Short Communication

Thermospermine suppresses auxin-inducible xylem differentiation in *Arabidopsis thaliana*

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Abstract

Thermospermine, a structural isomer of spermine, is synthesized by a thermospermine synthase designated ACAULIS5 (ACL5). Thermospermine-deficient *acl5* mutant of *Arabidopsis thaliana* shows severe dwarfism and excessive xylem differentiation. By screening for compounds that affect xylem differentiation in the *acl5* mutant, we identified auxin analogs that remarkably enhanced xylem vessel differentiation in the *acl5* mutant but not in the wild type. The xylem-inducing effect of auxin analogs was clearly suppressed by thermospermine, indicating that auxin-inducible xylem differentiation is normally limited by thermospermine. Here, we further characterized xylem-inducing effect of auxin analogs in various organs. Auxin analogs promoted protoxylem differentiation in roots and cotyledons in the *acl5* mutant. Our results indicate that the opposite action between thermospermine and auxin in xylem differentiation is common in different organs and also suggest that thermospermine might be required for the suppression of protoxylem differentiation.

TEXT

Xylem is a major conducting tissue for water, minerals and signaling molecules in vascular plants. Previous studies revealed that multiple signaling molecules regulate the differentiation of xylem vessel elements.[1] Among these signals, auxin plays a pivotal role in xylem development and auxin polar transport might be required to determine the pattern of xylem differentiation. In the auxin canalization hypothesis, polar auxin transport generates local auxin flow, which in turn specifies procambial cell fate.[2-4] Auxin may interact with other signals for spatial and temporal regulation of xylem vessel differentiation, while the interaction mechanisms are not well understood.[5]

Thermospermine is a structural isomer of a major polyamine, spermine, and has recently been identified as another plant growth regulator that represses xylem differentiation and promotes stem elongation in *Arabidopsis thaliana*.[6-7] The *acaulis5* (*acl5*) mutant of *A. thaliana* exhibits excessive differentiation of xylem tissues and severe dwarfism[8-10] and shows a deficient biosynthesis of thermospermine.[6] Exogenously supplied thermospermine remarkably suppresses xylem vessel differentiation in *A. thaliana* and *Zinnia elegans*.[7] Although the mode of action of thermospermine remains unclear, genetic analyses of *suppressor of acl5* (*sac*) mutants have suggested that thermospermine suppresses the inhibitory effect of an upstream open reading frame (uORF) located in the 5' leader of the *SAC51* mRNA on its translation.[11-12] Consequently, thermospermine enhances translation of *SAC51*, which encodes a basic helix-loop-helix (bHLH) transcription factor, and SAC51 in turn may restrict xylem differentiation and promote stem elongation.

To investigate how thermospermine or *SAC51*-mediated thermospermine signaling regulates xylem differentiation, we screened for chemicals that affect xylem development in the *acl5* mutant and identified the isooctyl ester of 2,4-dichlorophenoxyacetic acid (2,4-D IOE) as a potent inducer of xylem vessel differentiation (Fig. 1A, B).[13] Our experiments using other auxin analogs indicated that 2,4-D, 2,4-D analogs, and IAA analogs also induced excessive xylem differentiation in *acl5-1* but not in the wild type. The effectiveness of auxin analogs may be dependent on their cellular accumulation, tissue permeability and metabolic stability. For example, metabolism-resistant 4-Cl-IAA remarkably induced xylem differentiation in *acl5* while IAA did not,[13] suggesting that the high level of auxin is required to overcome the

threshold for xylem induction.

Savaldi-Goldstein et al.[14] (2008) have also found that 2,4-D analogs with an amide linkage promote hypocotyl elongation in *A. thaliana*. These compounds act as “proauxins”, which diffuse efficiently to the tissue, where they undergo cleavage and release functional auxins.[14-15] Our results suggested that 2,4-D IOE might permeate efficiently into the tissue and release active 2,4-D, which promotes xylem vessel differentiation through $SCF^{TIR1/AFB}$ auxin signaling pathway.[13] To confirm this idea, we analyzed the effect of a bipartite pro-auxin named 533[14] on xylem vessel differentiation in the *acl5* mutant (Fig. 1A, B). 533 induced excessive xylem differentiation in *acl5-1*, indicating that pro-auxin is a potent inducer of xylem vessel differentiation probably due to its high tissue permeability.

Next, we addressed whether auxin analogs promote protoxylem differentiation or metaxylem differentiation in the *acl5* mutant. Close-up observation of the cotyledons of *acl5-1* indicated that 2,4-D IOE strongly promoted protoxylem differentiation (Fig. 1B, lower panels). This is consistent with the previous report that protoxylem-like vessel elements were predominant in the hypocotyls of the *acl5* mutant.[16] The xylem-inducing effect of 2,4-D IOE was also found in the roots and leaves as well as in the cotyledons (Fig. 1C-F). 2,4-D IOE remarkably induced excessive xylem differentiation in roots of *acl5-1* but not in those of the wild type (Fig. 1C). The roots of *acl5-1* grown with 2,4-D IOE had the increased number of protoxylem cell files and less metaxylem vessels (Fig. 1D, E). 2,4-D IOE also promoted xylem differentiation in the leaf tip margin of *acl5-1* (Fig. 1F). In addition, effect of 2,4-D IOE on xylem differentiation was quantitatively analyzed by measuring the width of xylem in the cotyledon

midvein (Fig. 1G). 2,4-D IOE induced about 3-fold increase in the width of xylem in *acl5-1* but not in the wild type. These results indicate that thermospermine mainly suppresses protoxylem differentiation that can be stimulated by auxin (Fig. 1H). In addition, the opposite action between auxin and thermospermine may commonly regulate xylem differentiation in various organs.

In summary, our results demonstrate that auxin analogs show an inducing effect on protoxylem vessel differentiation in the absence of thermospermine. Thermospermine might be an opposing factor to auxin in the regulation of the timing and spatial pattern of protoxylem differentiation (Fig. 1 H). Because *ACL5* is expressed in provascular cells and xylem vessels and is up-regulated by auxin,[9-10, 16] thermospermine may be synthesized in developing vascular cells in response to auxin and form a gradient around the auxin maxima to limit auxin-inducible xylem differentiation (Fig. 1 H). Taking into account that transcription factors required for xylem differentiation are remarkably up-regulated by 2,4-D in the *acl5* mutant,[13] thermospermine is critically required for suppressing the inductive effect of auxin on gene expression involved in xylem differentiation. Further analysis of the effect of auxin analogs and thermospermine using mutants related to auxin and thermospermine signaling will provide new insight into the molecular mechanism of xylem differentiation.

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Figure Legend

Figure 1

Auxin analogs induce excessive xylem differentiation in the *acl5* mutant.

(A) Structure of 2,4-D IOE and 533. Arrows indicate the putative cleavage sites by esterases. (B) 533 and 2,4-D IOE induce excessive xylem differentiation in *acl5-1*. The *acl5-1* mutant was grown for 7 days in the MS liquid medium in the absence (Mock) or presence of 10 μM 533 or 2,4-D IOE, and analyzed for xylem vessel differentiation in the cotyledons. (C-F) 2,4-D IOE promotes xylem differentiation in the *acl5-1* mutant but not in the wild type. Wild type (Wt) and the *acl5-1* mutant were grown for 10 days on the MS agar medium in the absence (Mock) or presence of 0.3 μM 2,4-D IOE. Xylem vessels in the root tips (upper panels in C, D, E), the basal parts of roots (lower panels in C) or leaf tips (F) were observed under a

microscope. White and red arrowheads in D and E point to the protoxylem vessels and metaxylem vessels, respectively. Arrow in F indicates excessive xylem differentiation in *acl5-1*. Bars = 100 μm (B, C, F) or 50 μm (D, E). (G) Effect of 2,4-D IOE on the width of xylem in the cotyledon midvein. Wild type (Wt) and the *acl5-1* mutant were grown for 7 days on the MS agar medium in the absence (Mock) or presence of 0.3 μM 2,4-D IOE. Values indicate means ± SEs (n = 9). Values designated by the same letter are not significantly different at the $P = 0.05$ level in the Student's *t*-test. (H) A model of interaction between thermospermine and auxin in xylem development. The upper panels show that thermospermine ("T") limits xylem differentiation (black line). In the lower panels, the black, red and blue lines represent auxin gradient, thermospremine (Tspm) gradient and the threshold for xylem differentiation, respectively. Arrows indicate xylem differentiation.