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Gene Note

Spatio-temporal expression analysis of *Arabidopsis thaliana* spermine synthase gene promoter

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Abstract Of the polyamines (PAs), plants contain at least two kinds of tetraamines, spermine (Spm) and thermospermine (T-Spm). They seem to have different functions in plants because the stem growth defect shown by the loss-of-function mutant plant of *ACL5* (encoding T-Spm synthase) was partially complemented by T-Spm but not by Spm in *Arabidopsis thaliana.* Here we examined the localization of *Spm synthase* (*SPMS*) gene promoter activity using the *SPMS* promoter-β-glucronidase (*GUS*) gene expressed in transgenic Arabidopsis plants and compared it with that of *ACL5* promoter-*GUS* expression. *SPMS* promoter activity was detected in almost all organs at all developmental growth stages, while *ACL5* promoter activity was only detected in the vascular systems. Upon high salt stress, *SPMS* promoter activity was highly enhanced in all organs except cotyledons whereas the *ACL5* promoter activity was reduced which is consistent with the reduced levels of *ACL5* transcripts. The result indicates that *SPMS* expression is different from that of *ACL5* in respect to tissue specificity and stress response, suggesting corresponding differences in functions of Spm and T-Spm.

Key words: *Arabidopsis thaliana*, polyamine, promoter, spermine synthase, thermospermine synthase.

Polyamines are low molecular cationic compounds with two or more primary amino groups. Major components are diamine putrescine, triamine spermidine (Spd), tetraamines spermine (Spm) and thermospermine (T-Spm) in plants (Alcázar et al. 2010). Spm and T-Spm are synthesized from Spd and decarboxylated *S*-adenosyl methionine, which acts as an aminopropyl donor, by Spm synthase (SPMS) encoded by *SPMS* gene and T-Spm synthase encoded by *ACAULIS5* (*ACL5*) gene, respectively, in *Arabidopsis thaliana* (Knott et al. 2007; Kusano et al. 2008). Because *SPMS* and *ACL5* genes respond differentially to various abiotic and biotic stresses (Mitsuya et al. 2009; Naka et al. 2010) it is strongly suggested that the two tetraamines, Spm and T-Spm, have distinct functions. It was further evidenced that the stem growth defect displayed by the loss-of-function mutant *acl5* was partially complemented by exogenously applied T-Spm but not by Spm (Kakehi et al. 2008). We also introduced the *SPMS*- and *ACL5*-coding regions under the control of Cauliflower Mosaic Virus *35S* promoter into *acl5* mutant plants (Hanzawa et al. 2000), respectively, and observed the stem growth of the transgenic plants. The *acl5* plant overexpressing *ACL5* partially recovered the stem growth, whereas the *acl5* plant overexpressing *SPMS* showed no change in stem growth compared to the parental mutant plant (Sagor et al. data not shown), confirming the conclusion drawn by Kakehi et al. (2008).

Here we examined the localization of *SPMS* promoter activity in various developmental stages of *A. thaliana*, and compared it with the location of *ACL5* promoter activity. The Arabidopsis transgenic plants carrying *SPMS* promoter-β-glucuronidase (*GUS*) gene was generated as follows: The fragment containing the Arabidopsis *SPMS* promoter (−1919 to +1) region was amplified with the following primer pair: pSpms-F, 5-ATTTTCGTCGACCTAGTCCTCTTTTATTTT-3, *Sal*I site is underlined, and pSpms-R, 5-CCATAGTGGATCC-ATTCTGGCATACCAAACCTATTC-3, *Bam*HI site is underlined, and cloned into pGEM T-easy vector for sequencing. The clone with correct sequence was digested with *Sal*I and *Bam*HI, and the resulting *SPMS* promoter fragment was cloned into the corresponding restriction enzyme sites of the binary vector pBI101. The

Abbreviations: *ACL5*, *ACAULIS5*; GUS, β-glucuronidase; Spd, Spermidine; Spm, spermine; *SPMS*, Spm synthase gene; T-Spm, thermospermine

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resulting construct was introduced into *Agrobacterium tumefaciens* strain GV3101, provided by Dr. David Baulcombe (Sainsbury Laboratory). Then the transgenic *A. thaliana* seeds were obtained using the floral dip method (Clough and Bent 1998) with the *Agrobacterium* transformant. The seedlings carrying the transgene were selected on agarose plates consisting of half strength MS medium, supplemented with 10 g l^{-1} sucrose, 50 mg l^{-1} kanamycin and 50 mg l^{-1} carbenicillin. Out of more than 10 independent lines, five were grown as T2 plants, and representative results generated with two of them were shown in this study. *ACL5* promoter-*GUS* transgenic seeds (Clay and Nelson 2005) were provided by Dr. Timothy Nelson (Yale University).

While *ACL5* promoter activity was weakly detected in vascular cylinders of germinating roots (Figure 1A-c), strong *SPMS* activity was detected in whole regions of germinated seeds including lateral roots (Figures 1A-a, b). In embryos, the relative *GUS* transcript levels of the *SPMS* promoter-*GUS* transgenics were ca. 7-fold higher than those of the *ACL5* promoter-*GUS* transgenics (Figure 1B). At 3 days after imbibition, *SPMS* promoter activity was observed in whole body of seedlings and more intensely in roots especially in root tips (Figures 1A-d, e). At the same developmental stage, *ACL5* promoter activity was detected in above ground parts especially meristematic regions, tips of cotyledons and vascular systems of roots and root tips (Figure 1A-f).

Next histochemical GUS staining was performed with the *SPMS* promoter-*GUS* transgenic plants of later growth stages. *SPMS* promoter activity was detected in whole plant body and higher activity was detected in the vascular system of 7-d-old- and 10-d-old-seedlings (Figures 2A-a, c), in contrast *ACL5* activity was predominantly detected in the vascular systems (Figures 2A-b, d). Even with mature rosette leaves of 30-d-old plants, similar results were obtained (Figures 2A-e, f). In inflorescence, *SPMS* promoter activity was strongly detected in the flower organs including petals, androecium (anther and filament), gynoecium (ovary, style and stigma) but not in sepals (Figures 2A-g, i), while the activity of *ACL5* promoter was detected only in the anther, stigma and was restricted to the vascular parts of the gynoecium (Figures 2A-h, j). In young siliques, *SPMS* promoter activity was found only in basal and tip portions, whereas *ACL5* promoter activity was weakly detected in the basal portions (data not shown). A strong activity of *SPMS* promoter was also detected in whole regions of 10-d-old seedlings roots and with higher activity in root meristematic regions, and the activity of *ACL5* promoter was higher in root apical meristems and again in vascular cylinders (Figures 2A-k, m). In stems, *SPMS* promoter-*GUS* activity was strongly observed in whole regions including piths (Figures 2A-l, o), whereas *ACL5* promoter-*GUS* expression was only in the vascular bundles (Figures 2A-n, p). In all tissues tested, the expression levels of *GUS* transcripts in the *SPMS* promoter-*GUS* transgenics were higher than those in the *ACL5* promoter-*GUS* transgenics (Figure 2B; Supplemental Figure 1). Those expressional profiles of *SPMS* and *ACL5* were consistent with the results available at The Bio-Array Resource for Plant Biology

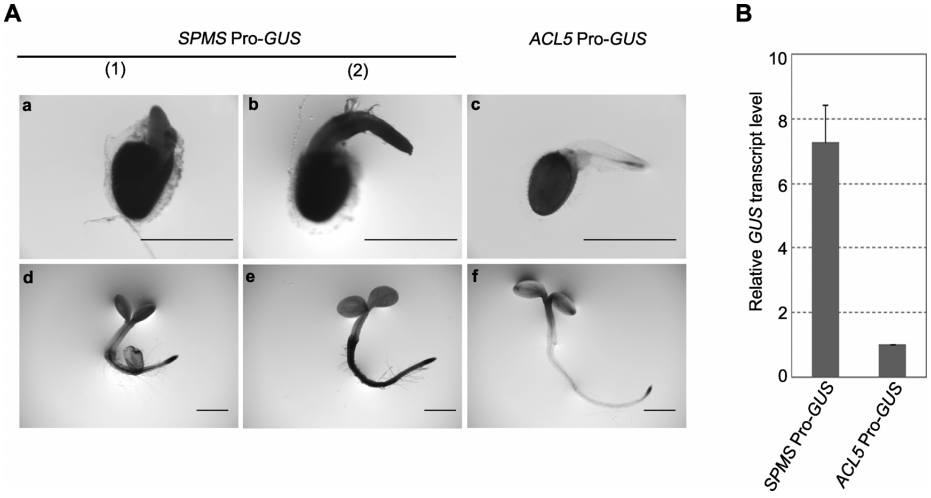

Figure 1. *SPMS* promoter activity in germination and early seedling stages. (A) *SPMS* promotor-driven GUS localization in germinating seeds, one day after imbibition (a, b), and 3-d-old seedlings (d, e) compared with *ACL5* promoter-driven GUS localization in germinating seed, one day after imbibition (c) and 3-d-old seedling (f). Bars = 1 mm. Histochemical GUS assays were performed as described by Jefferson et al. (1987). In brief, plant samples were fixed in 90% acetone for 15 min, rinsed with 100 mM phosphate buffer (pH 7.0) and then incubated for 24 h with the GUS staining solution [0.5 ml ml^{-1} X-gluc, 0.5 mM potassium ferrocyanide, 0.5 mM potassium ferricyanide, 0.1% Triton-X, 100 mM phosphate buffer (pH 7.0), 10 mM EDTA] in a weak negative pressure using a vacuum pump for 15 min. Then the samples were further incubated in the same solution overnight at 37°C, fixed with 70% ethanol, and then observed under a light microscope (OLYMPUS SZX16). (B) Relative *GUS* transcript levels in various growth stages and various organs of *SPMS* promoter *GUS*- and *ACL5* promoter-*GUS* transgenic plants. The *GUS* transcript levels detected in *ACL5* promoter-*GUS* transgenics were set as 1. Quantitative real time PCR was performed as described (Naka et al. 2010).

(BAR) webpage http://bbc.botany.utoronto.ca/efp/cgi-bin/efpWeb.cgi). The above results indicated that the expression sites and levels of the two promoters responsible for the synthesis of the two different tetraamines, Spm and T-Spm, were differentiated in *A. thaliana*.

Finally 10-d-old-seedlings of the *SPMS* promoter transgenics and the *ACL5* promoter transgenics were exposed to high salt stress for 24 h and assayed for GUS staining. In *ACL5* promoter-*GUS* transgenics, the intensity of GUS staining was weakened (Figures 3A-c, f) in consistent with the decreased levels of *GUS*- and endogenous *ACL5* transcripts (Figure 3A-i). In the *SPMS* transgenics, the GUS staining was intensed in whole seedlings except cotyledons of 10-d-old seedlings upon high salt stress, reflecting the increased expression of *GUS*-transcripts and endogenous *SPMS* transcripts (Figures 3A-a, b, d, e, g and h). It is interesting to refer that cotyledons where *SPMS* promoter was not induced upon high salt stress showed chlorosis whereas main leaves where *SPMS* promoter was activated were still greenish and vital (Figure 3B, Yamaguchi et al. 2006).

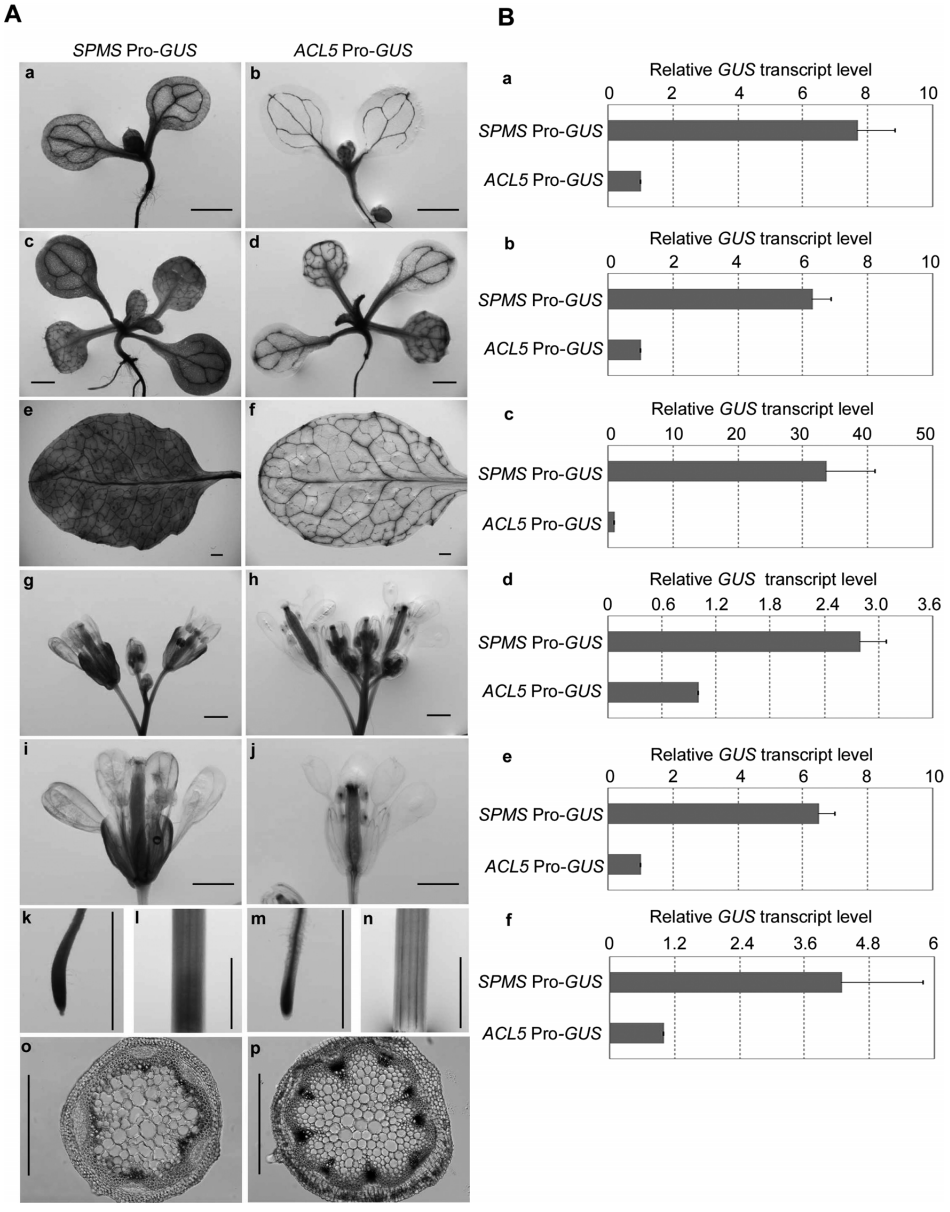

Figure 2. Localization of *SPMS* promoter activity along with Arabidopsis plant growth development. (A) Samples were incubated for 24 h with the GUS staining solution. The activity was showed comparatively with that of *ACL5* promoter activity. (a, b) 7-d-old seedlings; (c, d) 10-d-old seedlings; (e, f) mature rosette leaves of 30-d-old plants; (g, h) inflorescence; (i, j) flower; (k, m) root of 10-d-old seedling; (l, n) stem of 30-d-old plants; (o, p) transverse section of stem of 30-d-old plant. Bars = 1 mm. (B) Relative *GUS* transcript levels in various growth stages and various organs of *SPMS* promoter *GUS*- and *ACL5* promoter- *GUS* transgenic plants. The *GUS* transcript levels detected in *ACL5* promoter-*GUS* transgenics were set as 1. (a) 7-d-old seedlings; (b) 10-d-old seedlings; (c) mature rosette leaves of 30-d-old plants; (d) flowers; (e) root of 10-d-old seedling; (f) stem of 30-d-old plants.

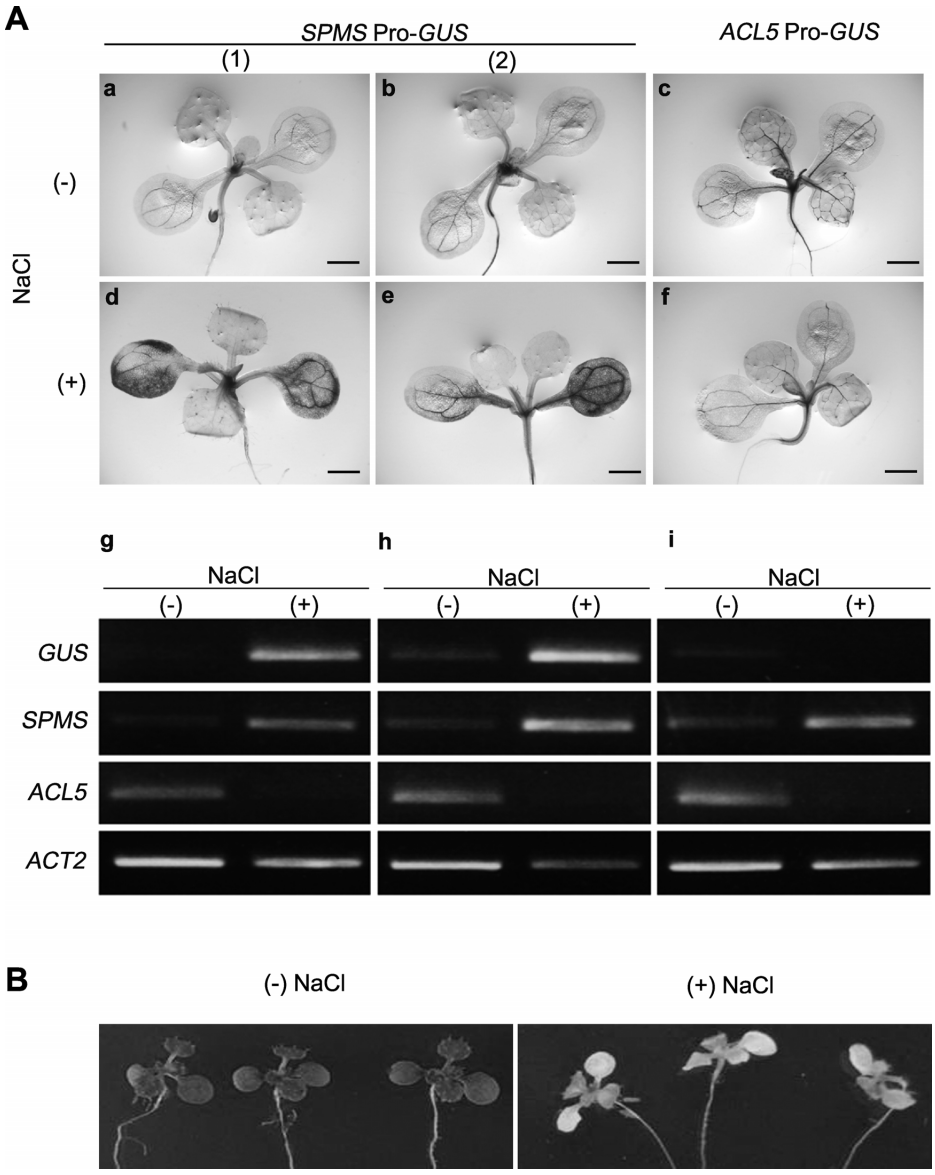

Figure 3. High salt-induced *SPMS* expression analyzed by GUS staining of *SPMS* promoter-*GUS* transgenic plants. (A) Two *SPMS* promoter-*GUS* transgenic lines were used. The sterilized seeds were placed on half strength MS agar media containing 1 g l^{-1} sucrose at 22°C with a 14 h light/10 h dark photocycle. After 10 days, the seedlings were transferred to the same media with or without containing 225 mM NaCl and further incubated for 24 h. Then the seedlings were subjected to GUS staining or total RNA extraction. For GUS staining plant samples were incubated for 4 h with the GUS staining solution. Total RNA from plants was extracted using Sepasol-RNA I Super (Nacalai tesque). Then first-strand cDNA was synthesized with Rever Tra Ace (Toyobo Co. Ltd., Osaka, Japan) and oligo-dT primers. (a, b, d, e) Histological GUS assays on *SPMS* promoter:*GUS* transgenic seedlings; (c, f) Histological GUS assays on *ACL5* promoter:*GUS* transgenic seedlings; (a, b, c) seedlings without salt treatment, (d, e, f) 225 mM NaCl-treated ones. Bars = 1 mm. (g, h, i) RT-PCR analysis of *GUS* transcripts, endogenous *SPMS*- and *ACL5*-transcripts in *SPMS* promoter transgenic and *ACL5* promoter transgenic seedlings with or without NaCl treatment. (g) cDNA prepared from the same line of *SPMS* promoter transgenic seedlings (a, d) was used; (h) cDNA prepared from the same *SPMS* promoter transgenic seedlings (b, e) was used; (i) cDNA prepared from the same *ACL5* promoter transgenic seedlings (c, f) was used. *ACT2* was used for a loading control. The following primers were used for PCR analysis: *GUS* forward, 5-TACCTCGCATTACCCTTACGCTGA-3, *GUS* reverse, 5-CAGCCATGCACACTGATACTCTTC-3; *SPMS* forward, 5-ACACTGGCTAGAGCGTTGAAGCCT-3, *SPMS* reverse, 5-GAGCCAAACAGAAGTCAAGAAGCC-3; *ACL5* forward, 5-TACGTGAAGGCTTACACAGCACAT-3, *ACL5* reverse, 5-AAGACAACACCCGGTTCTGTGCGG-3; *ACT2* forward, 5-TGTGCTCAGTGGTGGAACCACTATGTTCTCAGGTA-3, *ACT2* reverse, 5-AACAAAAGGAATAAAGAGGCATCAATTCGATCACT-3. PCR was performed as follows; 95°C for 30 s, 55°C for 30 s, followed by 72°C for 30 s with 25 cycles for all primer pairs. (B) Phenotypes of Arabidopsis exposed to high salt stress. Ten-d-old seedlings of *A. thaliana* ecotype Col-0 were transferred onto half-strength MS agar media with or without 225 mM NaCl. Photographs were taken at 4 days after transfer.

Counting all the data presented in this study, we could conclude that the two tetraamine polyamines, Spm and T-Spm, were produced in different space and tissues with different expressional degrees and that they were assigned with distinct roles in Arabidopsis, possibly also in other plant species.

Acknowledgements

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