GUS gene under the control of a CaMV 35S promoter was used as a positive control (pCAM-35S-GUS).

Table 5 represents data from three separate trials, each representing an average of six plates that were shot. All plasmids showed a fairly high level of expression (in terms of number of blue cells) when compared to the pCAM-35S-GUS. Lower levels of expression were seen in pCAM-SPDS3-A, pCAM-SPDS3-B, and pCAM-SPMS-A with an average number of 202, 278, and 288 blue cells, respectively. The rest of the plasmid expression levels were slightly higher and comparable to the pCAM-35S-GUS with pCAM-SPDS3-C, pCAM-SPMS-B, and pCAM-SPMS-C having 327, 376, and 395 blue cells, respectively. The pCAM-35S produced an average of 365 blue cells.

Table 5. Transient expression of the pCAM-SPDS3 and pCAM-SPMS plasmids in poplar cells. The values for each plasmid are the mean number of blue cells from six separate plates in three different experiments (trials).

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Transformation and Screening of *Agrobacterium tumefaciens*

All of the pCAM-SPDS3 and pCAM-SPMS plasmids were purified from *E. coli* and transformed into *Agrobacterium tumefaciens* GV3101 by electroporation. Colonies were selected on LB plates supplemented with kanamycin (100 µg/ml). Plates were incubated at 28°C for 36-48 h. For each plasmid, 4 colonies were selected and PCR screened for the presence of the *A. thaliana* promoter using the same primer combinations used for cloning (Table 4). PCR products were analyzed by gel electrophoresis (Figure 24) indicating that all Agrobacterium colonies were positive.

Glycerol stocks were prepared from colonies #1 and #2 for each plasmid and each were appropriately named according to the primer combination used for cloning: SPDS3 F1-R2B (SPDS3-A), F2-R1B (SPDS3-B), F1-5UTR (SPDS3-C), and SPMS F2-R1B (SPMS-A), F3-R1B (SPMS-B), and F2-5UTR (SPMS-C).

![Figure 24. PCR screening of transformed *A. tumefaciens* colonies.](image)

Colonies were screened with the promoter cloning primers to indicate the presence of the SPDS3 and SPMS pCAMBIA plasmids. A) SPDS3-A (lanes 1-4) PCR product contains an ~1.9 Kb fragment, the SPDS3-B (lanes 5-8) product is ~1.3 Kb, and SPDS3-C (lanes 9-12) is ~1.8 Kb. B) SPMS-A PCR products are ~1.6 Kb, SPMS-B ~1.1 Kb, and SPMS-C (lanes 9-13) ~1.5 Kb.
Transformation and Screening of *Arabidopsis*

*Arabidopsis* (ecotype Columbia) plants were transformed by the floral dip method with *A. tumefaciens* containing the various pCAM-SPDS3 and pCAM-SPMS plasmids (Figures 11 and 16). A total of eight pots containing approximately 10 plants each were dipped twice (one week apart) for each construct and allowed to produce seed 2-3 weeks later. The T1 seeds from each pot were harvested, desiccated, and sterilized. Sterile seeds were placed on selective germination medium supplemented with 50 µg/ml kanamycin. After two weeks, surviving plants were transferred to soil and named according to treatment, pot number, and plant number. For example, line “SPDS3-A 1-4” refers to the pCAM-SPDS3-A treatment from pot #1, plant #4. Once plants had bolted, various organs (such as rosette and cauline leaves, flowers, and secondary bolts) were excised and checked for GUS activity by staining. In addition, a rosette leaf was removed and used for isolation of genomic DNA. Subsequently, the DNA was PCR screened for the GUS and NPTII transgenes using the appropriate primer combinations (Table 4). Plants which tested positive for GUS activity and the presence of both transgenes were deemed successfully transformed and the seeds from these plants were harvested (T2 seeds). The T2 seeds and T2 plants were subjected to the same selection and confirmation procedure as the T1 plants (antibiotic selection, GUS activity, and PCR screen). Confirmed positive T2 plants were allowed to produce T3 seeds which were used for experiments.

For T1 selection, a total of 18 plants were found to be kanamycin resistant for SPDS3-A and SPDS3-B, and 15 plants for SPDS3-C. A total of 16 plants were kanamycin resistant for SPMS-A, 22 plants for SPMS-B, and 19 plants for SPMS-C. The
kanamycin resistant plants were further screened for GUS activity and the presence of the GUS and NPTII transgenes (Figure 25). Both SPDS3-A and SPDS3-B had 12 plants which tested positive for GUS staining and PCR of both transgenes, whereas SPDS3-C had 9 plants. The SPMS putative transformants showed less GUS activity as SPMS-A had only 2 plants with GUS activity and PCR confirmation; SPMS-B had 5 plants with confirmed GUS activity and transgene presence. However, there were a number of plants which did not show GUS activity, yet did show presence of the GUS and NPTII transgene. Seeds were harvested from the 2 SPMS-A lines with GUS activity, as well as 3 additional lines without GUS activity that showed the presence of the two transgenes. SPMS-C had 14 plants with positive GUS and PCR confirmation. Seeds (T₂) were collected from 5 lines of each construct and further screened. Table 6 depicts a summary of screening for the T₁ lines.
Figure 25. Results of PCR screening of transformed *A. thaliana* T$_1$ plants. The genomic DNA of T$_1$ plants was PCR screened for the presence of the *NPTII* and *GUS* transgenes. PCR products of 12 *SPDS3*-A (A & D), 18 *SPDS3*-B (B & E), 15 *SPDS3*-C (C & F), 16 *SPMS*-A (G & J), 16 *SPMS*-B (H & K), and 19 *SPMS*-C (I & L) lines were analyzed on a 1% gel for the 0.7 kb *NPTII* product and the 1 kb *GUS* product. All water controls (-) were negative (not shown for all gels). The NEB 2-Log DNA ladder was used for all gels.
Table 6. Summary of T₁ Arabidopsis plant screening for A) SPDS3 and B) SPMS.
Plants were screened for GUS activity in various organs (Stain), and PCR screened for the NPTII and GUS transgenes. "#" refers to the PCR # analyzed on an agarose gel in Figure 25 (e.g. the PCR product from SPMS-A plant 1-3 is in lane 3 of Figure 25G and J. (+) indicates a positive result, (-) indicates a negative result, and (/) indicates that either the screen was not performed or the plant had died. Lines annotated with an “L” indicate a last harvest as seeds were collected and combined from all plants in a tray (as seen in SPDS3-C lines). An asterisk (*) indicates lines chosen for further screening.
The T<sub>2</sub> seeds from the confirmed T<sub>1</sub> transformed lines were collected, sterilized, and plated on germination medium containing kanamycin (50 μg/ml). A total of 18 plants were found to be kanamycin resistant for SPDS3-A, 15 for SPDS3-B, and 16 plants for SPDS3-C. A total of 23 plants were kanamycin resistant for SPMS-A and 21 plants for each SPMS-B and SPMS-C. Surviving plants were transferred to soil and stained for GUS activity in various organs. Genomic DNA was, once again, PCR screened for the presence of GUS and NPTII (Figure 26). Plants were confirmed to be transformed if staining and PCR screening for both transgenes were positive. Seeds were collected and named according to the previous pot number and T<sub>1</sub> plant, as well as the T<sub>2</sub> plant. For example, T<sub>3</sub> seeds collected from the SPDS3-A line 1-3-1 were derived from pot 1 dipped with the pCAM-SPDS3-C-Agro, T<sub>1</sub> plant #3, and T<sub>2</sub> plant #1. Both SPDS3-A and SPDS3-B had 8 plants each which tested positive for GUS staining and PCR of both transgenes, whereas SPDS3-C had 7 plants. SPMS-A had 5 plants with GUS staining, yet 8 lines were PCR screened and tested positive. One line was chosen for experimental use which tested negative for GUS staining, but was positive for PCR screening. This line was chosen despite positive screening because all other lines showed very low levels of staining. A total of 16 plants were positive for SPMS-B and 12 for SPMS-C. T<sub>3</sub> seeds were collected from 5 separate lines for each construct for use in further experiments. Table 7 displays a summary of the T<sub>2</sub> selection.
Figure 26. Results of PCR screening of transformed *A. thaliana* T$_2$ plants. The genomic DNA of T$_2$ plants was PCR screened for the presence of the *NPTII* and *GUS* transgenes. PCR products of 8 *SPDS3*-A (A & D), 8 *SPDS3*-B (B & E), 7 *SPDS3*-C (C & F), 8 *SPMS*-A (G & J), 16 *SPMS*-B (H & K), and 12 *SPMS*-C (I & L) lines were analyzed on a 1% gel for the 0.7 Kb *NPTII* product and the 1 Kb *GUS* product. Water controls (-) were negative and plasmid controls (+) were positive (not shown for all gels). The NEB 2-Log DNA ladder was used for all gels.
Table 7. Summary of T2 Arabidopsis plant screening for A) SPDS3 and B) SPMS.

Plants were screened for GUS activity in various organs (Stain), and PCR screened for the NPTII and GUS transgenes. "#" refers to the PCR # analyzed on an agarose gel in Figure 26 (e.g. the PCR product from SPMS-A plant 2-5-4 is in lane 3 of Figure 26G and J. (+) indicates a positive result, (-) indicates a negative result, and (/) indicates that either the screen was not performed or the plant had died.

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Developmental Expression Profile of *SPDS3* and *SPMS* in *Arabidopsis*

To observe the developmental expression profile of *SPDS3* and *SPMS*, approximately 200 T3 seeds from five confirmed transformed lines for each construct were sterilized and plated on germination medium supplemented with 50 μg/ml kanamycin. A non-transformed control line was also sterilized and plated on germination medium without kanamycin. The plates were placed at 4°C for 48 h for seed stratification and then placed at 25°C under fluorescent light. The first samples were collected when the majority of seeds had germinated, approximately 48 h after cold treatment. This was labeled as 0 time (DPG 0). Samples were removed from GM plates and submerged in GUS substrate solution. Following incubation, the localization of blue color (indicating GUS activity) in various organs was counted and tabulated. Plants grown in petri plates were collected and stained at germination (time 0) and 1, 3, 5, 7, 9, 11, 13, and 15 days post germination (DPG). For each construct, a total of 10 samples were collected for each of the 5 transformed lines, providing 50 samples at each developmental time point. Seeds were also sown in pots at the same time as on germination medium. Following 15 DPG, samples from pots were collected and stained at 18, 21, 24, 28, 32, 38, and 45 DPG. The experiment was repeated in its entirety (Trial A and B).

At germination, seedlings were surveyed for the presence of blue color in the apex, cotyledons, hypocotyl, root, root tip, and vascular tissue of cotyledons, hypocotyl, and root (Table 8). The total number of plants that stained blue in a particular organ were counted and tabulated as a percentage based on the number of plants showing GUS activity compared to the total number of plants observed. For the first 15 days of
development, a total of 50 plants were observed at each time point for each construct.
The percent of plants showing staining was averaged between the two trials to get a
single value as shown in Table 8.

On the first day of observation, GUS staining was seen in all organs of all three
SPDS3 constructs (Figures 27A, B, and C). However, more intense and widespread
activity was observed in SPDS3-C as compared to SPDS3-A and SPDS3-B; SPDS3-A
activity was higher than SPDS3-B. In SPMS transformants, GUS activity was much
lower than SPDS3::GUS transformants and also varied among the three constructs at
DPG 0 (Figures 27D, E, and F). Activity was particularly high in the cotyledons and
vascular regions of the cotyledons, hypocotyl, and root for SPMS-C. There was much
less activity in the SPMS-A and SPMS-B transformants. SPMS-A transformants showed
faint staining in the vascular tissue of the cotyledons. SPMS-B also showed faint staining
in the vascular tissue of the cotyledons with more intense staining at the cotyledon distal
tips. GUS staining in SPMS-C was the most intense, and the activity was localized in the
vascular tissue of the cotyledons, as well as the hypocotyl and roots.

At 1 and 3 DPG, GUS activity was surveyed in the cotyledons, hypocotyls
(upper, middle, lower), root, the root tip, and the veins of the cotyledons, hypocotyl, and
root (Tables 9 and 10) with the addition of root hair at 3 DPG. Activity remained high in
the SPDS3-A and SPDS3-C transformants, particularly in the root tissue, root tip, and
vascular region of the cotyledons, hypocotyls, and roots at both developmental stages.
GUS activity was more localized in the cotyledon veins of SPDS3-A when compared to
SPDS3-C which showed activity in the veins as well as surrounding tissue (Figures 28A
and C; 29A and C). There was less activity on DPG1 and 3 in SPDS3-B transformants as
compared to SPDS3-A and SPDS3-C. In SPDS3-B transformants, GUS activity was localized in the veins of the cotyledons and roots, yet, continued to be less intense than the other two constructs (Figure 28B and 29B). The SPMS transformants displayed a similar GUS staining pattern at 1 and 3 DPG as DPG 0. GUS activity was high near the tip of the cotyledons and vascular region of the cotyledons, hypocotyls, and root in SPMS-C transformants (Figures 28F and 29F). Once again, much less activity was observed in the SPMS-A and SPMS-B transformants as some activity was observed in the distal tip of the cotyledons for both constructs. Both SPMS-A and SPMS-C transformants showed activity in the root veins (Figures 28E and F & 29E and F). No activity was seen in the root tips of any SPMS construct. Activity was, once again, dramatically higher in SPDS3 transformants when compared to SPMS plants, particularly in the cotyledons and root tissues (Figures 28 and 29).

At 5 DPG, distribution of GUS activity was observed in all organs, including new emerging primary leaf tissue, tip of leaf, and vascular tissue (Table 11). By 5 DPG, the percentage of plants showing GUS staining in some organs was higher than that on 0 or 1 DPG, approaching 100% in root tips, cotyledon veins, and roots. High GUS activity continued in SPDS3-A and SPDS3-C transformants (Figures 30A and C). Activity was observed in the vascular tissue of the cotyledons, hypocotyl, and root, as well as the root tip and root tissue. SPDS3-C transformants displayed activity in the cotyledon tissue as well as the new primary leaf distal tip, however, the activity was low in the leaf tissue. Much less activity was observed in SPDS3-B plants, as activity was only observed in the root tips and vascular tissue of the cotyledons, hypocotyl, and root (Figure 30B). Very little GUS activity was observed in the new emerging primary leaves of SPDS3-A plants.
In contrast, most SPMS plants showed a high level of activity in the primary leaves, particularly in the distal tip of the leaf. SPMS-A, SPMS-B, and SPMS-C plants showed higher GUS activity in the primary leaf distal tip and cotyledon hydathode; yet, little activity was observed elsewhere in SPMS-A and SPMS-B plants (Figures 30E and F). SPMS-C plants continued higher expression in the vascular tissue of the cotyledons, hypocotyl, and root (Figures 30G and H).

Similar expression patterns continued at 7 and 9 DPG. Seedlings were surveyed for GUS activity in more detail than before in the apex, cotyledons, hypocotyl (upper, middle, lower), root tissue, root tip, root hair, vascular tissue of the cotyledons, hypocotyl, root, as well as the primary leaf tissue, distal tip, and vascular tissue. In addition, secondary roots began to develop and were also surveyed (Tables 12 and 13). GUS activity continued to be present in the vascular tissue of SPDS3 transformants, particularly in the cotyledons, hypocotyl, and the root. Activity was still widely distributed in the entire cotyledon tissue for SPDS3-C transformants (Figures 31C and 32C), whereas staining remained localized to only the veins of the cotyledons for SPDS3-A and SPDS3-B plants (Figures 31A and B, 32A and B). The intensity of GUS staining in SPDS3-B was noticeably higher in DPG 7 and 9 plants when compared to younger seedlings, yet GUS activity was still lower than that in SPDS3-C plants. GUS staining was intense in the secondary root veins, root tissue, and the root tips. Staining continued to be sparse in SPMS-A and SPMS-B transformants (Figures 31E and F, 32E and F), but activity was still observed in the cotyledon and primary distal tip. There was also staining in the vascular tissue of the SPMS-A roots. SPMS-C transformants showed intense staining in the vascular tissue of the cotyledons, hypocotyl, and primary leaf. No
staining was observed in the root tips (Figures 31G and 32G), yet there was staining in the initial lateral roots for *SPMS*-A and *SPMS*-C plants.

GUS activity remained unchanged at 11 and 13 DPG (Tables 14 and 15). Additional tissue categories were added at 11 DPG as secondary leaves began to emerge. *SPDS3* transformants continued a pattern of GUS activity in the vascular tissue of the cotyledons, yet little activity was observed in the leaves of about 20% of *SPDS3*-C plants (Figures 33C and 34C). This trend continued, as activity decreased in the leaves through development (Figures 33A, B, C and 34A, B, C). Activity remained high in the primary, as well as secondary lateral roots. GUS activity also continued to decrease in all organs of the *SPMS*-A and *SPMS*-B plants (Figures 33E and F, 34E and F). *SPMS*-C transformants retained high GUS activity in the vascular tissue of the cotyledons, hypocotyl, root, and the primary leaves. Staining was also observed in the secondary tissue, particularly at the tip of the leaf and the vein of the roots (Figures 33G and 34G).

The final time point for seedlings on medium was at 15 DPG. The secondary leaf was characterized at the tip, vein, and leaf tissue (Table 16). Similar trends in GUS staining among the three *SPDS3* constructs were observed. Lower activity was observed in the leaf veins when compared to the cotyledons. Very little staining was observed in the younger secondary leaf of *SPDS3*-C (Figure 35C). High GUS activity remained in the primary and secondary root tissue and veins (Figure 35A, B, C). Activity continued to decrease in the *SPMS*-A and *SPMS*-B transformants (Figures 35E and F). In *SPMS*-C, however, GUS activity remained consistent in the vascular tissue and distal tips of the cotyledons, primary and secondary leaves (Figure 35G). A summary and timeline of GUS activity in early developing *SPDS3* and *SPMS* transformants is provided in Figure 36.
Table 8. Summary of GUS activity in *A. thaliana* plants at germination.
The numbers indicate a % value based on the # of plants showing GUS activity compared to the total # of plants observed (50). The % also represents an average of two separate experiments. Wild type plants showed no GUS activity at any stage of development.

<table>
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<tr>
<th>Tissue/Organ</th>
<th>SPDS3-A</th>
<th>SPDS3-B</th>
<th>SPDS3-C</th>
<th>SPMS-A</th>
<th>SPMS-B</th>
<th>SPMS-C</th>
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Figure 27. GUS staining of representative plants at germination.
A) *SPDS3*-A  B) *SPDS3*-B  C) *SPDS3*-C  D) *SPMS*-A  E) *SPMS*-B  F) *SPMS*-C

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Table 9. Summary of GUS activity in *A. thaliana* plants at 1 DPG.

<table>
<thead>
<tr>
<th>Tissue/Organ</th>
<th>SPDS3-A</th>
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<th>SPDS3-C</th>
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<td>80</td>
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<td>Hypocotyl – upper</td>
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<td>76</td>
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<td>80</td>
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<tr>
<td>Vein - root</td>
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<td>90</td>
<td>52</td>
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<td>72</td>
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</table>

Figure 28. GUS staining of representative plants at 1 DPG.
A) SPDS3-A  B) SPDS3-B  C) SPDS3-C  D) SPMS-A  E) SPMS-B  F) SPMS-C

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Table 10. Summary of GUS activity in *A. thaliana* plants at 3 DPG.

<table>
<thead>
<tr>
<th>Tissue/Organ</th>
<th>SPDS3-A</th>
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<th>SPDS3-C</th>
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<td>64</td>
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<td>80</td>
<td>36</td>
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<td>Hypocotyl - Upper</td>
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<td>0</td>
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<td>80</td>
<td>0</td>
<td>0</td>
<td>40</td>
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<td>Hypocotyl - Lower</td>
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<td>80</td>
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<td>6</td>
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<tr>
<td>Root tip</td>
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<td>96</td>
<td>0</td>
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<td>Vein - cotyledon</td>
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<td>92</td>
<td>40</td>
<td>0</td>
<td>80</td>
</tr>
<tr>
<td>Vein - hypocotyl</td>
<td>88</td>
<td>30</td>
<td>92</td>
<td>2</td>
<td>0</td>
<td>54</td>
</tr>
<tr>
<td>Vein - root</td>
<td>100</td>
<td>92</td>
<td>92</td>
<td>0</td>
<td>44</td>
<td>56</td>
</tr>
<tr>
<td>Root hair</td>
<td>92</td>
<td>60</td>
<td>82</td>
<td>0</td>
<td>0</td>
<td>0</td>
</tr>
</tbody>
</table>

Figure 29. GUS staining of representative plants at 3 DPG.
A) *SPDS3*-A  B) *SPDS3*-B  C) *SPDS3*-C  D) *SPMS*-A  E) *SPMS*-B  F) *SPMS*-C
Table 11. Summary of GUS activity in *A. thaliana* plants at 5 DPG.

<table>
<thead>
<tr>
<th>Tissue/Organ</th>
<th>SPDS3-A</th>
<th>SPDS3-B</th>
<th>SPDS3-C</th>
<th>SPMS-A</th>
<th>SPMS-B</th>
<th>SPMS-C</th>
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</thead>
<tbody>
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<td>Apex</td>
<td>88</td>
<td>24</td>
<td>80</td>
<td>0</td>
<td>0</td>
<td>60</td>
</tr>
<tr>
<td>Cotyledon</td>
<td>12</td>
<td>16</td>
<td>90</td>
<td>24</td>
<td>24</td>
<td>80</td>
</tr>
<tr>
<td>Hypocotyl - upper</td>
<td>14</td>
<td>12</td>
<td>36</td>
<td>0</td>
<td>0</td>
<td>10</td>
</tr>
<tr>
<td>Hypocotyl - middle</td>
<td>14</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>Hypocotyl - lower</td>
<td>90</td>
<td>42</td>
<td>80</td>
<td>0</td>
<td>0</td>
<td>54</td>
</tr>
<tr>
<td>Root tissue</td>
<td>80</td>
<td>24</td>
<td>80</td>
<td>0</td>
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</tr>
<tr>
<td>Root tip</td>
<td>100</td>
<td>62</td>
<td>100</td>
<td>0</td>
<td>0</td>
<td>0</td>
</tr>
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<td>Vein - cotyledon</td>
<td>100</td>
<td>78</td>
<td>96</td>
<td>14</td>
<td>22</td>
<td>80</td>
</tr>
<tr>
<td>Vein - hypocotyl</td>
<td>100</td>
<td>28</td>
<td>96</td>
<td>0</td>
<td>0</td>
<td>68</td>
</tr>
<tr>
<td>Vein - root</td>
<td>100</td>
<td>86</td>
<td>96</td>
<td>44</td>
<td>0</td>
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<td>Primary leaf vein</td>
<td>0</td>
<td>8</td>
<td>14</td>
<td>24</td>
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<td>60</td>
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<td>50</td>
<td>34</td>
<td>68</td>
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<td>84</td>
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<td>20</td>
<td>0</td>
<td>26</td>
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<td>38</td>
</tr>
</tbody>
</table>

Figure 30. GUS staining of representative plants at 5 DPG.
Table 12. Summary of GUS activity in *A. thaliana* plants at 7 DPG.

<table>
<thead>
<tr>
<th>Tissue/Organ</th>
<th>SPDS3-A</th>
<th>SPDS3-B</th>
<th>SPDS3-C</th>
<th>SPMS-A</th>
<th>SPMS-B</th>
<th>SPMS-C</th>
</tr>
</thead>
<tbody>
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<td>Apex</td>
<td>82</td>
<td>40</td>
<td>80</td>
<td>0</td>
<td>0</td>
<td>56</td>
</tr>
<tr>
<td>Cotyledon</td>
<td>38</td>
<td>8</td>
<td>82</td>
<td>50</td>
<td>36</td>
<td>84</td>
</tr>
<tr>
<td>Hypocotyl - upper</td>
<td>0</td>
<td>0</td>
<td>20</td>
<td>0</td>
<td>0</td>
<td>18</td>
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<tr>
<td>Hypocotyl - middle</td>
<td>18</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>Hypocotyl - lower</td>
<td>60</td>
<td>24</td>
<td>80</td>
<td>0</td>
<td>0</td>
<td>64</td>
</tr>
<tr>
<td>Root tissue</td>
<td>78</td>
<td>20</td>
<td>80</td>
<td>0</td>
<td>0</td>
<td>16</td>
</tr>
<tr>
<td>Root tip</td>
<td>98</td>
<td>72</td>
<td>86</td>
<td>0</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>Vein - cotyledon</td>
<td>90</td>
<td>66</td>
<td>88</td>
<td>30</td>
<td>22</td>
<td>90</td>
</tr>
<tr>
<td>Vein - hypocotyl</td>
<td>88</td>
<td>32</td>
<td>68</td>
<td>0</td>
<td>2</td>
<td>74</td>
</tr>
<tr>
<td>Vein - root</td>
<td>100</td>
<td>68</td>
<td>80</td>
<td>38</td>
<td>8</td>
<td>60</td>
</tr>
<tr>
<td>Root hair</td>
<td>92</td>
<td>48</td>
<td>78</td>
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<td>Primary leaf vein</td>
<td>70</td>
<td>82</td>
<td>50</td>
<td>58</td>
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<td>60</td>
</tr>
<tr>
<td>Primary leaf tip</td>
<td>40</td>
<td>74</td>
<td>68</td>
<td>42</td>
<td>44</td>
<td>72</td>
</tr>
<tr>
<td>Primary leaf tissue</td>
<td>0</td>
<td>18</td>
<td>34</td>
<td>20</td>
<td>0</td>
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<tr>
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<td>68</td>
<td>54</td>
<td>40</td>
<td>24</td>
<td>8</td>
<td>46</td>
</tr>
</tbody>
</table>

Figure 31. GUS staining of representative plants at 7 DPG.
A) SPDS3-A  B) SPDS3-B  C) SPDS3-C  D) SPMS-A  E) SPMS-B  F) SPMS-C
Table 13. Summary of GUS activity in *A. thaliana* plants at 9 DPG.

<table>
<thead>
<tr>
<th>Tissue/Organ</th>
<th>SPDS3-A</th>
<th>SPDS3-B</th>
<th>SPDS3-C</th>
<th>SPMS-A</th>
<th>SPMS-B</th>
<th>SPMS-C</th>
</tr>
</thead>
<tbody>
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<td>Apex</td>
<td>92</td>
<td>20</td>
<td>62</td>
<td>18</td>
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<td>72</td>
</tr>
<tr>
<td>Cotyledon</td>
<td>8</td>
<td>44</td>
<td>96</td>
<td>42</td>
<td>50</td>
<td>88</td>
</tr>
<tr>
<td>Hypocotyl - upper</td>
<td>36</td>
<td>10</td>
<td>44</td>
<td>0</td>
<td>8</td>
<td>8</td>
</tr>
<tr>
<td>Hypocotyl - middle</td>
<td>0</td>
<td>6</td>
<td>22</td>
<td>0</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>Hypocotyl - lower</td>
<td>80</td>
<td>44</td>
<td>82</td>
<td>0</td>
<td>0</td>
<td>76</td>
</tr>
<tr>
<td>Root tissue</td>
<td>86</td>
<td>38</td>
<td>78</td>
<td>0</td>
<td>0</td>
<td>26</td>
</tr>
<tr>
<td>Root tip</td>
<td>80</td>
<td>68</td>
<td>92</td>
<td>0</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>Vein - cotyledon</td>
<td>92</td>
<td>80</td>
<td>94</td>
<td>44</td>
<td>52</td>
<td>74</td>
</tr>
<tr>
<td>Vein - hypocotyl</td>
<td>64</td>
<td>32</td>
<td>48</td>
<td>0</td>
<td>0</td>
<td>64</td>
</tr>
<tr>
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<td>100</td>
<td>80</td>
<td>80</td>
<td>50</td>
<td>12</td>
<td>58</td>
</tr>
<tr>
<td>Root hair</td>
<td>52</td>
<td>56</td>
<td>70</td>
<td>0</td>
<td>0</td>
<td>0</td>
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<td>Primary leaf vein</td>
<td>56</td>
<td>74</td>
<td>64</td>
<td>56</td>
<td>26</td>
<td>80</td>
</tr>
<tr>
<td>Primary leaf tip</td>
<td>34</td>
<td>36</td>
<td>28</td>
<td>36</td>
<td>38</td>
<td>52</td>
</tr>
<tr>
<td>Primary leaf tissue</td>
<td>0</td>
<td>0</td>
<td>20</td>
<td>48</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>Secondary root</td>
<td>88</td>
<td>56</td>
<td>48</td>
<td>20</td>
<td>0</td>
<td>64</td>
</tr>
</tbody>
</table>

Figure 32. GUS staining of representative plants at 9 DPG.
A) *SPDS3-A*  B) *SPDS3-B*  C) *SPDS3-C*  D) *SPDS3-C* root tissue  E) *SPMS-A*
F) *SPMS-B*  G) *SPMS-C*  H) *SPMS-C* root tissue

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Table 14. Summary of GUS activity in *A. thaliana* plants at 11 DPG.

<table>
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<tr>
<th>Tissue/Organ</th>
<th>SPDS3-A</th>
<th>SPDS3-B</th>
<th>SPDS3-C</th>
<th>SPMS-A</th>
<th>SPMS-B</th>
<th>SPMS-C</th>
</tr>
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<td>0</td>
<td>0</td>
<td>62</td>
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<tr>
<td>Cotyledon</td>
<td>20</td>
<td>0</td>
<td>88</td>
<td>30</td>
<td>40</td>
<td>66</td>
</tr>
<tr>
<td>Hypocotyl - upper</td>
<td>0</td>
<td>14</td>
<td>22</td>
<td>0</td>
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</tr>
<tr>
<td>Hypocotyl - middle</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>Hypocotyl - lower</td>
<td>88</td>
<td>36</td>
<td>80</td>
<td>0</td>
<td>0</td>
<td>70</td>
</tr>
<tr>
<td>Root tissue</td>
<td>82</td>
<td>36</td>
<td>42</td>
<td>0</td>
<td>0</td>
<td>8</td>
</tr>
<tr>
<td>Root tip</td>
<td>92</td>
<td>78</td>
<td>62</td>
<td>0</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>Vein - cotyledon</td>
<td>90</td>
<td>72</td>
<td>90</td>
<td>0</td>
<td>30</td>
<td>76</td>
</tr>
<tr>
<td>Vein - hypocotyl</td>
<td>56</td>
<td>0</td>
<td>44</td>
<td>0</td>
<td>0</td>
<td>40</td>
</tr>
<tr>
<td>Vein - root</td>
<td>90</td>
<td>52</td>
<td>80</td>
<td>26</td>
<td>0</td>
<td>66</td>
</tr>
<tr>
<td>Root hair</td>
<td>64</td>
<td>52</td>
<td>52</td>
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<td>0</td>
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<tr>
<td>Primary leaf vein</td>
<td>72</td>
<td>52</td>
<td>54</td>
<td>0</td>
<td>8</td>
<td>72</td>
</tr>
<tr>
<td>Primary leaf tip</td>
<td>24</td>
<td>46</td>
<td>0</td>
<td>50</td>
<td>22</td>
<td>54</td>
</tr>
<tr>
<td>Primary leaf tissue</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>10</td>
<td>2</td>
<td>8</td>
</tr>
<tr>
<td>Secondary root</td>
<td>68</td>
<td>16</td>
<td>50</td>
<td>0</td>
<td>0</td>
<td>30</td>
</tr>
<tr>
<td>Secondary leaf tissue</td>
<td>24</td>
<td>0</td>
<td>34</td>
<td>54</td>
<td>0</td>
<td>72</td>
</tr>
</tbody>
</table>

Figure 33. GUS staining of representative plants at 11 DPG.

Reproduced with permission of the copyright owner. Further reproduction prohibited without permission.
Table 15. Summary of GUS activity in *A. thaliana* plants at 13 DPG.

<table>
<thead>
<tr>
<th>Tissue/Organ</th>
<th><em>SPDS3</em>-A</th>
<th><em>SPDS3</em>-B</th>
<th><em>SPDS3</em>-C</th>
<th><em>SPMS</em>-A</th>
<th><em>SPMS</em>-B</th>
<th><em>SPMS</em>-C</th>
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<td>14</td>
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<td>0</td>
<td>0</td>
<td>66</td>
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<tr>
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<td>0</td>
<td>82</td>
<td>18</td>
<td>28</td>
<td>78</td>
</tr>
<tr>
<td>Hypocotyl - upper</td>
<td>0</td>
<td>28</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>16</td>
</tr>
<tr>
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<td>0</td>
<td>0</td>
<td>12</td>
<td>0</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>Hypocotyl - lower</td>
<td>92</td>
<td>20</td>
<td>66</td>
<td>0</td>
<td>0</td>
<td>68</td>
</tr>
<tr>
<td>Root tissue</td>
<td>64</td>
<td>30</td>
<td>68</td>
<td>0</td>
<td>0</td>
<td>38</td>
</tr>
<tr>
<td>Root tip</td>
<td>70</td>
<td>70</td>
<td>56</td>
<td>0</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>Vein - cotyledon</td>
<td>100</td>
<td>78</td>
<td>94</td>
<td>0</td>
<td>0</td>
<td>74</td>
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<td>4</td>
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<tr>
<td>Vein - root</td>
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<td>70</td>
<td>74</td>
<td>18</td>
<td>0</td>
<td>52</td>
</tr>
<tr>
<td>Root hair</td>
<td>76</td>
<td>26</td>
<td>74</td>
<td>0</td>
<td>0</td>
<td>0</td>
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<tr>
<td>Primary leaf vein</td>
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<td>48</td>
<td>52</td>
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<td>24</td>
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<td>32</td>
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<td>20</td>
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<td>0</td>
<td>0</td>
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<tr>
<td>Secondary root</td>
<td>76</td>
<td>18</td>
<td>48</td>
<td>0</td>
<td>0</td>
<td>0</td>
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<tr>
<td>Secondary leaf tissue</td>
<td>0</td>
<td>6</td>
<td>12</td>
<td>14</td>
<td>0</td>
<td>38</td>
</tr>
</tbody>
</table>

Figure 34. GUS staining of representative plants at 13 DPG.
A) *SPDS3*-A  B) *SPDS3*-B  C) *SPDS3*-C  D) *SPMS*-A  E) *SPMS*-B  F) *SPMS*-C
Table 16. Summary of GUS activity in *A. thaliana* plants at 15 DPG.

<table>
<thead>
<tr>
<th>Tissue/Organ</th>
<th>SPDS3-A</th>
<th>SPDS3-B</th>
<th>SPDS3-C</th>
<th>SPMS-A</th>
<th>SPMS-B</th>
<th>SPMS-C</th>
</tr>
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<tbody>
<tr>
<td>Apex</td>
<td>100</td>
<td>30</td>
<td>80</td>
<td>0</td>
<td>0</td>
<td>54</td>
</tr>
<tr>
<td>Cotyledon</td>
<td>36</td>
<td>0</td>
<td>100</td>
<td>8</td>
<td>44</td>
<td>50</td>
</tr>
<tr>
<td>Hypocotyl - upper</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>24</td>
</tr>
<tr>
<td>Hypocotyl - middle</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>2</td>
</tr>
<tr>
<td>Hypocotyl - lower</td>
<td>100</td>
<td>20</td>
<td>68</td>
<td>0</td>
<td>0</td>
<td>66</td>
</tr>
<tr>
<td>Root tissue</td>
<td>68</td>
<td>32</td>
<td>68</td>
<td>0</td>
<td>0</td>
<td>34</td>
</tr>
<tr>
<td>Root tip</td>
<td>88</td>
<td>58</td>
<td>90</td>
<td>0</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>Vein - cotyledon</td>
<td>100</td>
<td>70</td>
<td>80</td>
<td>0</td>
<td>28</td>
<td>56</td>
</tr>
<tr>
<td>Vein - hypocotyl</td>
<td>28</td>
<td>0</td>
<td>2</td>
<td>0</td>
<td>0</td>
<td>24</td>
</tr>
<tr>
<td>Vein - root</td>
<td>84</td>
<td>52</td>
<td>78</td>
<td>10</td>
<td>0</td>
<td>70</td>
</tr>
<tr>
<td>Root hair</td>
<td>76</td>
<td>32</td>
<td>90</td>
<td>0</td>
<td>0</td>
<td>10</td>
</tr>
<tr>
<td>Primary leaf vein</td>
<td>38</td>
<td>42</td>
<td>48</td>
<td>0</td>
<td>24</td>
<td>54</td>
</tr>
<tr>
<td>Primary leaf apex</td>
<td>0</td>
<td>22</td>
<td>0</td>
<td>0</td>
<td>2</td>
<td>34</td>
</tr>
<tr>
<td>Primary leaf tissue</td>
<td>0</td>
<td>2</td>
<td>40</td>
<td>0</td>
<td>0</td>
<td>2</td>
</tr>
<tr>
<td>Secondary root</td>
<td>74</td>
<td>20</td>
<td>66</td>
<td>0</td>
<td>0</td>
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</tr>
<tr>
<td>Secondary leaf vein</td>
<td>30</td>
<td>28</td>
<td>20</td>
<td>10</td>
<td>0</td>
<td>26</td>
</tr>
<tr>
<td>Secondary leaf apex</td>
<td>0</td>
<td>12</td>
<td>0</td>
<td>10</td>
<td>14</td>
<td>50</td>
</tr>
<tr>
<td>Secondary leaf tissue</td>
<td>0</td>
<td>16</td>
<td>12</td>
<td>6</td>
<td>0</td>
<td>46</td>
</tr>
</tbody>
</table>

Figure 35. GUS staining of representative plants at 15 DPG.
A) SPDS3-A  B) SPDS3-B  C) SPDS3-C  D) SPDS3-C root tissue  E) SPMS-A  F) SPMS-B  G) SPMS-C  H) SPMS-C root tissue
<table>
<thead>
<tr>
<th></th>
<th>SPDS3-A</th>
<th>SPDS3-B</th>
<th>SPDS3-C</th>
<th>SPMS-A</th>
<th>SPMS-B</th>
<th>SPMS-C</th>
</tr>
</thead>
<tbody>
<tr>
<td>0 DPG</td>
<td><img src="0_DPG.jpg" alt="Image" /></td>
<td><img src="0_DPG.jpg" alt="Image" /></td>
<td><img src="0_DPG.jpg" alt="Image" /></td>
<td><img src="0_DPG.jpg" alt="Image" /></td>
<td><img src="0_DPG.jpg" alt="Image" /></td>
<td><img src="0_DPG.jpg" alt="Image" /></td>
</tr>
<tr>
<td>1 DPG</td>
<td><img src="1_DPG.jpg" alt="Image" /></td>
<td><img src="1_DPG.jpg" alt="Image" /></td>
<td><img src="1_DPG.jpg" alt="Image" /></td>
<td><img src="1_DPG.jpg" alt="Image" /></td>
<td><img src="1_DPG.jpg" alt="Image" /></td>
<td><img src="1_DPG.jpg" alt="Image" /></td>
</tr>
<tr>
<td>5 DPG</td>
<td><img src="5_DPG.jpg" alt="Image" /></td>
<td><img src="5_DPG.jpg" alt="Image" /></td>
<td><img src="5_DPG.jpg" alt="Image" /></td>
<td><img src="5_DPG.jpg" alt="Image" /></td>
<td><img src="5_DPG.jpg" alt="Image" /></td>
<td><img src="5_DPG.jpg" alt="Image" /></td>
</tr>
<tr>
<td>9 DPG</td>
<td><img src="9_DPG.jpg" alt="Image" /></td>
<td><img src="9_DPG.jpg" alt="Image" /></td>
<td><img src="9_DPG.jpg" alt="Image" /></td>
<td><img src="9_DPG.jpg" alt="Image" /></td>
<td><img src="9_DPG.jpg" alt="Image" /></td>
<td><img src="9_DPG.jpg" alt="Image" /></td>
</tr>
<tr>
<td>15 DPG</td>
<td><img src="15_DPG.jpg" alt="Image" /></td>
<td><img src="15_DPG.jpg" alt="Image" /></td>
<td><img src="15_DPG.jpg" alt="Image" /></td>
<td><img src="15_DPG.jpg" alt="Image" /></td>
<td><img src="15_DPG.jpg" alt="Image" /></td>
<td><img src="15_DPG.jpg" alt="Image" /></td>
</tr>
</tbody>
</table>

Figure 36. Summary of GUS activity in SPDS3 and SPMS transformants during the early stages of development.
Following the 15 DPG, plants grown in soil were collected at 18, 21, 24, 28, 32, 38, and 45 DPG. However, staining did not vary among these time points, therefore the observations were combined to represent mature plant organs, providing a total of 75 samples per trial. In mature plants, the roots, stem, flower, rosette and cauline leaves were observed for the presence of GUS activity. The counts for each tissue were combined and tabulated as a percentage of plants with a blue color in a particular anatomical location.

The first part of the mature plants surveyed was the roots. The roots were further divided into the primary root (just below the rosette), secondary roots, vasculature, and root tip (Table 17). The majority of secondary roots were destroyed or excised as the plants were removed from the soil. However, staining appeared to be consistent among remaining intact roots as compared to the young developing plants. In SPDS3 transformants, the roots displayed a high level of GUS staining, particularly in the SPDS3-C plants which had high activity in the root tissue as well as the root vein (84% and 76%, respectively; Figure 37C). The staining trend among the three constructs continued as the SPDS3-C plants showed a higher level of staining compared to the other constructs and the SPDS3-A (Figure 37A) transformants had higher GUS activity than the SPDS3-B plants (Figure 37B). The root tips of secondary roots continued to show high GUS activity in all constructs. In the SPMS transformants, a moderate level of GUS activity was observed in the SPMS-A and SPMS-B plants (Figures 37D and E), whereas a relatively high level of activity continued in the SPMS-C plants (Figure 37F). No staining was observed in the root tips of SPMS transformants.
The rosette leaves were surveyed for GUS activity and were further subdivided into the leaf blade, petiole, central and secondary veins, leaf margin, leaf tip, leaf base, and the trichomes (Table 18). GUS activity was high in the veins of the petioles in \textit{SPDS3} transformants with sparse staining of the leaf tissue in \textit{SPDS3-B} plants (Figure 38B) when compared to \textit{SPDS3-A} (Figure 38A) which, in turn, was less intense than \textit{SPDS3-C} (Figure 38C). Staining was very low and sporadic in \textit{SPMS-A} (Figure 38D) and \textit{SPMS-B} (Figure 38E) rosette leaves with only 19\% of the leaf petioles of \textit{SPMS-A} showing GUS staining. \textit{SPMS-C} rosette leaves displayed strong GUS activity in the petioles (63\%) and the vascular tissue (Figure 38F). There was also a high level of GUS activity in the leaf margins or hydathodes, which was not seen in \textit{SPDS3} plants.

The cauline leaves were also subdivided into the same categories as the rosette leaves (Table 19) and showed similar GUS patterns. Slightly lower activity was seen in the petioles, central, and secondary veins of \textit{SPDS3} plants when compared to the rosette leaves, but localization of GUS activity was the same (Figures 39A, B, and C). There was dramatically more staining in the trichomes of rosette leaves than the cauline leaves (Table 18 and 19). The same trend was seen in \textit{SPMS} plants, as the localization was similar, but the activity decreased in all \textit{SPMS} constructs (Figures 39D, E, and F).

The stems were subdivided into the primary (stem ascending directly from rosette) and secondary (branches of primary stem) stems. Each category was further subdivided into the upper, middle, and lower regions. The rosette, cauline, and primary stem junctions (junction between secondary and primary stems) were observed, as well as, the stipules and trichomes (Table 20). The only observed GUS activity in the stems was at the rosette junction for all \textit{SPDS3} and \textit{SPMS} transformants (Figures 40A-F) and
the cauline leaf and primary stem junctions of the SPDS3 and SPMS-C plants. The GUS activity in the SPMS-A and SPMS-B plants was weak compared to the SPDS3 and SPMS-C transformants.

Flowers were individually observed for GUS activity in the pedicel, receptacle, petals, stamens, pistils, stigma, and the veins of the petals, pedicel, and pistils (Table 21). GUS activity was particularly high in the receptacle, anthers, and pollen grains of SPDS3 flowers (Figures 41A, B, C, and G). Moderate activity was observed in the veins of petals. No significant staining was observed in the pistil at any stage. The only significant staining in SPMS flowers (Figures 40D, E, and F) was the stigma and anther septum of SPMS-C (Figures 41F and H). No staining was observed in the pollen grains. GUS activity was seen in fertilized and unfertilized stigmas for SPMS-C transformants. SPMS-A and SPMS-B pollen and anther tissues were distinguished from SPDS3-C in showing a complete lack of GUS staining.

The final organ that was evaluated for GUS activity was the siliques. This organ was subdivided into the upper, middle, and lower valve tissue and septum, valve tip, valve base, the upper, middle and lower septum, the pedicel, and seeds (Table 22). The only noticeable staining in the siliques was observed in the valve base of SPDS3 transformants (Figures 42A, B, and C) and SPMS-C (Figure 42F). No staining was observed in the siliques of SPMS-A and SPMS-B (Figures 42D and E) except very low counts in the base (9.3%) and tip (4%) of SPMS-B. High levels of GUS activity was seen in the silique tip of SPMS-C (Figure 42H), but not in SPDS3 transformants (Figure 42G).
Table 17. Summary of GUS activity in roots of mature plants.

<table>
<thead>
<tr>
<th>Tissue</th>
<th>SPDS3-A</th>
<th>SPDS3-B</th>
<th>SPDS3-C</th>
<th>SPMS-A</th>
<th>SPMS-B</th>
<th>SPMS-C</th>
</tr>
</thead>
<tbody>
<tr>
<td>Primary Tap Root</td>
<td>84</td>
<td>36</td>
<td>84</td>
<td>32</td>
<td>51</td>
<td>52</td>
</tr>
<tr>
<td>Secondary Roots</td>
<td>81</td>
<td>60</td>
<td>84</td>
<td>40</td>
<td>17</td>
<td>73</td>
</tr>
<tr>
<td>Vein</td>
<td>84</td>
<td>76</td>
<td>76</td>
<td>33</td>
<td>27</td>
<td>68</td>
</tr>
<tr>
<td>Root Tips</td>
<td>95</td>
<td>60</td>
<td>73</td>
<td>0</td>
<td>0</td>
<td>0</td>
</tr>
</tbody>
</table>

Figure 37. GUS staining of mature roots.
A) SPDS3-A  B) SPDS3-B  C) SPDS3-C  D) SPMS-A  E) SPMS-B  F) SPMS-C

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Table 18. Summary of GUS activity in rosette leaves of mature plants.

<table>
<thead>
<tr>
<th>Tissue</th>
<th>SPDS3-A</th>
<th>SPDS3-B</th>
<th>SPDS3-C</th>
<th>SPMS-A</th>
<th>SPMS-B</th>
<th>SPMS-C</th>
</tr>
</thead>
<tbody>
<tr>
<td>Leaf blade</td>
<td>0</td>
<td>0</td>
<td>44</td>
<td>0</td>
<td>0</td>
<td>4</td>
</tr>
<tr>
<td>Petiole</td>
<td>91</td>
<td>69</td>
<td>84</td>
<td>19</td>
<td>2.7</td>
<td>63</td>
</tr>
<tr>
<td>Vein – central</td>
<td>77</td>
<td>44</td>
<td>67</td>
<td>9.3</td>
<td>1.3</td>
<td>60</td>
</tr>
<tr>
<td>Vein – secondary</td>
<td>59</td>
<td>59</td>
<td>79</td>
<td>4</td>
<td>0</td>
<td>49</td>
</tr>
<tr>
<td>Leaf margin</td>
<td>0</td>
<td>5.3</td>
<td>20</td>
<td>4</td>
<td>8</td>
<td>67</td>
</tr>
<tr>
<td>Leaf tip</td>
<td>0</td>
<td>0</td>
<td>1.3</td>
<td>0</td>
<td>0</td>
<td>2.7</td>
</tr>
<tr>
<td>Leaf base</td>
<td>0</td>
<td>0</td>
<td>1.3</td>
<td>0</td>
<td>0</td>
<td>2.7</td>
</tr>
<tr>
<td>Trichome</td>
<td>33</td>
<td>29</td>
<td>31</td>
<td>2.7</td>
<td>13</td>
<td>8</td>
</tr>
</tbody>
</table>

Figure 38. GUS staining of mature rosette leaves.
A) SPDS3-A  B) SPDS3-B  C) SPDS3-C  D) SPMS-A  E) SPMS-B  F) SPMS-C
Table 19. Summary of GUS activity in cauline leaves of mature plants.

<table>
<thead>
<tr>
<th>Tissue</th>
<th>SPDS3-A</th>
<th>SPDS3-B</th>
<th>SPDS3-C</th>
<th>SPMS-A</th>
<th>SPMS-B</th>
<th>SPMS-C</th>
</tr>
</thead>
<tbody>
<tr>
<td>Leaf blade</td>
<td>5.3</td>
<td>1.3</td>
<td>55</td>
<td>1.3</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>Petiole</td>
<td>60</td>
<td>33</td>
<td>52</td>
<td>8</td>
<td>0</td>
<td>77</td>
</tr>
<tr>
<td>Vein – central</td>
<td>73</td>
<td>36</td>
<td>51</td>
<td>8</td>
<td>0</td>
<td>59</td>
</tr>
<tr>
<td>Vein – secondary</td>
<td>63</td>
<td>43</td>
<td>53</td>
<td>5.3</td>
<td>0</td>
<td>36</td>
</tr>
<tr>
<td>Leaf margin</td>
<td>1.3</td>
<td>1.3</td>
<td>19</td>
<td>0</td>
<td>5.3</td>
<td>36</td>
</tr>
<tr>
<td>Leaf tip</td>
<td>20</td>
<td>0</td>
<td>9.3</td>
<td>4</td>
<td>5.3</td>
<td>2.7</td>
</tr>
<tr>
<td>Leaf base</td>
<td>17</td>
<td>0</td>
<td>13</td>
<td>0</td>
<td>0</td>
<td>24</td>
</tr>
<tr>
<td>Trichome</td>
<td>17</td>
<td>16</td>
<td>19</td>
<td>8</td>
<td>6.7</td>
<td>9.3</td>
</tr>
</tbody>
</table>

Figure 39. GUS staining of mature cauline leaves.
A) SPDS3-A  B) SPDS3-B  C) SPDS3-C  D) SPMS-A  E) SPMS-B  F) SPMS-C

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Table 20. Summary of GUS activity in the stems of mature plants

<table>
<thead>
<tr>
<th>Tissue</th>
<th>SPDS3-A</th>
<th>SPDS3-B</th>
<th>SPDS3-C</th>
<th>SPMS-A</th>
<th>SPMS-B</th>
<th>SPMS-C</th>
</tr>
</thead>
<tbody>
<tr>
<td>1° stem - upper</td>
<td>0</td>
<td>0</td>
<td>1.3</td>
<td>0</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>1° stem - middle</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>1° stem - lower</td>
<td>0</td>
<td>1.3</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>Rosette junction</td>
<td>95</td>
<td>76</td>
<td>85</td>
<td>32</td>
<td>63</td>
<td>80</td>
</tr>
<tr>
<td>2° stem - upper</td>
<td>4</td>
<td>0</td>
<td>6.7</td>
<td>2.7</td>
<td>0</td>
<td>2.7</td>
</tr>
<tr>
<td>2° stem - middle</td>
<td>2.7</td>
<td>0</td>
<td>1.3</td>
<td>4</td>
<td>0</td>
<td>2.7</td>
</tr>
<tr>
<td>2° stem - lower</td>
<td>2.7</td>
<td>2.7</td>
<td>5.3</td>
<td>0</td>
<td>0</td>
<td>8</td>
</tr>
<tr>
<td>1° stem junction</td>
<td>51</td>
<td>11</td>
<td>40</td>
<td>16</td>
<td>4</td>
<td>43</td>
</tr>
<tr>
<td>Cauline leaf junction</td>
<td>49</td>
<td>48</td>
<td>35</td>
<td>15</td>
<td>0</td>
<td>48</td>
</tr>
<tr>
<td>Stipule</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>1.3</td>
</tr>
<tr>
<td>Trichome</td>
<td>4</td>
<td>11</td>
<td>4</td>
<td>2.7</td>
<td>4</td>
<td>6.7</td>
</tr>
</tbody>
</table>

Figure 40. GUS staining of the rosette junction.
A) SPDS3-A B) SPDS3-B C) SPDS3-C D) SPMS-A E) SPMS-B F) SPMS-C
Table 21. Summary of GUS activity in flowers of mature plants.

<table>
<thead>
<tr>
<th>Tissue</th>
<th>SPDS3-A</th>
<th>SPDS3-B</th>
<th>SPDS3-C</th>
<th>SPMS-A</th>
<th>SPMS-B</th>
<th>SPMS-C</th>
</tr>
</thead>
<tbody>
<tr>
<td>Pedicel</td>
<td>5.3</td>
<td>2.7</td>
<td>2.7</td>
<td>2.7</td>
<td>0</td>
<td>6.7</td>
</tr>
<tr>
<td>Receptacle</td>
<td>76</td>
<td>43</td>
<td>61</td>
<td>0</td>
<td>16</td>
<td>17</td>
</tr>
<tr>
<td>Petal</td>
<td>0</td>
<td>13</td>
<td>21</td>
<td>0</td>
<td>0</td>
<td>4</td>
</tr>
<tr>
<td>Anther</td>
<td>57</td>
<td>69</td>
<td>89</td>
<td>17</td>
<td>6.7</td>
<td>20</td>
</tr>
<tr>
<td>Filament</td>
<td>11</td>
<td>29</td>
<td>33</td>
<td>0</td>
<td>0</td>
<td>13</td>
</tr>
<tr>
<td>Pollen grains</td>
<td>97</td>
<td>85</td>
<td>89</td>
<td>21</td>
<td>15</td>
<td>17</td>
</tr>
<tr>
<td>Style</td>
<td>0</td>
<td>0</td>
<td>2.7</td>
<td>0</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>Stigma</td>
<td>2.7</td>
<td>2.7</td>
<td>25</td>
<td>0</td>
<td>0</td>
<td>49</td>
</tr>
<tr>
<td>Vein - petal</td>
<td>52</td>
<td>29</td>
<td>49</td>
<td>4</td>
<td>0</td>
<td>36</td>
</tr>
<tr>
<td>Vein - pedicel</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>Vein - pistil</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
</tr>
</tbody>
</table>

Figure 41. GUS staining of mature flowers.
A) SPDS3-A  B) SPDS3-B  C) SPDS3-C  D) SPMS-A  E) SPMS-B  F) SPMS-C
G) SPDS3-C stigma and pollen grains  H) SPMS-C anther and stigma

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Table 22. Summary of GUS activity in mature siliques.

<table>
<thead>
<tr>
<th>Tissue</th>
<th>SPDS3-A</th>
<th>SPDS3-B</th>
<th>SPDS3-C</th>
<th>SPMS-A</th>
<th>SPMS-B</th>
<th>SPMS-C</th>
</tr>
</thead>
<tbody>
<tr>
<td>Seeds</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>Valve - tip</td>
<td>2.7</td>
<td>0</td>
<td>6.7</td>
<td>0</td>
<td>4</td>
<td>89</td>
</tr>
<tr>
<td>Valve - upper</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>Valve - middle</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>Valve - lower</td>
<td>6.7</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>Valve - base</td>
<td>95</td>
<td>76</td>
<td>89</td>
<td>0</td>
<td>9.3</td>
<td>48</td>
</tr>
<tr>
<td>Septum - upper</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>Septum - middle</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>1.3</td>
</tr>
<tr>
<td>Septum - lower</td>
<td>0</td>
<td>5.3</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>1.3</td>
</tr>
<tr>
<td>Pedicel</td>
<td>9.3</td>
<td>0</td>
<td>8</td>
<td>0</td>
<td>0</td>
<td>1.3</td>
</tr>
</tbody>
</table>

Figure 42. GUS staining of mature siliques.
A) SPDS3-A  B) SPDS3-B  C) SPDS3-C  D) SPMS-A  E) SPMS-B  F) SPMS-C  
G) SPDS3 silique valve tip  H) SPMS-C silique valve tip
RT-PCR Screen of *SPDS3, SPMS, and GUS* Transcripts

To further confirm the organ-specific expression profiles of *SPDS3* and *SPMS*, RT-PCR was performed to correlate the presence or absence of *SPDS3* or *SPMS* transcripts with the *GUS* transcripts. Specific RT-PCR primers were designed such that the different paralogs of SPDS could be distinguished from each other; whereas only one set of primers were used for SPMS and GUS (Table 4). Primers were tested for specificity using full length cDNA clones of the *SPDS1* (ABRC stock# 104K18), *SPDS2* (220B5), *SPDS3* (202J6), and *SPMS* (147N21) in the pZL1 plasmid. Primer sets for each transcript were also tested against all other cDNAs listed above. Each set was positive for its respective sequence and negative when used with the other cDNA templates, which confirmed specificity for the corresponding transcripts (Figure 43). *GUS* primers were screened using the pCAMBIA 2381 vector and against all the cDNA clones mentioned above.

For testing of transcripts of different genes, cDNA was prepared from RNA isolated from 10 DPG whole seedlings, 20 DPG and 35 DPG rosette leaves, 35 DPG roots, and 35 DPG stems. RNA from two separate lines for each construct was isolated and screened using the *SPDS3, SPMS, and GUS* primers. The *SPDS3* transformants were screened for the *SPDS3* and *GUS* transcripts (Figure 44) and the *SPMS* transformants screened for the *SPMS* and *GUS* transcripts (Figure 45). *SPDS3* and *GUS* transcripts were detected in 10 DPG whole seedlings, 20 and 35 DPG rosette leaves for all constructs where GUS activity was observed. Similarly, staining in *SPMS-C* 10 DPG seedlings, 20 and 35 DPG rosette leaves was seen and corresponded well with the presence of both transcripts. A small amount of staining was observed in *SPMS-A* and
SPMS-B 10 DPG whole seedlings, which correlates with the presence of the SPMS and GUS transcripts. No staining was observed in the SPMS-A and SPMS-B 20 and 35 DPG rosette leaves or the SPDS3 and SPMS 35 DPG stems. However, RT-PCR revealed the presence of both the native and transgene transcripts in these tissues. A small amount of transcript was detected in the stem tissue where no GUS staining was observed for both SPDS3 and SPMS, except at the rosette and cauline leaf junctions. The results of the RT-PCR are summarized in Table 23 and 24.
Figure 43. RT-PCR primer specificity.

RT-PCR primers were screened with cDNA clones to confirm specificity. As described in the above table, all primers were positive for the corresponding cDNA sequences and negative when screened against the other cDNA clones. For example, the SPDS1 cDNA clone amplified with the SPDS1 primers (lane 1, 2) was positive by producing a 106 bp fragment, the water control was negative (lane 3), and the SPDS1 primers used to amplify the SPDS2 (lane 4), SPDS3 (lane 5), and SPMS (lane 6) cDNA clones were also negative. SPDS2 was positive at 101 bp, SPDS3 at 86 bp, and SPMS at 92 bp. The GUS primers (lane 25, 26) were screened against the pCAMBIA 2391 plasmid to give a positive band at 107 bp. Gels were analyzed with the NEB Low Molecular Weight DNA Ladder.
Figure 44. Results of SPDS3 RT-PCR screening of transformed A. thaliana T3 plants. *Arabidopsis* cDNA was prepared from two separate lines for each SPDS3 construct and screened for native SPDS3 and the transgene GUS transcripts. Transcripts were isolated and screened in A) 10 DPG whole seedlings, B) 20 DPG rosette leaves, C) 35 DPG rosette leaves, and D) 35 DPG primary stem. The 86 bp SPDS3 PCR product (lanes 1, 5, 9, 13, 17, 21) and the 107 bp GUS product (lanes 3, 7, 11, 15, 19, 23) was detected in all organs where GUS staining was observed. No staining was observed in stems, however, a small amount of transcript was detected (Gel D). All SPDS3 negative controls (lanes 2, 6, 10, 14, 18, 22) and GUS negative controls (lanes 4, 8, 12, 16, 20, 24) were performed using the DNase treated RNA to detect DNA contamination. All controls were negative, showing a band below 50 bp which is likely primer dimerization. The NEB low molecular weight DNA ladder was used in all gels. A summary of the gel can be found in Table 23.
Figure 45. Results of SPMS RT-PCR screening of transformed A. thaliana T₃ plants. Arabidopsis cDNA was prepared from two separate lines for each SPMS construct and screened for native SPMS and the transgene GUS transcripts. Transcripts were isolated and screened in A) 10 DPG whole seedlings, B) 20 DPG rosette leaves, C) 35 DPG rosette leaves, and D) 35 DPG primary stem. The 92 bp SPMS PCR product (lanes 1, 5, 9, 13, 17, 21) and the 107 bp GUS product (lanes 3, 7, 11, 15, 19, 23) was detected in all organs where GUS staining was observed. No staining was observed in stems, however, a small amount of transcript was detected (Gel D). All SPMS negative controls (lanes 2, 6, 10, 14, 18, 22) and GUS negative controls (lanes 4, 8, 12, 16, 20, 24) were performed using the DNase treated RNA to detect DNA contamination. All controls were negative. The NEB low molecular weight DNA ladder was used in all gels. A summary of the gels can be found in Table 24.
Table 23. Summary of SPDS3 RT-PCR of 10 DPG whole seedlings, 20 DPG and 35 DPG rosette leaves, and 35 DPG stems. All screens, analyzed on 1.5% gels, were loaded in the same order according to “Lane #” (Figure 44). All PCR screens were positive for the presence of SPDS3 and GUS transcripts.

<table>
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Table 24. Summary of SPMS RT-PCR of 10 DPG whole seedlings, 20 DPG and 35 DPG rosette leaves, and 35 DPG stems. All screens, analyzed on 1.5% gels, were loaded in the same order according to “Lane #” (Figure 45). All PCR screens were positive for the presence of SPMS and GUS transcripts.

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Sequence Analysis of SPDS3 and SPMS Promoter Regions
Revealing Putative Motifs

Promoter sequences of SPDS3 and SPMS were analyzed for the presence of putative transcription factor binding sites using the Athena software (O’Connor et al., 2005) to determine the presence or absence of these motifs in each promoter construct used in this study. The analysis revealed a number of putative developmental motifs in each promoter sequence (Table 25). Some of these motifs in the 935 bp SPDS3 fragment used in SPDS3-A and SPDS3-C, include the CARCCW8GAT motif (-244 to -235 bp upstream of the transcription start site), T-box (-946 to -941), and the gibberellic acid response factor (GAREAT) (-188 to -182). The MYB motifs, which have been suggested to respond to development and stress signals, include MYB1AT (-236 to -231; -622 to -617), MYB2AT (-435 to -430), and MYB4 (-387 to -381; -39 to -33). Other putative stress-related motifs found in the SPDS3 promoter are the DRE core (-927 to 922); DREB1A/CFB3 (-927 to -920), low temperature response factor (LTRE) (-927 to -921), and the W-box (-462 to -457). Also, a number of TATA boxes are present in each promoter (-251 to -246; -197 to -192; -112 to -107). The 214 bp promoter region used in SPDS3-B construct contains the GAREAT motif, MYB4, and two TATA boxes (Figure 46).

The 1416 bp SPMS promoter region used in SPMS-A and SPMS-C also contains a number of putative developmental motif binding sites, such as the auxin response factor (ARF) (-16 to -11), CARCCW8GAT motif (-1326 to -1317), GAREAT (-1423 to -1417; -612 to -606), GAP-box (-703 to -696; -83 to -76), hexamer motif (-160 to -155), T-box (-1040 to -1035), and the Box II motif (-615 to -610). The SPMS promoter also contains a number of MYB motifs including the MYB binding factor (-786 to -779), MYB1AT

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(-787 to -782; -735 to -730; -616 to -611; -522 to -517; -459 to -454), and the MYB4 site (-785 to -779). Similar to SPDS3, SPMS contains additional stress-related response factors such as MYC2 BS in RD22 (-1118 to -1113), MYCATERD1 (-1118 to -1113) and the W-box (-1066 to -1061). SPMS also has three TATA boxes (-257 to -252; -253 to -248; -33 to -28). The 939 bp SPMS-B promoter contains a number of these motifs, including ARF, Box II, GAREAT, GAP-box, hexamer, MYB, MYB1AT, MYB4, and the TATA box (Figure 46).

Table 25. Summary of putative motifs found in the SPDS3 and SPMS promoter sequences. Sequences were analyzed using the Athena software and identified by the presence of the consensus sequence. The physiological response of these motifs is based on the literature.

<table>
<thead>
<tr>
<th>Promoter Motifs</th>
<th>Consensus Sequence</th>
<th>Gene</th>
<th>Physiological Response</th>
</tr>
</thead>
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<tr>
<td>ARF</td>
<td>TGTCTC</td>
<td>SPMS</td>
<td>Auxin Response Factor</td>
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<tr>
<td>Box II</td>
<td>GGTTAA</td>
<td>SPMS</td>
<td>light activation</td>
</tr>
<tr>
<td>CARCCW8GAT</td>
<td>C(A/T)^8G</td>
<td>SPDS3, SPMS</td>
<td>AGL-15 site regulating embryogenesis</td>
</tr>
<tr>
<td>DREB1A/CBF3</td>
<td>(A/G)CCGACNT</td>
<td>SPDS3</td>
<td>drought, salt, cold, wounding</td>
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<tr>
<td>DRE CORE</td>
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<td>drought, salt, cold, wounding</td>
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<td>CAAATGAA(G/A)A</td>
<td>SPMS</td>
<td>light responsive element</td>
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<td>TAACAA(A/G)</td>
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<td>flower specific motif</td>
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<td>SPDS3, SPMS</td>
<td>drought, salt, cold, wounding</td>
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<td>MYB1AT</td>
<td>(A/T)AACCA</td>
<td>SPDS3, SPMS</td>
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<td>CACATG</td>
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<td>drought and ABA regulation</td>
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<td>G-3-PDH beta subunit</td>
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<td>TATAAA</td>
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<tr>
<td>W-box</td>
<td>TTGAC</td>
<td>SPDS3, SPMS</td>
<td>Wounding response</td>
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Figure 46. Location of putative motifs in the A) SPMS and B) SPDS3 promoter constructs. Diagrams indicate the presence and/or absence of putative motifs in the promoter sequences used in this study. Sequence analysis was performed using the Athena software, which searches Arabidopsis promoter sequences for putative transcription factor binding sites (www.bioinformatics2.wsu.edu).
Expression Analysis of \textit{SPDS3} and \textit{SPMS} in Response to Abiotic Stress

After completing the developmental expression profile of \textit{SPDS3} and \textit{SPMS}, expression analysis of transgenic plants was performed for induction or suppression of GUS activity by salt (NaCl) treatment, drought, chilling, and wounding; each treatment was performed for various lengths of time. Treatments were applied to T\textsubscript{3} plants at 10, 20, and 35 DPG. A total of 25 samples (5 samples from each of the 5 lines) for each construct were collected and assayed for GUS activity as previously described. Transformants maintained under normal growth conditions were used as a control for each stress experiment and collected at each time point along with the experimental sample. Variations in GUS activity were difficult to detect in 10 DPG plants due to the relatively high levels of activity of both the \textit{SPDS3} and \textit{SPMS} transformants at this developmental time point (Figure 33). Variations in response to stress were detectable in 20 and 35 DPG plants as expression had weakened, yet there were no differences between these two stages, therefore only the rosette leaves of 35 DPG samples were recorded. No changes were observed in any other organs aside from rosette and cauline leaves. As seen in the developmental profile, the \textit{SPDS3-A} and \textit{SPDS3-B} transformants displayed similar profiles with slightly weaker GUS intensity when compared to \textit{SPDS3-C}. Similar changes in GUS activity were observed for all three \textit{SPDS3} constructs during the stress experiments, therefore only the \textit{SPDS3-C} samples were recorded. \textit{SPMS-A} and \textit{SPMS-B} continued to show little GUS activity when compared to \textit{SPMS-C}, therefore, only \textit{SPMS-C} samples were recorded.

Two separate NaCl treatments (100 mM and 200 mM) were used on 10, 20, and 35 DPG plants. Approximately 100 seedlings at 10 DPG were removed from the plates

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and placed in 50 ml flasks containing liquid germination medium with either 100 mM or 200 mM NaCl concentrations. The same solutions were applied (approximately 20 ml) directly to the GM plates and allowed to fully soak into the medium. Samples from the flasks and plates were collected at 0 (just before treatment), 6, 12, 24, and 48 h post treatment. For the 20 and 35 DPG plants, seeds were sown directly in soil and allowed to reach the desired developmental stage. At this stage, both 100 mM and 200 mM NaCl solutions were applied directly to the soil until saturation. The control group for 10 DPG plants was soaked with liquid germination and the 20 and 35 DPG soil sown plants were saturated with water. Samples were once again collected at 0, 6, 12, 24, and 48 h. It is evident that in the presence of 100 mM NaCl, both \textit{SPDS3} and \textit{SPMS} are induced over time. After 12 h of salt treatment, there is a noticeable increase in GUS staining throughout the vascular region which intensifies at 24 and 48 h (Figure 47 and 48). A similar pattern was also observed in cauline leaves (results not shown), but no other organs (including stems and flowers). Induction was undetectable in root tissue as the developmental profile had previously revealed high levels of expression. In the 200 mM NaCl treatment, \textit{SPDS3} and \textit{SPMS} expression slightly increased after 12 h, but gradually decreased at 24 and 48 h. Highest GUS staining was seen in the hydathodes and no GUS was seen outside the vascular tissue. These results imply that either the 200 mM concentration inhibits expression, or the salt levels are toxic to cells preventing all protein function.

Drought treatments were performed on 20 and 35 DPG transformants planted directly in soil. Plants were removed from water for 96 h. Samples were collected at 0, 24, 48, 72, and 96 h and stained for GUS activity. Controls were kept at the normal
watering schedule, which is every 48 h, therefore, osmotic stress should occur at 72 and 96 h. The results indicate that this is the case as the 0 (not shown), 24, and 48 h GUS activity for both promoters appears to be relatively the same in the leaves at each time point (Figure 49 and 50) and resembles the pattern observed in the developmental profile. Yet, there is a noticeable increase in activity after 72 h of removed water, which increases at 96 h. The increase in activity is much more dramatic in SPDS3-C transformants (Figure 50), but the activity remains localized in the vascular region of the rosette and cauline leaves with intense staining at the hydathodes. No changes were observed in the stem and reproductive organs (data not shown). These results indicate that SPMS, and especially SPDS3, are induced during osmotic stress.

Chilling stress was applied to 10 DPG plants sown on germination medium and 20 and 35 DPG plants sown in soil. Transformants were placed at 4°C and samples collected and stained at 0, 4, 8, 12, 24, and 48 h. Controls were kept at regular growth conditions. GUS activity gradually increased between 0 and 12 h for both SPDS3 and SPMS, but activity decreased after 24 h and beyond (Figures 51 and 52). It appears that either SPDS3 and SPMS respond only to short-term chilling or the low temperature inhibits protein synthesis after 12 h.

Response to wounding was tested with 20 and 35 DPG plants. Various organs including the primary and secondary rachis, siliques, rosette and cauline leaves, were cut and/or scraped. Organs were either cut and placed under high humidity in a plastic bag or kept intact on the plant. Wounded and unwounded organs were collected and stained at 0, 6, 12, and 24 h after injury. There were no differences in expression levels between the injured intact organs and the removed organs placed in a plastic bag. The results
indicate that SPDS3 expression is increased in response to wounding, particularly in the stem, rosette, and cauline leaves. GUS activity gradually increased over 24 h. It also appears that after 24 h, SPDS3 has a "systemic" response as GUS activity increased not only at the injured site, but also the entire organ (Figure 53). SPMS transformants did not display any response to wounding (results not shown).
Figure 47. GUS staining of SPMS-C 35 DPG rosette leaves exposed to 100 mM NaCl for A) 0 h (control for both experiments), B) 12 h, C) 24 h, and D) 48 h and 200 mM NaCl for E) 12 h, F) 24 h, and G) 48h

Figure 48. GUS staining of SPDS3-C 35 DPG rosette leaves exposed to 100 mM NaCl for A) 0 h (control for both experiments), B) 12 h, C) 24 h, and D) 48 h and 200 mM NaCl for E) 12 h, F) 24 h, and G) 48h
Figure 49. GUS staining of SPMS-C 35 DPG rosette leaves under water stress for A) 24 h, B) 48 h, C) 72 h and D) 96 h.

Figure 50. GUS staining of SPDS3-C 35 DPG rosette leaves under water stress for A) 24 h, B) 48 h, C) 72 h and D) 96 h.
Figure 51. GUS staining of SPMS-C 35 DPG rosette leaves exposed to 4°C for A) 0 h, B) 12 h, C) 24 h and D) 48 h.

Figure 52. GUS staining of SPDS3-C 35 DPG rosette leaves exposed to 4°C for A) 0 h, B) 12 h, C) 24 h and D) 48 h.
Figure 53. GUS staining of wounded *SPDS3*-C organs. The A-D) rosette leaves, E-H) cauline leaves, and I-L) stems were cut and/or scraped and stained at 0 (immediately after wound), 6, 12, and 24 h after injury.
Cloning of SPDS1 and SPDS2 Promoter Regions

Primers to amplify promoter regions for SPDS1 and SPDS2 were designed and DNA was amplified in a manner similar to that used for SPDS3. A total of six separate constructs were designed for SPDS1. The putative promoter region of SPDS1 (accession #’s NM_102230, NM_202171; BAC clone #F508) was PCR amplified from A. thaliana genomic DNA using specific primers (Table 4) designed to amplify various regions of the promoter and, in some constructs part of the SPDS1 open reading frame (Figure 6). The promoter lengths were chosen by eliminating certain 5’ regulatory motifs in order to observe differences in the expression pattern to further help define the putative promoter for this gene. The SPDS1-A construct was designed using the F1 (forward) and RIB (reverse) primers which amplified a 1922 bp fragment including 1701 bp upstream from the transcription start site (putative promoter region), the entire 123 bp 5’UTR, and 98 bp of the SPDS1 ORF. The SPDS1-B construct was amplified using the F1 (forward) and 5’UTR (reverse) primers which amplified 1701 bp of the putative promoter and the 123 bp 5’UTR to provide an 1824 bp fragment. The 1088 bp SPDS1-C fragment was amplified using the F2 (forward) and RIB (reverse) primers providing 867 bp of the promoter, the entire 123 bp 5’UTR, and 98 bp of the ORF. The 990 bp SPDS1-D construct was designed with the F2 (forward) and 5’UTR (reverse) primers which amplified 876 bp of the promoter and the 123 bp 5’UTR. The SPDS1-E construct was designed with the F3 (forward) and RIB (reverse) primers which amplified 291 bp of the promoter, the entire 123 bp 5’UTR, and 98 bp of the ORF to provide a 512 bp fragment. The final construct, SPDS1-F was amplified with the F3 (forward) and 5’UTR (reverse) primers which provided 291 bp of the promoter and the entire 123 bp 5’UTR to produce a
414 bp fragment. The primers were designed to incorporate flanking restriction sites, as the F1, F2, and F3 primers included an EcoRI site. The R1B primer incorporated a BamHI site and the 5'UTR primer a SpeI site. The PCR products were analyzed on a 1% gel by electrophoresis (Figure 54A) to confirm amplification and the confirmed products were individually cloned into the pCR2.1 TOPO TA vector (Figure 55). The selected clones were confirmed by restriction enzyme analysis and sequenced using the M13, T7 primers, and the cloning primers (Table 4) to confirm the presence and orientation of the promoter region. The results in all cases gave the expected insert sizes (Figure 54B). The DNA sequence alignments of the SPDS1 promoter regions matched perfectly with the published sequence (Figure 56). Glycerol stocks were prepared from 2 positive colonies and named according to the primer combination used for cloning: SPDS1 F1-R1B (SPDS1-A), F1-5UTR (SPDS1-B), F2-R1B (SPDS1-C), F1-5UTR (SPDS1-D), F3-R1B (SPDS1-E), and F3-5UTR (SPDS1-F).

Four different fragments of the SPDS2 promoter (accession # NM_105699; BAC clone #F1707) were designed and amplified from genomic DNA using the primers shown in Table 4. The primer combination for the SPDS2-A construct was F1 (forward) and RIB (reverse) which provided an 1150 fragment including 987 bp upstream of the transcription start site (putative promoter), the entire 61 bp 5'UTR, and 111 bp of the ORF. The 1048 bp SPDS2-B fragment was amplified with the forward F1 primer and the 5'UTR (reverse) primer, containing 987 bp of the promoter and the 61 bp 5'UTR. The primers were also designed to incorporate flanking restriction sites as the F1 primer provided a BamHI site, the R1B primer incorporated a SalI site, and the 5'UTR primer a SpeI restriction site. The SPDS2-A and SPDS2-B PCR products were analyzed on a 1%
gel (Figure 54A) and individually cloned into the pCR2.1 TOPO TA vector (Figure 57) and the sequence confirmed using the M13, T7, and cloning primers (Figure 57). Confirmed clones were stored as glycerol stocks and named according to primer combination used for cloning: \textit{SPDS2} F1-R1B (\textit{SPDS2-A}) and F1-5UTR (\textit{SPDS2-B}).

There is an internal EcoRI site within the putative \textit{SPDS2} promoter, 567 bp upstream from the transcription start site. This site was used for the \textit{SPDS2-C} construct which will contain 567 bp of the promoter, the entire 61 bp 5'UTR, and 111 bp of the ORF (cloned with the RIB reverse primer). The positive \textit{SPDS2-A} clone will be digested with EcoRI and SalI to create the appropriate fragment. The \textit{SPDS2-D} construct was also designed with the internal EcoRI site and the 5'UTR reverse primer, providing 567 bp of the promoter and the 61 bp 5'UTR. This fragment will be created by digesting the positive \textit{SPDS2-B} clone with EcoRI and SpeI. The internal cut site for the \textit{SPDS2-C} and \textit{SPDS2-D} constructs were confirmed with a restriction enzyme digest of the TOPO clones using EcoRI and SalI or SpeI, respectively (Figure 54B).
Figure 54. Gel analysis of SPDS1 and SPDS2 cloned promoter fragments. A) PCR products of SPDS1 (Lane 1-6) and SPDS2 (Lane 7-8) using the primers from Table 4. PCR products were cloned into the B) TOPO TA vector, which was digested using restriction enzymes incorporated by the primers. SPDS2-A (Lane 7) was digested with EcoRI and SauI to create SPDS2-C (Lane 9). SPDS2-B (Lane 8) was digested with EcoRI and SpeI to create SPDS2-D (Lane 10). These restriction digests were analyzed to confirm cloning and transformation. SPDS2-C and SPDS2-D bands are depicted by the asterisk (*). The NEB 2-log DNA ladder was used on all gels. All PCR water controls were negative.
Figure 55. Summary of SPDS1 TOPO maps. Various regions of the SPDS1 promoter, the entire 5'UTR with or without a portion of the ORF were PCR amplified and cloned into the TOPO vector.
Figure 56. Alignment of SPDS1 TOPO clones with the SPDS1 genomic sequence used for cloning (NM_102230; NM_202171). The entire length of the insert was sequenced using the cloning and TOPO sequencing primers from Table 4. The black underline indicates part of the TOPO sequence. The red line indicates the restriction site incorporated by the cloning primers. The blue line indicates the location of the 123 bp
Figure 57. Summary of SPDS2 TOPO maps. Various regions of the SPDS2 promoter, the entire 5'UTR with or without a portion of the ORF were PCR amplified and cloned into the TOPO vector.
Figure 58. Alignment of SPDS2 TOPO clones with the SPDS2 genomic sequence used for cloning (NM_105699). The entire length of the insert was sequenced using the cloning and TOPO sequencing primers from Table 4. The black underline indicates part of the TOPO sequence. The red line indicates the restriction site incorporated by the cloning primers. The blue line indicates the location of the 61 bp 5'UTR. SPDS2-C and SPDS2-D were not cloned into the TOPO vector, rather, were created by digesting the SPDS2-A and SPDS2-B TOPO clones with the internal EcoRI site indicated by the black box.
Summary of Plasmids Prepared

- **SPDS3-A TOPO**: TOPO vector containing the 1867 bp *SPDS3-A* PCR product (Figure 9)
- **SPDS3-B TOPO**: TOPO vector containing the 1259 bp *SPDS3-B* PCR product (Figure 9)
- **SPDS3-C TOPO**: TOPO vector containing the 1810 bp *SPDS3-C* PCR product (Figure 9)
- **SPMS-A TOPO**: TOPO vector containing the 1567 bp *SPMS-A* PCR product (Figure 14)
- **SPMS-B TOPO**: TOPO vector containing the 1090 bp *SPMS-B* PCR product (Figure 14)
- **SPMS-C TOPO**: TOPO vector containing the 1506 bp *SPMS-C* PCR product (Figure 14)
- **pCAM-SPDS3-A**: This pCAM 2381 x C binary vector contains the *SPDS3-A* PCR product in control of the GUS reporter gene. This plasmid was transformed into *A. tumefaciens* and used to transform *A. thaliana* by floral dip (Figure 11)
- **pCAM-SPDS3-B**: This pCAM 2381 x C binary vector contains the *SPDS3-B* PCR product in control of the GUS reporter gene. This plasmid was transformed into *A. tumefaciens* and used to transform *A. thaliana* by floral dip (Figure 11)
- **pCAM-SPDS3-C**: This pCAM 2381 x C binary vector contains the *SPDS3-C* PCR product in control of the GUS reporter gene. This plasmid was transformed into *A. tumefaciens* and used to transform *A. thaliana* by floral dip (Figure 11)
- **pCAM-SPMS-A**: This pCAM 2381 x A binary vector contains the *SPMS-A* PCR product in control of the GUS reporter gene. This plasmid was transformed into *A. tumefaciens* and used to transform *A. thaliana* by floral dip (Figure 16)
- **pCAM-SPMS-B**: This pCAM 2381 x A binary vector contains the *SPMS-B* PCR product in control of the GUS reporter gene. This plasmid was transformed into *A. tumefaciens* and used to transform *A. thaliana* by floral dip (Figure 16)
- **pCAM-SPMS-C**: This pCAM 2381 x A binary vector contains the *SPMS*-C PCR product in control of the GUS reporter gene. This plasmid was transformed into *A. tumefaciens* and used to transform *A. thaliana* by floral dip (Figure 16).

- **SPDS1-A TOPO**: TOPO vector containing the 1922 bp *SPDS1*-A PCR product (Figure 49).
- **SPDS1-B TOPO**: TOPO vector containing the 1824 bp *SPDS1*-B PCR product (Figure 49).
- **SPDS1-C TOPO**: TOPO vector containing the 1088 bp *SPDS1*-C PCR product (Figure 49).
- **SPDS1-D TOPO**: TOPO vector containing the 990 bp *SPDS1*-D PCR product (Figure 49).
- **SPDS1-E TOPO**: TOPO vector containing the 512 bp *SPDS1*-E PCR product (Figure 49).
- **SPDS1-F TOPO**: TOPO vector containing the 414 bp *SPDS1*-F PCR product (Figure 49).
- **SPDS2-A TOPO**: TOPO vector containing the 1159 bp *SPDS2*-A PCR product (Figure 50).
- **SPDS2-B TOPO**: TOPO vector containing the 1048 bp *SPDS2*-B PCR product (Figure 50).

**Summary of Plasmids Used**

- **pCR2.1-TOPO**: This vector was supplied with the Invitrogen TOPO TA cloning kit. It was used in the topoisomerase cloning reaction with *Taq* polymerase-based PCR products (Figure 4).

- **pCAMBIA 2381 x A**: This binary vector was used to accept the *SPMS* cloned promoter regions containing a portion of the *SPMS* ORF. This frame shift vector provided an in-frame translational fusion between the *SPMS* ORF and the *GUS* ORF. This plasmid also has the *NPTII* gene under control of the CaMV 35S promoter in its T-DNA region for plant selection (Figure 5).

- **pCAMBIA 2381 x C**: This binary vector was used to accept the *SPDS3* cloned promoter regions containing a portion of the *SPDS3* ORF. This frame shift vector provided an in-frame translational fusion between the *SPDS3* ORF and the *GUS* ORF. This
plasmid also has the \textit{NPTII} gene under control of the CaMV 35S promoter in its T-DNA region for plant selection (Figure 5).

- pCAMBIA 2381 GUS+: This binary vector was used to accept the \textit{SPDS3-C} and \textit{SPMS-C} cloned promoter fragments which did not contain any part of the native gene’s ORF. This vector contains a GUS gene with its own ATG start site. This plasmid also has the \textit{NPTII} gene under control of the CaMV 35S promoter in its T-DNA region for plant selection (Figure 5).

\textbf{Summary of Bacterial Strains Prepared}

- \textit{E. coli} TOP 10 (\textit{SPDS3-A} TOPO)
- \textit{E. coli} TOP 10 (\textit{SPDS3-B} TOPO)
- \textit{E. coli} TOP 10 (\textit{SPDS3-C} TOPO)
- \textit{E. coli} TOP 10 (\textit{SPMS-A} TOPO)
- \textit{E. coli} TOP 10 (\textit{SPMS-B} TOPO)
- \textit{E. coli} TOP 10 (\textit{SPMS-C} TOPO)
- \textit{E. coli} TOP 10 (pCAM- \textit{SPDS3-A})
- \textit{E. coli} TOP 10 (pCAM- \textit{SPDS3-B})
- \textit{E. coli} TOP 10 (pCAM- \textit{SPDS3-C})
- \textit{E. coli} TOP 10 (pCAM- \textit{SPMS-A})
- \textit{E. coli} TOP 10 (pCAM- \textit{SPMS-B})
- \textit{E. coli} TOP 10 (pCAM- \textit{SPMS-C})
- \textit{E. coli} TOP 10 (\textit{SPDS1-A} TOPO)
- \textit{E. coli} TOP 10 (\textit{SPDS1-B} TOPO)
- \textit{E. coli} TOP 10 (\textit{SPDS1-C} TOPO)
• E. coli TOP 10 (SPDS1-D TOPO)
• E. coli TOP 10 (SPDS1-E TOPO)
• E. coli TOP 10 (SPDS1-F TOPO)
• E. coli TOP 10 (SPDS1-A TOPO)
• E. coli TOP 10 (SPDS1-B TOPO)
• A. tumefaciens GV3101 (pCAM-SPDS3-A)
• A. tumefaciens GV3101 (pCAM-SPDS3-B)
• A. tumefaciens GV3101 (pCAM-SPDS3-C)
• A. tumefaciens GV3101 (pCAM-SPMS-A)
• A. tumefaciens GV3101 (pCAM-SPMS-B)
• A. tumefaciens GV3101 (pCAM-SPMS-C)

Summary of Bacterial Strains Used

• E. coli TOP 10: This E. coli strain was used for routine plasmid DNA replication, cloning, and cryopreservation.

• A. tumefaciens GV3101: This Agrobacterium strain (carrying the pCAM-SPDS3 or pCAM-SPMS plasmids) was used for transformation of A. thaliana ecotype Columbia plants.
CHAPTER 3

DISCUSSION

The GUS reporter gene system is a powerful technique for studying gene expression. The idea behind this approach is to attach or replace the coding region of a gene of interest with the GUS reporter gene whose expression can be visualized within a tissue or an organ as blue color in the presence of the GUS substrate, X-gluc. The promoter::GUS gene fusion construct is transformed into the organism and the expression of the reporter gene supposedly mimics the expression pattern of the gene of interest and can be visualized at the cellular level by staining for GUS activity (Jefferson et al., 1987; Hanfrey et al., 2002; Mantis and Tague, 2000). Because the promoter region of a gene is often poorly defined, it is customary to include approximately 1000-2000 bp upstream of the transcription start site to ensure that most of the regulatory sequences are included (Guilfoyle, 1997). This region will, however, not include distant cis-acting elements.

The reporter gene system offers two major advantages over other techniques such as microarrays, northern blots, and reverse transcription-PCR (RT-PCR). First, it provides a high degree of specificity since it uses the native promoter region; thus eliminating problems when studying gene family members with high sequence homology. Second, the expression of a gene can be studied at the cellular level in all tissues, at any developmental time point without cumbersome RNA preparations. Since the regulation of expression of any gene is also affected by its location in the chromatin and may involve distant cis-acting elements, it is prudent to confirm the expression of the GUS gene under the control of specific a promoter with actual measurement of the transcripts of the gene of interest by RT-PCR or some other equally sensitive method. In the present
study, the GUS reporter gene system was used in conjunction with RT-PCR to analyze the expression of two important genes (SPDS3 and SPMS) in the polyamine biosynthetic pathway of Arabidopsis.

As mentioned earlier, polyamines have been implicated in a number of physiological responses in plants. Understanding the regulation of expression of key polyamine biosynthetic genes could provide valuable information about the mechanism and the functions of these ubiquitous molecules. This study is focused on two of these genes, SPDS3 and SPMS, whose contribution to the biosynthesis of higher polyamines (Spd and Spm) is still controversial (Hanzawa et al., 2002; Panicot et al., 2002, Imai et al., 2004a,b). Since the exact promoter sequence is poorly defined, three separate constructs were designed for each gene. The constructs varied in having different parts of the 3' and/or 5' regions of the putative promoter, with the 5'UTR, and in some cases, a small portion of the ORF (Figure 3).

**Transient Expression of the pCAM-SPDS3 and pCAM-SPMS Plasmids**

The functionality of the pCAM-SPDS3 and pCAM-SPMS plasmids was tested using transient assays in poplar (Populus nigra x maximowiczii) cells by biolistic bombardment. The assays were performed to check if the promoter::GUS fusions were capable of producing a functional GUS protein in a cellular system. Data presented in Table 5 show that the Arabidopsis promoter::GUS fusions (in the pCAMBIA vector) were functioning properly in a plant system; all plasmids produced fairly high numbers of blue cells per plate. The lowest levels of expression were seen in pCAM-SPDS3-A,
pCAM-SPDS-B, and pCAM-SPMS-A; the other plasmids showed higher than the 35S promoter-regulated expression.

The results suggest that both the SPDS3 and SPMS promoters are rather strong promoters in plant cells. The SPMS promoter constructs showed equally high expression when compared to the 35S::GUS, whereas the SPDS3 constructs were slightly lower. It should be noted that biolistic bombardment does not always provide consistent transformation efficiency. This method is susceptible to variation as the number of plasmid-coated projectiles varies for each shot. However, the data presented here represent the average of three separate experiments, each with six separate plates that were shot. The transient expression data, at the least, provide a preliminary indication of promoter behavior, although, there are studies which claim that transient assays can be used for quantitative functional analysis of different promoters and promoter regions. For example, viral promoters have been successfully studied using transient expression in various plant cells (Schenk et al., 1999), as well as human cells (Zhang et al., 2002) to determine the strength of promoter sequences. Regulatory regions of fruit-specific promoters were studied in strawberry using a similar approach of transient assays (Agius et al., 2005). Regulatory regions of Arabidopsis promoters have been studied in protoplasts (Abel and Theologis 1994) and ethylene responsive cis-elements in tomato (Xu et al., 1996) to define the function of specific regions of the promoter.

Expression Profile of SPDS3 and SPMS in Arabidopsis During Development

A major objective of my study was to generate an expression profile of SPDS3 and SPMS during the entire life cycle of Arabidopsis thaliana. Three separate
promoter::GUS constructs were designed for each gene, each containing different parts of
the promoter along with or without a small portion of the ORF in order to help define the
functional components of the promoter region. The constructs were introduced into
Arabidopsis (ecotype Columbia) via Agrobacterium-mediated transformation and five
lines were obtained through two generations of selection.

The sampling involved 50 plants representing the 5 independently transformed
lines in order to minimize the effect of the site of integration of T-DNA, as well as
genetic penetrance. Following 15 DPG, samples were collected at various time points
and the data were combined to represent mature organs, as the expression patterns did not
vary through late development. As the data were combined, a total of 75 plants were
surveyed to represent the expression pattern in mature organs. Overall, this study
presents a comprehensive expression profile of the two genes during the entire life cycle
of Arabidopsis in all major organs and tissues. A second objective of the study was to
analyze the response of these genes to various forms of abiotic stress, namely salinity,
water deprivation, and chilling, as well as the response to wounding.

Expression of SPDS3

It is apparent from the data presented here that SPDS3 is expressed throughout the
life of Arabidopsis, particularly during early stages of development. The expression of
all three SPDS genes in Arabidopsis was reported by Hanzawa et al. (2002) in 7 day old
seedlings and various organs of mature plants, such as the rosette leaves, stem internodes,
roots, inflorescences and siliques. The transcripts of the three SPDS genes were
examined using RNA gel blot hybridization using specific probes. The authors were able
to detect all three SPDS transcripts in the whole seedlings, leaves, stem internodes, roots, inflorescences, and siliques. SPDS1 and SPDS2 transcripts were predominant in root tissue and SPDS3 expression was predominant in stem internodes, flower buds, and roots. Similar findings were reported by Urano et al. (2003) in 6-week old Arabidopsis plants using RT-PCR and northern blots. The mature organs that were analyzed included whole flowers, buds, immature and mature siliques, the upper and lower stem, roots, and cauline and rosette leaves. SPDS1 and SPDS3 were constitutively expressed in all organs, whereas SPDS2 transcripts were absent in mature siliques and upper parts of the stem.

My findings are consistent with those of the previous reports, with a major exception that widespread GUS activity was not observed in the stems of mature plants (except at the rosette and cauline leaf junctions). The defining differences in my study are that the analysis not only covers the entire life cycle of Arabidopsis, but also provides tissue specificity of SPDS3 expression. RT-PCR and northern blots do not provide information about tissue localization and, as seen in the aforementioned studies, these techniques led to the conclusion of constitutive expression of SPDS3. Through the promoter::GUS fusion technique, I was able to detect the precise location of expression of this gene. For example, whereas transcripts were detected in the stems, flowers, siliques, rosette and cauline leaves by gel blot analysis, my study has detected GUS activity only in the cauline leaves and rosette junctions rather than the stem itself, the pollen grains rather than the whole flower, the base of the siliques, and the vascular region of the leaves. This localization of SPDS3 in the vascular region is consistent with the presumed role of spermidine as a source of H$_2$O$_2$ for lignification of cell walls (Sebela et al. 2001). While corroborating the previous findings, the data in this study go beyond
any previous expression profile provided for SPDS3, in covering developmental time points as well as tissue localization. As has been suggested, if polyamines are involved in growth and development, it is important to determine where (in what cells) and when the specific genes are expressed throughout plant development.

A bioinformatics approach was taken to further correlate the expression pattern of SPDS3 by recognition of specific regulatory motifs within the promoter. Sequence analysis of the SPDS3 promoter indicates a number of putative developmental and stress-related motifs which may help elucidate the function of this biosynthetic enzyme and provide explanation for the localization of its expression (Table 25). One such cis-element found in the promoter of SPDS3 is the GAREAT (Giberellic Acid Response Element) motif. Giberellic acid has long been known to be an inducer of seed germination. This motif may be responsible for the high expression of SPDS3 during seed germination (Figure 27 and 28) and in the developing seedlings (Figure 36). The SPDS3 promoter sequence also contains a number of abscisic acid (ABA) response elements (Table 25). ABA has been shown to be a mediator in signaling plant responses to environmental stresses (Wright 1978; Hartung et al., 1988; Zeevart and Creelman, 1988), and further plays a regulatory role during development. ABA is involved in several physiological processes such as stomatal closure (Jones and Manfield 1970; Davies et al., 1981), embryogenesis (Kermode 2005), seed development (Thomas 1993), synthesis of storage proteins and lipids (Thomas 1993; Rock and Quatrano 1995), seed germination (Narasimha and Swamy 1979; Korneef et al., 1989), and leaf senescence (Zeevart and Creelman 1988). ABA has also been implicated in the control of root elongation, lateral root development, control of root/shoot ratios, and geotropism, as well

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as in water uptake and ion transport by roots (reviewed by Pritchard 1994). The SPDS3 promoter has a number of ABA response elements, e.g. the MYB motifs (MYB2AT, MYB4, and MYB1AT). This may help explain the high levels of SPDS3 expression found in roots throughout development. The intensity of expression of SPDS3 in the roots is also in line with large titers of spermidine seen in the root tissue (Imai et al., 2004a; Urano et al., 2003; Hanzawa et al., 2002; Tassoni et al., 2000). A direct up-regulation of SPDS3 transcript by exogenous application of ABA was observed by both Hanzawa et al. (2002) and Urano et al. (2003) indicating a direct interaction between the gene and this plant hormone.

The CARCCW8GAT motif, an AGL-15 (AGAMOUS-like 15) regulatory site, is also present in the promoter of SPDS3. The AGL-15 is part of a regulatory family of proteins which often accumulate in proliferating tissues and organs, particularly during zygotic and somatic embryogenesis (Perry et al., 1999; Tang and Perry, 2003). This correlates well with the SPDS3 expression observed in germinating seeds and seedlings, as well as the pattern in the developing cotyledons and root tips, which are the sites of cell proliferation. The presence of this motif may also explain the expression of SPDS3 in the pollen grains. Perry et al. (1999) detected the AGL-15 protein in pollen grains during microsporogenesis of oilseed rape (Brassica napus). Imai et al. (2004a) showed that Arabidopsis double mutants lacking the SPDS1 and SPDS2 genes resulted in defective embryos at the heart stage, indicating that SPDS has a significant role in cell proliferation during embryogenesis. It has been shown that large amounts of spermidine and spermine accumulate during the transition of G1 to the S phase during the cell cycle in plants and animals (Fuller et al., 1977).
Numerous studies have shown that spermidine is essential for the survival of *Arabidopsis* (Hanzawa et al., 2002; Panicot et al., 2002; Imai et al., 2004a), yet there is a controversy with respect to the function of SPDS3 versus SPDS1 and SPDS2. Imai et al. (2004a) created *Arabidopsis* double mutants in the *SPDS1* and *SPDS2* genes. The resulting phenotype was embryonically lethal, indicating that spermidine is essential for embryo development, and thus survival. Similar findings by Hanzawa et al. (2002) and Panicot et al. (2002) confirmed the requirement of spermidine using yeast mutants lacking SPDS activity. The cDNAs for *Arabidopsis* *SPDS1*, *SPDS2*, and *SPDS3* were cloned into yeast expression vectors and subsequently transformed into mutant yeast cells (*spe3*) lacking SPDS activity. Using *spe3* mutants, Panicot et al. (2002) reported normal growth in cells expressing *Arabidopsis* *SPDS1* and *SPDS2* indicating that either of these SPDSs can complement the native SPDS deficiency. However, transformants containing *SPDS3* displayed growth arrest, indicating that either *SPDS3* does not code for a functional SPDS enzyme or that the *Arabidopsis* SPDS3 requires certain conditions for activity not met by the yeast cells. Further investigation by Panicot et al. (2002) of SPDS3 involved the use of the *spe4* mutants lacking SPMS activity. Mutant cells transformed with *Arabidopsis* *SPDS3* complemented the lack of spermine, indicating that SPDS3 could be a functional enzyme showing SPMS activity. Similar results were seen by Hanzawa et al. (2002) using Y480 mutant yeast cells with SPDS deficiency. In cells complemented with *Arabidopsis* *SPDS1* and *SPDS2*, there were large amounts of spermidine detected; however there was a small amount of spermidine accumulated in cells with *SPDS3*. Using *E. coli* cells, feeding experiments with radiolabeled spermidine indicated that SPDS3 does indeed convert spermidine into spermine. The authors suggest
that SPDS3 may have both spermidine and spermine synthase capabilities. This may be further supported by the expression profile of SPM observed in this study as both genes showed a similar expression pattern during the life cycle of Arabidopsis, and the promoters of both genes share a fairly high homology with 43% sequence identity (Figure 59).
Figure 59. Sequence alignment of SPDS3 and SPMS promoter region. The putative promoter regions (1000 bp upstream of the transcription start site) of SPDS3 and SPMS, were aligned and sequence identity calculated using the Bioedit software.
Expression of SPMS

In contrast to SPDS, only one copy of SPMS, annotated as ACL5 (TAIR and NCBI), was identified in Arabidopsis by Hanzawa et al. (1997) as a gene required for internodal growth and maintenance of proliferating activity of inflorescence meristems. In that study, acl5 mutants displayed a severe defect that restricted cell elongation specifically in the apical meristems. This gene was later characterized to encode spermine synthase (Hanzawa et al., 2000, 2002). RNA gel blot analysis revealed that the ACL5 transcripts accumulated in the stem internodes, flower buds, and root tissue, with much lower levels in other vegetative tissues. RT-PCR analysis of transcript levels revealed expression in immature siliques, cauline leaves, and roots (Urano et al., 2003). While my results agree with the reported expression patterns in the above studies, further tissue specificity is revealed, as expression was observed in the apical and marginal regions of the cotyledons, vascular region of mature rosette and cauline leaves, fertilized and unfertilized stigmas, and the tip of developing siliques (Figures 36, 38, 39, 41, and 42). These are regions of intense cell expansion and elongation, which corresponds with the need for spermine in these areas. Clay and Nelson (2005) studied expression of the putative 2.13 Kb ACL5 promoter-driven GUS reporter gene in early development of Arabidopsis. Expression was detected throughout embryogenesis until the bent cotyledon-stage, where the expression was delimited to procambial cells; the expression continued in the primary root and during leaf development. The expression of ACL5 in procambial cells of roots was earlier seen by RNA in situ hybridization (Birnbaum et al., 2003). The GUS activity in the week-old seedlings of the Clay and Nelson (2005) study was nearly identical to that observed in SPMS-C transformants of my study.
Sequence analysis of the *SPMS* promoter also reveals a number of regulatory motifs which further support the expression pattern observed in this study. The *SPMS* promoter has a putative Auxin Response Factor (ARF), a site where the auxin regulatory transcription factor, ARF1, has been shown to bind in response to auxin treatment (Ulmason et al., 1997; Guilfoyle et al., 1998; Perry et al., 1999). Among its many functions, auxin is a regulator of root growth (Guilfoyle et al., 1998; Walker and Estelle 1998; Davies 1995; Hobbie and Estelle 1994). This regulatory site within the promoter may explain the intense GUS activity observed in the root tissue during early development and its continuance through maturity. Auxin has also been shown to play a role in regulating cell division and elongation (Evans 1984; Gray et al., 1998, 1999). Hanzawa et al. (2002) reported an increase in *SPMS* expression with exogenous application of auxin, indicating a direct interaction between the expression of this gene and the hormone.

The leaf margins have been shown to be a major auxin sink in developing leaves (Mattsson et al., 1999; Steinmann et al., 1999). Mutants with deficiencies in polar auxin transport have shown similar phenotypes with a reduced rosette leaf size (Bennett et al., 1995; Carland and McHale 1996; Przemeck et al., 1996; Carland et al., 1999; Deyholos et al., 2000; Hobbie et al., 2000; Koizumi et al., 2000). The decrease in cell expansion and elongation would further suggest a direct interaction between auxin and *SPMS*. An interesting correlation between auxin and *ACL5* was observed by Clay and Nelson (2005) where a base pair deletion in exon 7 of the *ACL5* gene provided a premature stop codon and a knock-out with the similar dwarfed phenotype as seen by Hanzawa et al. (2000). In this mutation, however, plants developed thicker veins in leaves and inflorescence stems.
due to increase in the number of xylem and phloem cells, as well as procambial cells. This vein mutation (named tkv) was attributed to reduced auxin transport as these vascular features are also induced by the application of auxin transport inhibitors, e.g. 1-N-naphthylphthalamic acid (Mattson et al., 1999; Sieburth 1999). The authors suggest a relationship between polar auxin transport and vein definition with spermine, found in the tkv mutation caused by the ACL5 knockout, the inducible expression of ACL5 by auxin (Hanzawa et al., 2000), and detection of ACL5 expression localized in provascular/procambial cells (Birnbaum et al., 2003; Clay and Nelson 2005). The precise mode of action of spermine and auxin remains to be delineated.

The CARCCW8GAT motif, an AGL-15 (AGAMOUS-like 15) regulatory site, was also found in the promoter of SPMS. The AGL-15 protein is often found in elongating cells (Perry et al., 1999; Tang and Perry, 2003) which supports the expression of SPMS in the apical and marginal regions of Arabidopsis organs, as well as the tip of developing siliques. Another motif which is consistent with the observed pattern is the hexamer motif, which has has been shown to be essential for meristem-specific expression (Atanassova et al., 1992; Chaubet et al., 1996). Both the ARF and hexamer motifs support the expression pattern observed in the root tissue with the role of auxin as a root stimulator. Similar to SPDS3, the SPMS promoter contains the GA Response Element, GAREAT, which could be related to high level of GUS expression during germination and early development.

Despite the number of important putative developmental motifs and widespread expression pattern of SPMS, it has been suggested that SPMS may not be essential for survival of Arabidopsis (Imai et al., 2004b). In that study, double mutants for ACL5 and
the proposed functional SPMS gene, SPDS3, were created. There was no obvious phenotypic change in mutants lacking the SPDS3 gene. Double mutant (acl5-1/spds3-1) displayed no difference from the acl5-1 mutant which had the characteristic reduced stem growth. This suggested that either spermine was not essential for the survival of Arabidopsis or the role(s) of spermine could be compensated by spermidine and/or putrescine (Imai et al., 2004b). HPLC analysis of the spds3-1 mutants revealed a remarkable decrease in free and conjugated spermine when compared to wild type plants, down to 5.8% and 3.4% of control levels, respectively. However, there was no significant change in spermine levels in the acl5-1 mutants. This indicated that SPDS3 may play a role in spermine biosynthesis. This is supported by the expression pattern observed in this study, as SPDS3 expression appears to be widespread throughout development in a number of organs. The authors speculated that the heterodimerization between SPDS3 and SPDS2 (reported by Panicot et al., 2002) may favor SPDS3 as a SPMS taking into account that spermidine is a precursor to spermine. Yet, this raises the question of why ACL5 is solely involved in stem elongation and maintenance of meristematic activity?

It is interesting to note that the observed expression pattern in this study is similar for SPDS3 and SPMS, except at key apical and marginal regions of vegetative tissues and floral organs. As mentioned earlier, the apical and marginal regions of leaves are areas of intense cell proliferation and elongation. If both SPDS3 and SPMS have spermine synthase activity, then it can be postulated that these elongating regions may require additional spermine produced by SPMS found in these cells. This would predict that SPMS expression should be cell or tissue-specific. Since the expression of SPDS3 was
much more intense and slightly more widespread in all organs as compared to \textit{SPMS}, it can be hypothesized that \textit{SPDS3} may produce the majority of spermine for normal cell activity, whereas \textit{SPMS} produces spermine for cell elongation in particular tissues. While both genes share a similar expression pattern and the promoter regions share similar regulatory motifs and a sequence homology (43\% identity) between the promoter nucleotide sequences (Figure 59), there are some unique motifs which probably are responsible for differences in the expression patterns. For example, the \textit{SPMS} promoter contains a specific hexamer motif shown to be required for meristematic activity (Chaubet et al., 1996), a motif not found in the \textit{SPDS3} promoter. Also, the ARF motif (Auxin Responsive Factor) in the \textit{SPMS} promoter supports the expression pattern in the marginal regions of cotyledons and leaves as these regions have been shown to be auxin sinks (Clay and Nelson, 2005). Despite the cloning of the \textit{ACL5} gene and feeding experiments which showed direct evidence of spermine synthase activity (Hanzawa et al., 1997, 2000; Panicot et al., 2002), \textit{in vivo} studies have distinctly shown that similar phenotypes to the \textit{ACL5} mutants can be recreated with the exogenous addition of DL-\(\alpha\)-difluoromethylornithine (DFMO), an of putrescine production by ODC (Hanzawa et al., 2000). It should, however, be noted that \textit{Arabidopsis} does not have the ODC gene, but has been shown to have some ODC activity (Hanfrey et al., 2001). This indicates that spermine, in particular, and thus \textit{SPMS}, plays a defining role in cell elongation which is supported by the expression profile observed in this study.

It is obvious from the above discussions that there remain a number of questions as to the exact function of these two enzymes. While some studies suggest that \textit{SPDS3} functions as \textit{SPMS} (Hanzawa et al., 2002, Panicot et al., 2002; Imai et al., 2004a), others
suggest that SPMS is not an important polyamine biosynthetic enzyme due to the fact that its gene knock-out does not affect spermine titers (Imai et al., 2004b). Whereas the four putative aminopropyltransferases in Arabidopsis show a number of similarities in nucleotide sequences, gene structure, protein structure, and function (Hanzawa et al., 2002; Panicot et al., 2002, Ikeguchi et al., 2006), SPDS3 and SPMS together are more divergent when compared to SPDS1 and SPDS2. It would appear from the similar expression patterns, the shared regulatory motifs, and promoter sequence homology, that the two enzymes perform similar functions. Even more convincing, based on previous studies, it would appear that these two enzymes have multiple functions in the biosynthesis of the higher polyamines, which would support their divergence from SPDS1 and SPDS2. The complete expression profile of SPDS1 and SPDS2 would need to be observed to provide more insight into this homologous gene family.

**Construct Comparison**

Three separate promoter::GUS constructs were designed for each gene, each containing segments of the promoter region with or without part of the ORF in order to help define the role of some of the regulatory elements within this sequence. Cellular localization of expression among the three SPDS3 promoter constructs was similar, yet intensity of GUS staining was not. SPDS3-C expression was widespread throughout early development, particularly in the cotyledons and root tissue (Figure 36). Expression was more localized in mature plants, as seen in the vascular region of rosette and cauline leaves, pollen grains, and the base of the siliques (Figures 38, 39, 41, and 42). Staining remained intense in the root tissue (Figure 37). Similar profiles were seen in SPDS3-A,
yet staining was more localized in the vascular region of cotyledons with high expression remaining in the root tissue (Figure 37). Staining was similar in \textit{SPDS3-A} and \textit{SPDS3-C} mature plants with less intensity in the former. This pattern was also similar in \textit{SPDS3-B}, with less staining observed in the vascular region of the cotyledons and roots of young plants, as well as in mature organs.

As mentioned earlier, there are a number of regulatory motifs found in the 935 bp \textit{SPDS3} promoter used in this study, such as GAREAT and CARCCW8GAT (Table 25). These motifs may help explain the high expression pattern observed in \textit{SPDS3-A} and \textit{SPDS3-C} as both these constructs contained the entire 935 bp promoter region.

However, in \textit{SPDS3-B}, the promoter region only extends 214 bp, missing approximately 700 bp of the putative promoter region. Despite the small size, there was reasonable GUS expression in the transformants, similar in localization, but weaker in intensity. Motif analysis provides evidence that the 214 bp region contains the GAREAT motif, which helps explain the expression in young developing plants. Also, there remain two putative TATA boxes, ensuring that transcription can still occur; thus this region can be defined as a core promoter of this gene (Guilfoyle 1997). The MYB4 motif is also present, a motif shown to be responsive to ABA (Figure 46). As mentioned earlier, ABA is a regulator of root elongation, which may help explain the continuing expression observed in the roots of \textit{SPDS3-B} transformants.

The most intriguing observation is the disparity in expression between \textit{SPDS3-A} and \textit{SPDS3-C}. An examination of the sequences that were used shows that the downstream region of the promoter and the presence or absence of part of the ORF may play a larger role in regulating expression of this gene. The difference in the intensity of
GUS staining between SPDS3-A and SPDS3-C indicates that there may be either a potential translational inhibitory region within the ORF or the presence of the encoded polypeptide may affect GUS activity. As mentioned above, one major difference between these two constructs is the 57 bp ORF in the SPDS3-A construct. Gene regulatory regions have been shown to be located throughout the genome, within the introns, the coding sequences, or thousands of base pairs up/downstream from the gene.

Sieburth and Meyerowitz (1997) found that regulatory sequences that control expression of the floral-specific gene, AGAMOUS, of Arabidopsis lie within the transcribed region of the gene and are not exclusively in the promoter region. These authors showed that correct “mimicking” of GUS expression requires a large upstream region of the AGAMOUS gene and a 3.8 Kb intragenic region from within the AGAMOUS transcriptional unit. Similar findings were presented by Callis et al. (1987) who observed increased reporter gene expression with the presence of intron sequences from specific maize genes. Similarly, the leader intron and certain 3’ sequences of the potato sucrose synthase 4 gene have been shown to contain a major enhancer of reporter genes (Fu et al., 1995). Currently, there is no software for identifying enhancer and/or inhibitor regulatory sequences within the ORF, therefore it is unclear whether or not the 57 bp SPDS3 ORF region actually contains an inhibitory element. The SPDS3-B construct also contained 170 bases of the ORF, but it is not possible to determine if the decrease in expression was due to the ORF or the lack of 5’ region of the promoter sequence.

Whereas the inhibitory role of the N terminus of SPDS is speculative, disparity among the three SPMS constructs was much more evident. Very little expression was
observed in SPMS-A and SPMS-B constructs as compared to SPMS-C. It appears that the
61 bp region of the SPMS ORF fused to the GUS ORF dramatically decreased GUS
activity (Figure 3). In all constructs, there was expression seen in the marginal regions of
developing cotyledons as well as the rosette junction of the stem and roots, indicating that
the disruption is not at the transcriptional level, and that the fusion protein (between the
part of the SPMS ORF and the GUS protein) is a functional enzyme (Figures 38 and 40).
Nucleotide sequencing of the fusion confirms the in-frame fusion between the part of
SPMS ORF and GUS ORF (Figures 21-23). It can be argued that the presence of the 30
amino acid sequence coded for by the SPMS ORF may negatively affect GUS activity,
resulting in the lack of staining. However, results from the transient expression in poplar
cells indicate that the constructs are functional in a plant system (Table 5).

The above arguments support the idea that there is a tissue-specific or species-
specific inhibitory region within the ORF of both SPDS3 and SPMS. However, another
possibility is that there is a species-specific amino acid sequence-based effect on the
stability of the GUS protein. Whereas the fusion protein was not affected in the poplar
system, the stability of the protein may be affected in Arabidopsis. Perhaps an equally
good explanation for the disparity among the various SPDS3 and SPMS constructs may
be due to the vector used for transformation. The SPDS3-A, SPDS3-B, SPMS-A, and
SPMS-B promoter and ORF regions were all cloned into the same pCAMBIA 2381
vector to obtain the GUS fusion. However, because the SPDS3-C and SPMS-C clones
did not contain an ATG start site provided by the respective ORF, a pCAMBIA vector
containing a GUS gene with its own ATG was needed (pCAMBIA GUS+). This
provided no protein fusion in SPDS3-C and SPMS-C transformants, whereas the other

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constructs with the ORF::GUS fusion not only contain amino acids from the native protein, but also a small amount of the vector sequence left over from the restriction cutting and ligation (Figures 18-23). As mentioned, the protein fusions are in-frame as shown by sequencing, transient expression, and the small amount of staining observed in the Arabidopsis transformants. Perhaps the combination of the vector sequence and ORF sequence provided an amino acid composition which interferes with protein stability and/or GUS activity. As proposed earlier, to further test this hypothesis, a 35S promoter ligated into the same pCAMBIA vector could be used to rule out the vector's role in inhibition. In such an experiment, the strong 35S promoter should drive expression of the GUS gene indicating if the problem is at the transcriptional or translational level.

At a closer look, the addition of the vector to the SPMS-C and SPDS3-C may not affect the final protein product as is the case with the protein fusion constructs, but it may affect the mRNA. In particular, the vector sequence of the SPMS-C and SPDS3-C constructs extends the 5'UTR, which also displaces the Kozak sequence typically surrounding the AUG start site; a sequence which plays a crucial role in translational efficiency (Kozak, 1987, 1991, 2001; Joshi, 1987; Lutcke et al., 1987). This sequence displacement and extended leader sequence may contribute to enhanced expression levels observed in the SPDS3-C and SPMS-C transformants. A number of studies have shown that extending the 5' leader sequence or changing single base pairs within the Kozak sequence may greatly affect translation (Taylor et al., 1987; Lutcke et al., 1987; Kozak, 1999, 2002; Kirsi and William, 1990; Guerineau et al., 1992; Dinesh-Kumar and Miller, 1993).
Another speculation to the differences in the expression patterns can be attributed to the half-life of the GUS protein. The half-life of a protein in a living cell can range from a few seconds to days. One aspect of the \textit{in vivo} half-life of a protein is related to the "N-end rule", a pathway which involves the N-terminal amino acid residue as a degradation signal (Bachmair et al., 1986; Varshavsky, 1996, 1997). The N-terminal protein processing pathway is an essential mechanism found in all organisms in which the typical terminal methionine is removed by the enzyme methionine aminopeptidase (MAP; Bradshaw et al., 1998; Giglione et al., 2000). This exposes the second amino acid which is thought to be targeted by various proteolytic pathways (Nishizawa et al., 1992; Varshavsky, 1996, 1997; Giglione 2000, 2003). Essentially, then it is the second codon that determines the stability of a protein. This is an interesting concept that could be explored in the transgenes produced in this study. In looking at the \textit{SPDS3}-A and \textit{SPDS3}-B sequence (Figures 18 and 19), the native gene's second codon, "GAG", codes for a glutamine. This amino acid has been shown to lower the half-life of a protein (to 30-60 min) in eukaryotes (Bachmair and Varshavsky, 1989; Varshavsky, 1997) and more than 10 h in \textit{E. coli} (Tobias et al., 1991). However, the \textit{SPDS3}-C construct contains the GUS gene with an AUG start site, therefore, the native \textit{SPDS3} ORF is not incorporated (Figure 20). The second codon in this construct is "GTA", which codes for valine; an amino acid shown to lengthen the half-life in eukaryotic proteins (to 30-100 h) and \textit{E. coli} (greater than 10 h). Based on this concept, it would appear that the \textit{SPDS3}-C GUS enzyme would have a much longer half-life than the \textit{SPDS3}-A and \textit{SPDS3}-B transformants. This may explain the increased intensity of GUS staining due to the extended life-span of the GUS enzyme. As mentioned earlier, the localization was the
same among the constructs, however, the intensity was much greater in the \textit{SPDS3-C} transformants. The same is true for the \textit{SPMS} as the \textit{SPMS-A} and \textit{SPMS-B} constructs have a “GGT” as second codon adding a fairly stable amino acid residue i.e. glycine (20-30 hr in eukaryotes). However, the \textit{SPMS-C} construct also has the valine residue providing a highly stable protein product. Whereas the exact function and characterization of this mechanism is not fully understood, it provides an interesting speculation based on the observations of this study.

To further correlate the expression profile of \textit{SPDS3} and \textit{SPMS} observed by GUS staining with the expression of the native gene, RT-PCR was performed on RNA from various tissues. The results show that in most cases there is a positive correlation between the GUS transgene expression and the presence or absence of the native gene transcripts. The presence of both GUS and native SPMS transcripts in 20 and 35 DPG rosette leaves in which no GUS activity was observed in \textit{SPMS-A} and \textit{SPMS-B} transformants, suggests that there probably is a translational or a protein stability problem in these constructs rather than transcriptional inhibition as previously proposed (Figure 45). The fact that \textit{GUS} and \textit{SPMS} transcripts were detected in all \textit{SPMS} transformants suggests that the staining pattern observed in \textit{SPMS-C} is likely more accurate as GUS enzyme activity is likely inhibited in \textit{SPMS-A} and \textit{SPMS-B} transformants. Because the localization of staining among the three \textit{SPDS3} constructs was similar, it was expected that transcripts would be detected in all organs tested. This was indeed seen in the RT-PCR results as \textit{GUS} and \textit{SPDS3} transcripts were detected in all organs surveyed in which GUS staining was observed (Figure 44). However, a major discrepancy observed in the RT-PCR was in the 35 DPG stem samples. In the developmental profile, very little GUS
activity was observed in the primary stem, except at the rosette junction and cauline leaf junctions (Figures 39 and 40). In collection of stem tissue for RNA isolation, all cauline leaves, flowers and secondary stems were removed. This may explain the presence of GUS and SPDS3 transcripts in the stem RT-PCR screen as a small amount of the rosette and cauline leaf junction tissue was used as a source of RNA. In addition, the wounding experiments (discussed later) indicate induction of SPDS3 expression immediately following injury. This may also help explain the detection of SPDS3 transcripts in the stem RT-PCR results. Similarly, SPMS and GUS transcripts were observed in the primary stem. The expression of SPMS-C shows GUS activity in the rosette and cauline leaf junctions which also shows the presence of transcripts. The RT-PCR results would imply that the profiles of SPDS3-C and SPMS-C are likely more accurate as the GUS staining and the presence of native gene transcripts correlate better with each other in these transformants. The literature also strongly supports the expression pattern of SPDS3-C and SPMS-C observed here. As mentioned earlier, the expression pattern of SPMS-C is nearly identical to that observed by Clay and Nelson (2005) who used 2.13 Kb of the putative ACL5 promoter to drive GUS expression in Arabidopsis.

Whereas my study may not have fully defined the limits of the promoter region of the two genes, this study provides one of the most in-depth expression profiles of any gene in the polyamine pathway and reveals distinct tissue localization not seen in any RT-PCR, RNA gel blot, or microarray analysis.
Expression of SPDS3 and SPMS in Response to Abiotic Stress

To further identify the functions of SPDS3 and SPMS, the expression of the promoter::GUS constructs was studied in response to three forms of abiotic stress, namely salt (NaCl), drought, and chilling. In addition, response to wounding was studied. Depending on the experiment, treatments were applied to T3 plants of all constructs at 10, 20, and 35 DPG. A total of 25 samples (5 samples from each of the 5 independent lines) for each construct, per treatment, were collected and stained for GUS activity. Plants maintained under normal growth conditions were used as controls for each stress experiment and collected at each time point along with the experimental samples. Variations in GUS activity were difficult to detect in 10 DPG plants due to the relatively high expression in both the SPDS3 and SPMS transformants at this time point (Figures 32 and 33). Changes in GUS staining in response to stress treatments were detectable in 20 and 35 DPG plants as expression had weakened when compared to 10 DPG. Since there were no differences between the two mature stages (20 and 35 DPG), only the results with rosette leaves of 35 DPG samples were recorded. No changes were observed in any other organ aside from rosette and cauline leaves. Any changes in root tissue were also undetectable due to the high levels of expression during development. As seen in the developmental profile, the SPDS3-A and SPDS3-B transformants displayed similar profiles with slightly weaker GUS intensity when compared to SPDS3-C. Similar changes in GUS staining were observed for all three SPDS3 constructs during the stress experiments, therefore only the SPDS3-C samples were recorded in detail. SPMS-A and SPMS-B continued to show little GUS activity when compared to SPMS-C, therefore, only SPMS-C samples were recorded in detail.
There were a number of interesting trends observed in the stress experiments. There was obvious induction of both \textit{SPDS3} and \textit{SPMS} in response to 100 mM salt treatment (Figures 47 and 48) as well as osmotic stress (Figures 49 and 50). Only for the 200 mM NaCl treatment, \textit{SPDS3} and \textit{SPMS} expression slightly increased after 12 h, but GUS staining was lower at 24 and 48 h. This may have resulted from either suppression of the expression of these two genes, or, more likely, the high salt levels may be toxic to cells and inhibit enzyme activity. In response to short term chilling, it appears that there was an induction of \textit{SPDS3} and \textit{SPMS} after 13 h at 4°C (Figures 51 and 52). Yet after 24 h, there was a noticeable decrease in GUS activity which would suggest again that the low temperature may inhibit GUS enzyme activity or stability. In response to wounding, \textit{SPDS3} displayed a wound response, particularly in the stem, rosette, and cauline leaves (Figure 53). Expression gradually increased up to 24 h after injury. It also appears that after 24 h, \textit{SPDS3} has a “systemic” response as GUS staining increased not only at the injured site, but throughout the entire organ. However, GUS was not detected at the wound site in \textit{SPMS} transformants (results not shown) indicating that \textit{SPMS} is not involved in the wounding response.

The observations, thus far, indicate that both of these genes are induced early in response to high salt, drought, and chilling, however, only \textit{SPDS3} appears to be involved in wounding, and not \textit{SPMS}. It should also be noted that the change in GUS activity of \textit{SPDS3} transformants was much more evident than \textit{SPMS}. There are obvious increases in GUS activity during the treatments, particularly noticeable during drought and salt stress, which are two of the best characterized abiotic stress responses in plants.
Plant cells undergo a variety of changes during both salinity and drought stress, initially involving the lowering of the extracellular water potential. Plant cells often respond to such cellular dehydration by a process called osmoregulation, which involves the production of cytosolic, low molecular weight, organic compounds and accumulation of ions in the vacuole (Leshem and Kuiper, 1996). This response aids in lowering of cellular water potential to attempt to restore the turgor pressure. During stress, the formation of reactive oxygen species (ROS) is a common event within the cytoplasm. These molecules are very reactive and often inhibit enzyme functions and oxidize membranes. During long-term salt stress, ion toxicity and energy imbalances greatly affect the cell, as NaCl has been shown to cause extensive damage to membrane integrity (Greenway and Munns, 1980) and interfere with multiple biochemical reactions; e.g. respiration, photosynthesis, protein and nucleic acid metabolism (Kakkar and Rai, 1993).

Drought and salinity stress have been a common focus of polyamine research. Early salt treatment experiments focused on the increased putrescine levels in plants. Strogonov (1964) observed putrescine accumulation in salt stressed leaves of *Gossypium herbaceum* and with similar findings in *Vicia faba* (Strogonov et al., 1972). Shevyakova (1981) observed an increase in putrescine in pea (*Pisum sativum* L.) and *Vicia faba* with the addition of 50 mM NaCl. Basu et al. (1988) reported an increase in all polyamine levels of salt-treated rice (*Oryza sativa* L.) seedlings with similar findings by Krishnamurthy and Bhagwat (1989). Prakash and Prathapasenan (1988) observed a 56% increase in growth in salt-treated rice seedlings when exogenous putrescine was applied. Chattopadhyay et al. (1997) compared the use of ADC and ODC pathways during salt stress in salt-sensitive and salt-tolerant rice (*Oryza sativa* L.). Their results support other
studies which suggest that in some plant species the ADC pathway is active during both normal and stress conditions, whereas the ODC pathway is functioning only during stress leading to the increased putrescine titers (Aziz et al., 1998).

Similar responses have been reported in response to drought. Flores and Galston (1984) found high levels of putrescine and ADC activity during sorbitol-induced and conventional drought in detached oat (Avena sativa) leaves. Capell et al. (2004) studied stress response of transgenic rice (Oryza sativa L.) over-expressing various polyamine biosynthetic genes (rice ADC, Datura stramonium ADC or rice SAMDC). The transgenic plants showed increased cellular polyamine levels and tolerance to drought. The authors proposed that the ability of plants to tolerate abiotic stresses relies heavily on the production of the higher polyamines, spermidine and spermine. The sudden burst of putrescine during abiotic stress may play a much greater role than just being a simple precursor to the higher polyamines. However, over-accumulation of putrescine in normal, unstressed plants has resulted in cellular toxicity as shown in transgenic tobacco with inducible over-expression of oat ADC which displayed a decrease in vegetative growth (Masgrau et al., 1997). Over-expression of Arabidopsis ADC2 (resulting in overaccumulation of putrescine) caused dwarfism and late-flowering (Alcazar et al., 2005). Putrescine has also been reported to cause depolarization of membranes and increased potassium leakage (Tiburcio et al., 1990). In some plants, the application of exogenous putrescine leads to the loss of turgor and causes necrotic spots (Flores et al., 1991). The physiological response to increased putrescine included chlorophyll loss and accelerated senescence (Capell et al., 1993), which were attributed to the depolarization of membranes. Generally, it appears that the best equipped stress tolerant plants are those
that can efficiently convert the high levels of putrescine to the higher polyamines, spermidine and spermine. In order to convert the potentially high levels of putrescine into spermidine and spermine, the biosynthetic enzymes would need to be present in high amounts. This would explain the increase in expression of the two biosynthetic genes during abiotic stress observed in this study.

The role of spermidine synthase during stress was studied by Kasukabe et al. (2004) in Arabidopsis transformed with the figleaf gourd (Cucurbita ficifolia) SPDS gene under the control of a 35S promoter. The transgenic lines were shown to have a significant increase in SPDS activity and spermidine content in the leaves which coincided with their enhanced tolerance to chilling, freezing, hyperosmosis, paraquat toxicity (oxidative stress), drought, and salinity. In addition, a cDNA microarray analysis revealed that there were a number of genes transcribed in the transgenic lines during stress that were not expressed in the wild type plants. These genes included stress responsive transcription factors such as DREB and the protective protein rd29A. This suggests that polyamines play a role as a signaling molecule in stress response. This correlates with similar studies which found polyamines promoting gene expression and increasing the DNA-binding activity of other transcription factors (Gupta et al., 1998; Childs et al., 2003). Kasukabe et al. (2004) showed a direct effect of increased polyamine levels during stress. The observations in the present study are in agreement with past studies, except going further in observing the actual expression of two important stress-induced polyamine biosynthetic genes.

Sequence analysis of the SPDS3 and SPM5 promoters also reveals a number of stress-related motifs (Table 25). Two of the most significant and well-characterized
motifs found in the *SPDS3* promoter are the dehydration-responsive elements (DRE), DREB1A/CBF3 and DRE CORE. These motifs have been identified in the promoters of a number of drought and cold-stress inducible genes (Wang et al., 1995; Iwasaki et al., 1997; Kasuga et al., 1999; Chen et al., 2002), most notably the well-characterized *rd29A* gene shown to be induced during drought, cold, and salt stress (Yamaguchi-Shinozaki and Shinozaki 1994). Wang et al. (1995) reported a microarray analysis and potential functions of 402 putative transcription factors. The DRE factors were categorized in Group I, containing 21 genes that are induced by abiotic stress (high salt and/or osmoticum, cold, and jasmonic acid treatment). This group includes the DREB1A/CBF3 that was previously shown to be activated by cold stress (Liu et al., 1998; Medina et al., 1999), but has been linked to a number of other stress-related (both abiotic and biotic) responses as well, e.g. high salt, chilling, drought, and mechanical wounding (Seki et al., 2001; Cheong et al., 2002). The presence of this motif may explain the increase in expression of *SPDS3* during drought, high salt, and wounding.

The MYB family of transcription factors contains a number of *cis*-acting elements activated during stress (Martin and Paz-Ares, 1997; Kranz et al., 1998). The MYB binding motifs found in both the *SPDS3* and *SPMS* promoter are MYB4 and MYB1AT. The MYB4 transcription factor apparently regulates genes during the wound response (Martin and Paz-Ares 1997; Cheong et al., 2002). The MYB1AT has been shown to be a drought response element (Yamaguchi-Shinozaki and Shinozaki, 1993; Abe et al., 2003). A similar motif, MYB2AT, found in the *SPDS3* promoter, has also been shown to be activated during drought stress and in response to ABA (Yamaguchi-Shinozaki and Shinozaki, 1993; Abe et al., 2003). Similar motifs found in the *SPMS* promoter are
Myc2 bs in RD22 and MYCATERD1, which respond to water stress and ABA (Simpson et al., 2003). The RD22 gene is a dehydration-response gene induced by water stress and the application of exogenous ABA (Yamaguchi-Shinozaki and Shinozaki, 1993). This RD22 promoter contains both the MYB2 and MYC2 recognition sites characterized as cis-elements during drought response (Abe et al., 1997; Abe et al., 2003; Simpson et al., 2003). Another cold response element found in the SPDS3 promoter is the LTRE motif (Low Temperature Response Element) found in the promoters of two Arabidopsis cold-responsive genes, LTI78 and LTI65 (Nordin et al., 1993). These genes were also regulated in response to drought and ABA. Finally, the W box motif has been shown to be activated by WRKY proteins, which regulate gene expression of plant defense genes (Maleck et al., 2000; Chen et al., 2002). The WRKY proteins are a unique family of proteins only found in plants (Robatzek and Somssich 2001) defined by the highly conserved amino acid site WRKY. WRKY genes have been shown to be up-regulated by a variety of conditions including pathogen attack, wounding, and senescence (Eulgem et al., 1999, 2000). The W box motif was found in both SPDS3 and SPMS promoter sequences.

It is evident from the stress experiments and sequence analysis that both SPDS3 and SPMS may respond to a variety of stress treatments, whether abiotic or mechanical injury. The obvious transcriptional up-regulation of the promoter::GUS construct and the numerous stress-related cis-elements in the promoter sequence provide a strong indication that these genes are induced as part of the overall plant response to stress. As mentioned, often times these response pathways are linked, as seen in a number of microarray studies (Chen et al., 2002, Cheong et al., 2002; Seki et al., 2001) and
indicated by the presence of multifunctional motifs. The transcripts of these genes have also been analyzed in response to various stress conditions. Urano et al. (2003) provided a characterization of all the key polyamine biosynthetic genes in *Arabidopsis* (Table 1) during various abiotic stress conditions via RT-PCR. The expression profiles of these genes in response to NaCl, dehydration, and abscisic acid treatments were separated into three categories: stress-inducible, which includes *ADC2*, *SAMDC2*, and *SPDS3*; constitutive, which includes *ADC1*, *SAMDC1*, *SPDS1*, and *SPDS2*; and stress-repressible, including *SPMS*. The induction of *SPDS3* is consistent with the observations in this study, however, SPMS was not repressible in 100 mM NaCl and drought.

There was an obvious increase in GUS activity in *SPDS3* transformants during treatments of 100 mM NaCl, drought, and 12 h of chilling. All stress experiments of Urano et al. (2003) involved a 24 h exposure, indicating that SPDS3 is involved in a rapid response to stress. *SPDS3* transcripts also increased with the exogenous application of ABA (100 μM). This finding is consistent with the report of Hanzawa et al. (2002) who saw a decrease in *SPDS1* mRNA (detected via RNA gel blot hybridization) and an increase in *SPDS3* in response to ABA application. This indicates that SPDS3 may play a major role in production of spermidine during stress conditions, whereas *SPDS1* may be the significant SPDS gene during development. It is interesting to note that Urano et al. (2003) observed *SPMS* transcripts gradually decreasing during a 24 h NaCl and drought treatment, yet transcripts increased during the 24 h cold treatment. *SPMS* appeared to be unaffected by the exogenous application of ABA (100 μm). It should be noted, in that study a 250 mM NaCl treatment was used, which is more than double the concentration used in my study in which an increase in *SPMS* was observed at 100 mM. The results of
The 200 mM NaCl treatment in my study are similar to the observations of Urano et al. (2003); the GUS staining decreased at 24 and 48 h. The induction of SPMS in response to 100 mM NaCl indicates that perhaps SPMS is inducible at 100 mM NaCl but repressed at higher concentrations, such as 200 and 250 mM, or perhaps the high salt is toxic to cells inhibiting enzyme activity/stability.

An interesting observation provided by Urano et al. (2003) was that SPMS transcripts increased during 10 h rehydration period of drought-treated plants. This would suggest that SPMS perhaps plays a role in the recovery of stressed plants. We can speculate that the decrease in SPMS transcripts during salt and drought were detected because the Urano et al. (2003) stress experiments were short and drastic (involving high NaCl and intense drought under light over a 24 h period). It could be that the experiments were so short that the recovery time was not observed. In my study, the measurements were spread over a longer period of time (0, 6, 12, 24, 48 h). These time points may have missed the decrease in SPMS expression and instead measured SPMS during the recovery period which takes place during stress. According to the Urano et al. (2003) study, it appears that it takes approximately 5-10 h for induction of SPMS, which would support the increased GUS activity after 12 h. The GUS staining method may not be sensitive enough to detect the decrease after 6 h of stress, to truly detect an increase or decrease in intensity, a quantitative assay of GUS (MUG assay) should be performed.

This method quantifies the activity of GUS to provide a true indication of induction or repression of the GUS gene under control of a native promoter.

Overall it appears that from the literature, promoter analysis, and the stress experiments in this study, SPDS3 plays a major role during the stress response in plants.
The role of SPMS would be a matter of debate. However, the results of my experiments and the fact that large numbers of stress-related motifs are present in the SPMS promoter would indicate that SPMS is expressed during stress. However, its precise role during the stress period remains unclear.
CONCLUSIONS

- Expression of SPDS3 and SPMS was high in young, developing tissues. Expression was weaker and more localized in the vascular tissues of mature organs.

- Expression of SPMS was weaker than SPDS3, but with similar tissue localization. SPMS expression was particularly noticeable in the marginal regions of cotyledons, and rosette and cauline leaves.

- Preliminary results on stress response indicate that both genes are induced during the early stages of drought and salinity stress. SPDS3 induction was also observed during wounding, particularly at the site of injury.

- Sequence analysis of the two promoter regions reveal a number of regulatory and stress-related motifs which support the expression patterns observed during development and in response to stress.

- The expression profiles of these two genes observed in this study support previous studies which indicate that polyamines, and the genes involved in their biosynthesis, are vital to growth and development and play a key role in the stress response.

- The results of this study correlate with previous expression profiles of these two genes; however, this study goes beyond previous studies in providing tissue localization and a complete profile throughout development.
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