Running title: Opposite action between thermospermine and auxin

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A Chemical Biology Approach Reveals an Opposite Action between Thermospermine and Auxin in Xylem Development in *Arabidopsis thaliana*

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Abbreviations: ACL5, ACAULIS5; AMP1, ALTERED MERISTEM PROGRAM 1; ARF, AUXIN RESPONSE FACTOR; ATHB, ARABIDOPSIS THALIANA HOMEOBOX PROTEIN; BDL, BODENLOS; bHLH, basic helix-loop-helix; 4-Cl-IAA, 4-chloro IAA; 4Cl-PAA 4-chlorophenoxy propionic acid; CNA, CORONA; 2,3-D, 2,3-dichlorophenoxyacetic acid; 2,4-D, 2,4-dichlorophenoxyacetic acid; 2,4-DB, 2,4-dichlorophenoxy butyric acid; 5,6-DC-IAA, 5,6-dichloro IAA; DMSO, dimethyl sulfoxide; 2,4-DP, 2-(2,4-dichlorophenoxy)-propionic acid; 6-F-IAA, 6-fluoro IAA; HD-Zip III, class III homeodomain leucine-zipper; IBA, indole-3-butyric acid; IOE, isooctyl ester; MCPA, 2-methyl-4-chlorophenoxyacetic acid; MP, MONOPTEROS; MS, Murashige-Skoog; NAA, Naphthalene-1-acetic acid; PCIB, *p*-chlorophenoxyisobutyric acid; PEO-IAA, α-(phenyl ethyl-2-one)-IAA; PIN1, PIN-FORMED1; RPL10A, ribosomal protein L10a; RT-PCR, reverse transcription-PCR; SAC, SUPPRESSOR OF ACAULIS5; TDIF, tracheary

element differentiation inhibitory factor; TDR, TDIF receptor; TIR1/AFB, TRANSPORT INHIBITOR RESPONSE1/AUXIN-BINDING F-BOX PROTEIN; TSPM, thermospermine; VND7, VASCULAR NAM, ATAF1/2 and CUC2 DOMAIN7

Footnotes: The nucleotide sequence reported in this paper can be found in the Arabidopsis Genome Initiative or GenBank/EMBL databases under accession numbers; AT5G19530 (ACL5), AT1G52150 (ATHB15), AT5G64340 (SAC51), AT1G14320 (SAC52) and AT1G71930 (VND7). We would like to dedicate this paper to the memory of Prof. Atsushi Komamine (1929-2011).

Abstract: Thermospermine, a structural isomer of spermine, is produced through the action of ACAULIS5 (ACL5) and suppresses xylem differentiation in *Arabidopsis thaliana*. To elucidate the molecular basis of the function of thermospermine, we screened chemical libraries for compounds that can modulate xylem differentiation in the *acl5* mutant, which is deficient in thermospermine and shows a severe dwarf phenotype associated with excessive proliferation of xylem vessels. We found that the isooctyl ester of a synthetic auxin, 2,4-dichlorophenoxyacetic acid (2,4-D), remarkably enhanced xylem vessel differentiation in *acl5* seedlings. 2,4-D, 2,4-D analogs and IAA analogs, including 4-chloro IAA (4-Cl-IAA) and IAA ethyl ester, also enhanced xylem vessel formation while IAA alone had little or no obvious effect on xylem differentiation. These effects of auxin analogs were observed only in the *acl5* mutant but not in the wild type, and were suppressed by the anti-auxin, *p*-chlorophenoxyisobutyric acid (PCIB) and α-(phenyl ethyl-2-one)-IAA (PEO-IAA) and also by thermospermine. Furthermore, the *suppressor of acaulis51-d* (*sac51-d*) mutation, which causes *SAC51* overexpression in the absence of thermospermine and suppresses the dwarf phenotype of *acl5*, also suppressed the effect of auxin analogs in *acl5*. These results suggest that the auxin signaling that promotes xylem differentiation is normally limited by *SAC51*-mediated thermospermine signaling but can be continually stimulated by exogenous auxin analogs in the absence of thermospermine. The opposite action between thermospermine and auxin may fine-tune the timing and spatial pattern of xylem differentiation.

Key words: ACAULIS5, *Arabidopsis thaliana*, Auxin, 2,4-D, Thermospermine, Xylem differentiation

Introduction

Xylem is a specialized conductive tissue for water, nutrients and signaling molecules in vascular plants. The developmental mechanism of xylem vessel elements has been well studied as an excellent model for plant cell differentiation. Previous studies of tracheary element differentiation from isolated mesophyll cells of *Zinnia elegans* revealed that xylem development is regulated by multiple signals (reviewed in Motose et al. 2001, Fukuda et al. 2007). Among these signals, auxin plays a central role by activating key genes involved in xylem development and those required for auxin transport. According to the auxin canalization hypothesis, polar auxin transport generates local auxin flow, which in turn specifies procambial cell patterning (Sachs 1991, Aloni et al. 2003, Mattsson et al. 2003, Scarpella et al. 2006, 2010). Other signals including brassinosteroids (Yamamoto et al. 1997), xylogen, which is a kind of arabinogalactan proteins (Motose et al. 2004, Kobayashi et al. 2011), and phytosulfokine (Matsubayashi et al. 1999, Motose et al. 2009) also promote differentiation of tracheary elements, while cytokinin negatively regulates protoxylem differentiation (Mähönen et al. 2006). A peptide hormone designated tracheary element differentiation inhibitory factor (TDIF) is secreted from the phloem and suppresses xylem differentiation and promotes axillary bud formation through the binding to a receptor kinase, TDIF receptor (TDR) (Ito et al. 2006, Hirakawa et al. 2008, Yaginuma et al. 2011). These signals may interact with each other for spatial and temporal regulation of xylem vessel differentiation, although the interaction mechanisms are far from being completely understood (Lehesranta et al. 2010). Furthermore, thermospermine has recently been identified as another plant growth regulator that represses xylem differentiation and promotes stem elongation in *Arabidopsis thaliana* (Kakehi et al. 2008).

Thermospermine is a structural isomer of a major polyamine, spermine, detected in most eukaryotes, and was first discovered in a thermophilic bacterium, *Thermus thermophilus* (Oshima 1979). In contrast to animals where thermospermine is sporadically detected, most plants may have thermospermine (Minguet et al. 2008). The *acaulis5* (*acl5*) mutant of *A. thaliana* exhibits excessive differentiation of xylem tissues and severe dwarfism (Hanzawa et al. 1997, 2000, Clay and Nelson 2005, Muñiz et al. 2008) and was shown to be deficient in the synthesis of thermospermine (Kakehi et al. 2008), based on the catalytic activity of the recombinant ACL5 protein (Knott et al. 2007). Exogenously supplied thermospermine remarkably suppresses xylem vessel differentiation in *A. thaliana* and tracheary element differentiation of isolated mesophyll cells of *Z. elegans* (Kakehi et al. 2010). Because these effects of thermospermine can not be mimicked by spermine but by norspermine (Kakehi et al. 2010), the NC_3NC_3N arrangement of carbon chains present in thermospermine ($NC_3NC_3NC_4N$) and norspermine ($NC_3NC_3NC_3N$) but not in spermine ($NC_3NC_4NC_3N$) may be important for the action of these polyamines. Although the mode of action of thermospermine remains unclear, isolation and 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 (Imai et al. 2006, 2008). This is consistent with the fact that cellular polyamines generally interact with RNA molecules and influence their translation (Igarashi and Kashiwagi 2010). Consequently, thermospermine enhances translation of *SAC51*, which encodes a basic helix-loop-helix (bHLH) transcription factor, and *SAC51* in turn may function in restricting proliferation of xylem cells and in promoting stem elongation. To investigate how thermospermine or *SAC51*-mediated thermospermine signaling participates in the regulatory

mechanism of xylem differentiation, we screened for chemicals that affect xylem developmet in *acl5* mutants and identified the isooctyl ester of 2,4-dichlorophenoxyacetic acid (2,4-D IOE; Fig. 1A) as a potent enhancer of xylem vessel differentiation. Our results suggest a critical requirement for thermospermine as an opposing factor to auxin in the regulation of the timing and spatial pattern of xylem differentiation.

Results

Chemical screening identifies 2,4-D IOE with a potent xylem-inducing activity in *acl5*

The *acl5* mutant seedling has thicker veins in cotyledons compared to the wild-type seedling (Fig. 1B). As an alternative to genetic screening for suppressor or enhancer mutants of *acl5*, we screened a chemical library containing 1680 compounds to find molecules that influence the vein phenotype in cotyledons of *acl5*. The *acl5-1* plants were grown for 7 d in liquid medium supplemented with different chemicals at 10 μM, and one compound, 2,4-D IOE, was identified for its activity in modulating the mutant phenotype. 2,4-D IOE induced ectopic xylem formation around veins and resulted in disordered clusters of xylem vessel elements in *acl5-1* cotyledons, while 2,4-D IOE had no apparent effect on wild-type cotyledons (Fig. 1B). We examined the effect of different concentrations of 2,4-D IOE on the mutant phenotype. 2,4-D IOE at concentrations of 1, 3, and 30 μM remarkably induced excessive xylem vessel differentiation (Fig. 1C). Even at the concentration of 0.3 μM, 2,4-D IOE had a subtle but obvious enhancing effect on the formation of extra xylem vessels around veins.

2,4-D also induces extra xylem vessels in *acl5*

We examined the effect of 2,4-D IOE on the expression of the auxin-responsive *DR5:GFP*

transgene expression in the wild-type background and confirmed that the GFP fluorescence was greatly increased by growing the seedlings in the presence of 2,4-D IOE (Supplementary Fig. S1), suggesting that 2,4-D IOE possesses auxin activity or is converted to 2,4-D. We then examined the effect of 2,4-D on xylem differentiation in *acl5-1* and found that 2,4-D also enhanced excessive xylem vessel formation (Fig. 2A). To investigate changes in the responsiveness to 2,4-D during vascular development in cotyledons, we added 2,4-D at various time of culture. 2,4-D added by the second day of culture could enhance excessive xylem differentiation in *acl5-1* whereas 2,4-D added after the third day of culture was less effective (Fig. 2B), indicating that the mutant cotyledons immediately after germination are responsive to 2,4-D.

We next compared the effect of 2,4-D IOE and 2,4-D on the growth of *acl5-1* seedlings grown on agar plates. Although they were similarly effective in inducing extra xylem vessels, 2,4-D IOE was much less toxic to the growth of seedlings than 2,4-D, which severely interfered with cotyledon expansion, hypocotyl elongation, and root development at 0.05 μM (Fig. 3A, B). Application of 2,4-D at 0.3 μM no longer resulted in development of foliage leaves (Fig. 3A), while 2,4-D IOE at 0.3 μM caused a change in the venation pattern of leaves both in wild type and *acl5-1* (enhanced vascularization along the leaf margin and more secondary veins branched from the base of the leaf, Fig. 3C). We also confirmed that, in wild-type seedlings, both 2,4-D IOE and 2,4-D at 0.3 μM were toxic to the growth but had no enhancing effect on xylem development (Figs. 1, 2, 3).

2,4-D up-regulates the genes down-regulated by thermospermine

We further examined the effect of 2,4-D on the expression of *ACL5*, *ARABIDOPSIS*

THARIANA HOMEOBOX15/*CORONA* (*ATHB15*/*CNA*), which is a member of the class III homeodomain leucine-zipper (HD-Zip III) gene family and plays a pivotal role in transcriptional regulation of the genes responsible for early vascular development (Ohashi-Ito and Fukuda 2003, Green et al. 2005), and *VASCULAR NAM, ATAF1/2 and CUC2 DOMAIN7* (*VND7*), which encodes a master transcription factor for xylem vessel differentiation (Kubo et al. 2005, Yamaguchi et al. 2008). These genes show increased expression in *acl5-1* compared to the wild type and are down-regulated by exogenous thermospermine (Imai et al. 2006, Kakehi et al. 2008). Our experiments revealed that the transcript levels of *ACL5*, *ATHB15 /CNA*, and *VND7* were up-regulated in both *acl5-1* and wild-type seedlings grown with 2,4-D compared to those grown without 2,4-D (Fig. 4).

Auxin analogs enhance xylem differentiation in *acl5*

Other 2,4-D analogs including 4-chlorophenoxy propionic acid (4Cl-PAA), 2,3-dichlorophenoxyacetic acid (2,3-D), 2-(2,4-dichlorophenoxy)-propionic acid (2,4-DP), 2-methyl-4-chlorophenoxyacetic acid (MCPA), 2,4-dichlorophenoxy butyric acid (2,4-DB), 2,4-dichlorophenoxy butyric acid methyl ester, and a member of the picolinate class of auxins, picloram, were also tested and found to induce extra xylem vessels in *acl5-1*, although the degree of the effect varied (Fig. 5, Supplementary Fig. S2). These auxin analogs did not induce extra xylem vessels in the wild type (Supplementary Fig. S3). Among them, 4Cl-PAA particularly had a strong effect on excessive xylem vessel formation, suggesting that 4Cl-PAA represents a core structure for ectopic xylem-inducing activity in *acl5-1*. Furthermore, we found that the isooctyl ester of a natural auxin, indole-3-acetic acid (IAA IOE), resulted in excessive xylem vessel formation in *acl5-1* but the original IAA had no effect at 10 μM and

only little effect on the mutant phenotype even at the concentration of 100 μM (Fig. 5). Naphthalene-1-acetic acid (NAA) and indole-3-butyric acid (IBA) did not induce extra xylem vessels in *acl5-1* (Supplementary Fig. S4).

The less effectiveness of IAA might be due to efficient secretion of IAA by the auxin efflux carrier (Delbarre et al., 1996), low permeability into the plants (Savaldi-Goldstein et al. 2008), and/or auxin homeostasis and metabolism including inactivation of excess IAA by conjugation to amino acids (Staswick et al. 2005, Mravec et al. 2009, Mashiguchi et al. 2011). Therefore, we analyzed effect of halogenated IAA analogs, which are resistant to auxin metabolism such as amino acid conjugation, on xylem differentiation in *acl5-1* (Fig. 6). A natural auxin, 4-chloro-IAA (4-Cl-IAA), strongly induced xylem vessel differentiation in *acl5-1* as well as 2,4-D and 2,4-D IOE (Fig. 6A). 6-fluoro-IAA (6-F-IAA), 5,6-dichloro-IAA (5,6-DC-IAA) methyl ester and 4-Cl-IAA methyl ester also induced extra xylem vessels in *acl5-1* (Fig. 6B). These IAA analogs did not induce extra xylem vessels in the wild type (Supplementary Figs. S5). These results indicate that exogenously applied IAA may be rapidly inactivated by auxin metabolism.

In addition, we found that IAA ethyl ester and NAA ethyl ester also strongly induced xylem vessel differentiation in *acl5-1* (Fig. 7). Because the esterified auxins may diffuse efficiently to the tissue and undergo cleavage to release functional auxins (Qin et al. 2005, Gershater et al. 2006, 2007, Savaldi-Goldstein et al. 2008, Yang et al. 2008), the less effectiveness of IAA and NAA might be due to low permeability into the plants.

Excessive xylem differentiation is suppressed by anti-auxin and thermospermine

The anti-auxin, *p*-chlorophenoxyisobutyric acid (PCIB) is thought to antagonize and block

auxin-dependent degradation of Aux/IAA proteins (Oono et al. 2003). To know whether PCIB suppresses the effect of auxin analogs on xylem differentiation in *acl5-1* or not, we added PCIB to liquid medium with 2,4-D or 2,4-D IOE. PCIB significantly suppressed xylem differentiation induced by both 2,4-D and 2,4-D IOE (Fig. 8A), indicating that the effect of 2,4-D IOE is mediated by auxin signaling pathway blocked by PCIB.

α-alkyl-IAA, especially α-(phenyl ethyl-2-one)-IAA (PEO-IAA), is a potent and specific antagonist of auxin receptors; TRANSPORT INHIBITOR RESPONSE1/AUXIN-BINDING F-BOX PROTEIN (TIR1/AFB) family (Hayashi et al. 2008, 2012). To investigate whether effect of 2,4-D and 2,4-D IOE on xylem vessel differentiation is mediated by the auxin receptors or not, we added PEO-IAA to liquid medium with 2,4-D or 2,4-D IOE. PEO-IAA strongly suppressed xylem differentiation induced by both 2,4-D and 2,4-D IOE (Fig. 8B), indicating that the effect of 2,4-D and 2,4-D IOE is mediated by TIR1/AFB auxin receptor family.

We also examined the effect of exogenous thermospermine on the 2,4-D-induced extra xylem differentiation in *acl5-1*. Thermospermine added to liquid medium concomitantly with 2,4-D clearly suppressed xylem differentiation in *acl5-1* (Fig. 8C). The three polyamines, spermine, spermidine and putrescine did not suppress 2,4-D-induced extra xylem differentiation whereas norspermine suppressed extra xylem differentiation (Supplementary Fig. S6). This is consistent with our previous report that norspermine can substitute for thermospermine in repressing xylem differentiation (Kakehi et al. 2010). Finally, the effect of *sac51-d* and *sac52-d* mutations on the 2,4-D-induced xylem formation in *acl5-1* was investigated. *SAC51* and *SAC52* encode a bHLH-type transcription factor and a ribosomal protein L10a (RPL10A), respectively. *sac51-d* and *sac52-d* are both dominant alleles and

suppress the dwarf phenotype of *acl5-1* in the absence of thermospermine probably because of SAC51 overproduction (Imai et al. 2006, 2008). In both *acl5-1 sac51-d* and *acl5-1 sac52-d* double mutants, 2,4-D did not induce excessive xylem differentiation (Fig. 8D).

Discussion

2,4-D is a synthetic auxin with various biological effects on plant growth and development including root growth inhibition and lateral root induction (Estelle and Somerville, 1987). Because of its potent activity, 2,4-D has been used as an herbicide or an essential factor in culture system for cell proliferation (Nagata et al, 1992), somatic embryogenesis (Nomura and Komamine, 1986), and xylem differentiation (Fukuda and Komamine, 1980). In this study, we identified an esterified 2,4-D analog, 2,4-D IOE, as a compound that induces extra xylem vessel formation around veins in *acl5-1* seedlings of *A. thaliana*. Our experiments using other auxin analogs revealed that, although the degree may differ, 2,4-D, some 2,4-D analogs, and IAA analogs also had enhancing effects on xylem differentiation in *acl5-1*. A previous study using chemical genetics has identified 2,4-D analogs with an amide linkage as growth-promoting compounds that promote hypocotyl elongation in *A. thaliana* (Savaldi-Goldstein et al. 2008). These compounds, whether masked with an amide linkage or an ester linkage, may act as “proauxins”, which diffuse efficiently to the tissue, where they undergo cleavage and release functional auxins (Yang et al. 2005, Gershater et al. 2006, 2007, Savaldi-Goldstein et al. 2008, Qin et al. 2008). Indeed, 2,4-D IOE was shown to induce expression of the auxin responsive *DR5* reporter. Furthermore, anti-auxin PCIB and PEO-IAA suppressed xylem-inducing effect of 2,4-D and 2,4-D IOE. These results suggest that 2,4-D IOE may permeate efficiently into the tissue and release active 2,4-D, which promotes xylem

vessel differentiation through TIR1/AFB-mediated auxin signaling pathway. Because the xylem-inducing effect of auxin analogs is only observed in *acl5* mutants, the phenotype found in this study might not be due to a side effect of auxin analogs but instead to the specific opposite action between auxin and thermospermine.

IAA is the principal natural auxin and the level of active IAA is maintained strictly by auxin homeostasis including synthesis, transport, inactivation and degradation (Normanly 1997; Woodward and Bartel 2005; Mravec et al. 2009). Exogenously applied IAA or overexpression of auxin biosynthesis genes increased IAA conjugates, which are inactive forms for storage or degradation (Nordström et al. 1991; Mashiguchi et al. 2011). Therefore, it would not be surprising that exogenous IAA was not effective even at high concentration in this study and other reports (Katayama 2000; Ozga et al. 2009). Actually, metabolism-resistant IAA analogs induce differentiation of extra xylem vessels in *acl5-1*, suggesting that excess IAA is inactivated by auxin metabolism. In particular, a natural auxin 4-Cl-IAA has a strong xylem-inducing activity equivalent to 2,4-D and 2,4-D IOE. Because 4-Cl-IAA is a poor substrate for amino acid conjugation as well as 2,4-D (Staswick et al. 2005), the effect of auxin analogs may depend on their metabolic stability rather than their artificial side effects. Alternatively, but not exclusively, the differential effect of auxin could be attributed to differential diffusion and transport properties. The high xylem-inducing activity of ethyl ester of IAA and NAA may be due to their increased permeability into the tissue (Savaldi-Goldstein et al. 2008).

A previous study has shown that 2,4-D accumulates efficiently in the cytoplasm compared to IAA and NAA because of its preferential uptake by the influx carrier and no substantial secretion by the efflux carrier (Delbarre et al., 1996). Because the concentration of

2,4-D that causes extra xylem differentiation is also toxic to the growth of seedlings, it is clear that relatively high concentrations of auxin are required for extra xylem differentiation in *acl5*. Taking into account their local effect around veins in *acl5*, exogenously supplied 2,4-D and auxin analogs that cause the extra xylem phenotype may be continually accumulated in the cells around xylem precursor cells over the threshold for xylem differentiation in the absence of thermospermine.

Exogenous thermospermine also suppressed the xylem-inducing effect of 2,4-D in *acl5-1*. Unlike PCIB and PEO-IAA, however, thermospermine may not act in an antagonistic manner to auxin action. Although its precise mode of action remains to be elucidated, thermospermine activates translation of *SAC51*, which contains multiple uORFs (Imai et al. 2006). Both *sac51-d* in which the 4th uORF of *SAC51* contains a premature termination codon and *sac52-d*, a mutant of the ribosomal large subunit protein L10 gene *RPL10A*, may cause SAC51 overproduction, thereby uncoupling stem elongation and repression of xylem development from the action of thermospermine (Imai et al. 2008). Our results showed that both *sac51-d* and *sac52-d* also suppressed the xylem-inducing effect of 2,4-D in *acl5-1*. These findings suggest that the auxin signaling that promotes xylem differentiation is normally limited by *SAC51*-mediated thermosp**er**mine signaling but can be continually stimulated by exogenous auxin analogs in the absence of thermospermine (Fig. 9).

What is the biological significance of such opposite effects between thermospermine and auxin? Recent studies on embryo development in *A. thaliana* reveal that auxin activates *AUXIN RESPONSE FACTOR5/MONOPTEROS* (*ARF5/MP*), a master transcriptional regulator for vascular development, which in turn promotes expression of an auxin efflux protein gene *PIN-FORMED1* (*PIN1*) and an HD-Zip III gene *ATHB8* (Wenzel et

al. 2007, Donner et al. 2009). While ATHB8 plays a key role in procambial cell proliferation and xylem differentiation (Baima et al. 2001), PIN1 generates a directional flow of auxin by its polar localization in the plasma membrane (Kleine-Vehn and Friml 2008, Petrášek and Friml 2009), leading to a positive feedback where localized high concentrations of auxin attract more auxin. This scenario consistently explains the auxin canalization model of vascular pattern formation. However, this type of positive feedback loop would require a negative regulator to prevent excessive amplification. Indeed, it is well known that the expression of auxin response factors is under negative feedback control by auxin-inducible Aux/IAA proteins [in the case of *ARF5/MP* by *BODENLOS* (*BDL*)*/IAA12*, Hamann et al. 2002]. *ARF5/MP* has further been shown to counteract the differentiation-promoting activity of *ALTERED MERISTEM PROGRAM 1* (*AMP1*, Vidaurre et al. 2007). In the process of xylem vessel differentiation, which involves programmed cell death along with thickening of secondary cell walls, overproliferation of xylem precursor cells would be contradictory to organ expansion or elongation and must be properly restricted. *ACL5* is predominantly expressed in early developing vessel elements (Muñiz et al. 2008). Together with our results that *ACL5*, *ATHB15/ CNA*, and *VND7*, all of which are up-regulated in *acl5-1*, were further up-regulated by 2,4-D, it is suggested that thermospermine is critically required for limiting the positive feedback effect of auxin described above and consequently for tuning the timing and spatial pattern of xylem differentiation.

In summary, 2,4-D analogs and also IAA analogs were shown to have a profound effect on xylem vessel differentiation in the absence of thermospermine. Further analysis of the effect of these auxin analogs and thermospermine using mutants related to auxin signaling and transport and those suppressing thermospermine deficiency will provide insight into the

molecular mechanism of timing and patterning of xylem differentiation. The xylem-inducing auxin analogs may also be useful for agricultural applications.

Materials and Methods

Chemicals

All chemicals used in this study were purchased from Sigma-Aldrich (http://www.sigmaaldrich.com). Thermospermine was provided from Prof. Masaru Niitsu (Josai University). Auxin analogs were synthesized according to Hayashi et al. (2008, 2012), Kusaka et al. (2009), and Tsuda et al. (2011). Auxin analogs were dissolved in dimethyl sulfoxide (DMSO) and adjusted so that the final concentration of DMSO in each medium was less than 0.1%.

Plant materials and growth condition

The *acl5-1*, *acl5-1 sac51d*, and *acl5-1 sac52d* mutants were as previously described (Hanzawa et al., 1997; Imai et al., 2006; 2008). *DR5:GFP* line was as described in Friml et al. (2003) and provided from ABRC. Plants were grown in the modified Murashige-Skoog (MS) medium supplemented with 1% sucrose at 22 °C under continuous white light as described (Motose et al., 2008; 2011). The liquid MS medium was used unless otherwise described. For the auxin treatment, 500 µl of MS liquid medium supplemented with auxin was prepared in 24-well plates and five to ten surface-sterilized seeds were spotted in each well, stratified for 2 d, and observed at the 7th d after germination for xylem vessel differentiation. For the experiment described in Figure 6, PCIB or TSPM was added simultaneously with auxin. For the experiment using MS agar medium described in Figure 3, the *acl5-1* mutant seeds were

spotted and grown on the MS medium supplemented with 2,4-D or 2,4-D IOE and solidified with 1% agar.

Chemical screening.

A chemical library (The Spectrum Collection, MicroSource Discovery Systems Inc., http://www.msdiscovery.com/home.html) contained 720 natural products and 960 pharmaceuticals in a final concentration of 10 mM in DMSO. For screening, 500 μL of MS liquid medium supplemented with individual compounds at the concentration of 10 μM was prepared in 24-well plates. Five to ten surface-sterilized seeds of *acl5-1* were spotted in each well, stratified for 2 d, and observed at the 7th d after germination for xylem vessel differentiation.

Histology and microscopy

Seedlings were fixed in a 9:1 mixture of ethanol and acetic acid. Fixed samples were then cleared in a mixture of chloral hydrate, glycerol, and water solution (8 g : 1 ml : 2 ml) and observed under a light microscope equipped with a Nomarski optics (DM5000B, Leica). GFP fluorescence was observed under a fluorescent microscope (DM5000B, Leica).

Real-time reverse transcription (RT)-PCR

Total RNA was isolated as described (Motose et al., 2008; 2009) from the wild-type and *acl5-1* seedlings grown in liquid medium for 7 d. For each sample, 30 ng of total RNA was reverse transcribed to cDNA using ReverTra Ace reverse transcriptase (TOYOBO, http://www.toyobo.co.jp) according to the accompanying protocol. Real-time PCR was

performed in a DNA Engine Opticon2 System (Bio-Rad, http://www3.bio-rad.com) using the iQ SYBR Green Supermix (Bio-Rad) and gene-specific primers (5'-ACCGTTAACCAGCGATGCTTT-3' and 5'-CCGTTAACTCTCTCTTTGATTCTTCGATCC-3' for *ACL5*, 5'-GAAACTCCACATGTGCAAGA-3' and 5'-TCTCTCATACGAAACTGGTC-3' for *ATHB15*, 5'-CGATGCATCAATATGGCAAC-3' and 5'-AGGGAAGCATCCAAGAGAAT-3' for *VND7*, and 5'-GTGAGCCAGATCTTCATTCGTC-3' and 5'-TCTCTTGCTCGTAGTCGACAG-3' for *ACTIN8*) according to Kakehi et al (2008). Transcript levels of *ACTIN8* were used as a reference for normalization.

Supplementary data

Supplementary data are available at PCP online.

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Figure Legends

Fig. 1 2,4-D IOE induces excessive xylem differentiation in the *acl5-1* mutant. (A) Structure of 2,4-D IOE and 2,4-D. (B) Effect of 2,4-D IOE on xylem vessel differentiation. Wild type (Wt) and the *acl5-1* mutant were grown for 7 d in liquid medium in the absence (Mock) or presence of 10 μM 2,4-D IOE and xylem vessels in cotyledons were analyzed under a microscope. (C) Dose-dependent effect of 2,4-D IOE on xylem vessel differentiation in *acl5-1*. The *acl5-1* mutant was grown for 7 d in liquid medium supplemented with 0.3, 1, 3, or 30 μM 2,4-D IOE and xylem vessels in cotyledons were analyzed under a microscope. Scale bars represent 100 μm.

Fig. 2 2,4-D induces excessive xylem differentiation in the *acl5-1* mutant. (A) Effect of 2,4-D on xylem vessel differentiation. Wild type (Wt) and the *acl5-1* mutant were grown for 7 d in liquid medium supplemented with 10 μM 2,4-D and xylem vessels in cotyledons were analyzed under a microscope. (B) Changes in the responsiveness to 2,4-D during vascular development in cotyledons. 2,4-D at the concentration of 10 μM was added after the first day

(1 d), second day (2 d), third day (3 d), or fourth day (4 d) of liquid culture of *acl5-1* and xylem vessels in cotyledons were analyzed at 7 d of culture under a microscope. Scale bars represent 100 μm.

Fig. 3 Effects of 2,4-D and 2,4-D IOE on the growth of wild type and *acl5-1* seedlings. Wild type (Wt) and the *acl5-1* mutant were grown on the agar medium supplemented with 2,4-D or 2,4-D IOE at the concentration of 0.05, 0.3, or 3 μM. (A) Morphology of *acl5-1* grown for 7 d in the absence (Mock) or presence of 2,4-D or 2,4-D IOE. (B) Root length of *acl5-1* grown for 7 d in the absence (Mock) or presence of 2,4-D or 2,4-D IOE. The data are represented as the mean ± SD (n = 10). (C) Xylem vessels in leaves of Wt and *acl5-1* grown for 10 d with 2,4-D IOE at the concentration of 0.3 μM. Scale bar represents 100 μm.

Fig. 4 2,4-D promotes expression of *ACL5*, *ATHB15*, and *VND7*. Total RNA was prepared from the wild type (Wt) and *acl5-1* seedlings, which were grown for 7 d in liquid medium without 2,4-D (-) or with 3 μM 2,4-D (+). Transcript levels of *ACL5*, *ATHB15*, and *VND7* were quantified by real-time RT-PCR and relative values were normalized to the expression of *ACT8*. The level of the mock-treated wild-type sample was set at 1.0. The data are represented as the mean ± SD (n = 3).

Fig. 5 Effects of 2,4-D analogs, picloram, IAA, and IAA IOE on xylem differentiation in *acl5-1*. The *acl5-1* mutant was grown for 7 d in liquid medium supplemented with 4Cl-PAA, 2,3-D, picloram, IAA IOE, or IAA, and analyzed for xylem vessel differentiation in cotyledons. The concentration of 4Cl-PAA, 2,3-D, picloram, and IAA IOE is 10 μM and the

concentration of IAA is 100 μM. Scale bar represents 100 μm.

Fig. 6 Effects of halogenated IAA analogs on xylem differentiation in *acl5-1*. (A) The *acl5-1* mutant was grown for 7 d in liquid medium supplemented with various concentrations of 4-Cl-IAA and analyzed for xylem vessel differentiation in cotyledons. (B) The *acl5-1* mutant was grown for 7 d in liquid medium supplemented with 10 μM of 6-F-IAA, 5,6-DC-IAA methyl ester or 4-Cl-IAA methyl ester, and analyzed for xylem vessel differentiation in cotyledons. Scale bars represent 100 μm.

Fig. 7 Effects of IAA ethyl ester and NAA ethyl ester on xylem differentiation. The wild type and *acl5-1* mutant was grown for 7 d in liquid medium supplemented with 10 μM of IAA ethyl ester or NAA ethyl ester and analyzed for xylem vessel differentiation in cotyledons. Scale bar represents 100 μm.

Fig. 8 Anti-auxin, thermospermine and *sac* mutations suppress 2,4-D-inducible xylem differentiation in *acl5-1*. (A) PCIB suppresses inductive effect of 2,4-D-IOE and 2,4-D on xylem vessel differentiation. The *acl5-1* mutant was grown for 7 d in liquid medium supplemented with 3 μM 2,4-D or 2,4-D-IOE and 20 μM PCIB and xylem vessels in cotyledons were analyzed under a microscope. (B) PEO-IAA suppresses inductive effect of 2,4-D-IOE and 2,4-D on xylem vessel differentiation. The *acl5-1* mutant was grown for 7 d in liquid medium supplemented with 3 μM 2,4-D or 2,4-D-IOE and 100 μM PEO-IAA and xylem vessels in cotyledons were analyzed under a microscope. (C) Thermospermine suppresses inductive effect of 2,4-D on xylem vessel differentiation. The *acl5-1* mutant was

grown for 7 d in liquid medium supplemented with 10 μM thermospermine (TSPM) or with 10 μM TSPM and 3 μM 2,4-D (TSPM+2,4-D) and xylem vessels in cotyledons were analyzed under a microscope. (D) The *acl5-1 sac51-d* and *acl5-1 sac52-d* mutants were grown for 7 d in liquid medium supplemented with 3 μM 2,4-D and xylem vessels in cotyledons were analyzed under a microscope. Scale bars represent 100 μm.

Fig. 9 Proposed model of an opposite action of auxin and thermospermine in xylem vessel differentiation. (A) In the wild type, thermospermine biosynthesized by ACL5 promotes SAC51 translation and then SAC51 suppresses auxin-inducible xylem vessel differentiation program. (B) In the case of thermospermine-deficient *acl5* mutant, auxin signaling that promotes xylem differentiation can be continually stimulated by exogenous auxin analogs. (C) The *sac51-d* mutation causes SAC51 overproduction and suppresses xylem vessel differentiation program, which can be activated by auxin analogs.

Supplementary Fig. S1 2,4-D IOE and 2,4-D promote the auxin-response reporter *DR5:GFP* expression. *DR5:GFP* seedlings were grown for 7 d in liquid medium supplemented with 10 μM 2,4-D-IOE or 2,4-D and analyzed for GFP fluorescence. Scale bar represents 100 μm.

Supplementary Fig. S2 Effects of 2,4-D analogs on xylem differentiation in *acl5-1*. The *acl5-1* mutant was grown for 7 d in liquid medium supplemented with 2,4-DP, MCPA, 2,4-DB, or 2,4-DB methyl ester at the concentration of 10 μM, and analyzed for xylem vessel differentiation in cotyledons. Scale bar represents 100 μm.

Supplementary Fig. S3 Effects of 2,4-D analogs, picloram, IAA, and IAA IOE on xylem differentiation in the wild type. The wild type was grown for 7 d in liquid medium supplemented with 4Cl-PAA, 2,3-D, picloram, IAA IOE, or IAA, and analyzed for xylem vessel differentiation in cotyledons. The concentration of 4Cl-PAA, 2,3-D, picloram, and IAA IOE is 10 μM and the concentration of IAA is 100 μM. Scale bar represents 100 μm.

Supplementary Fig. S4 Effects of NAA and IBA on xylem differentiation in *acl5-1*. The *acl5-1* mutant was grown for 7 d in liquid medium supplemented with NAA or IBA at the concentration of 100 μM and analyzed for xylem vessel differentiation in cotyledons. Scale bar represents 100 μm.

Supplementary Fig. S5 Effects of IAA analogs on xylem differentiation in the wild type. (A) The wild type was grown for 7 d in liquid medium supplemented with various concentrations of 4-Cl-IAA and analyzed for xylem vessel differentiation in cotyledons. (B) The wild type was grown for 7 d in liquid medium supplemented with 10 μM of 6-F-IAA, 5,6-DC-IAA methyl ester or 4-Cl-IAA methyl ester, and analyzed for xylem vessel differentiation in cotyledons. Scale bars represent 100 μm.

Supplementary Fig. S6 2,4-D-inducible xylem differentiation in *acl5-1* is suppressed by norspermine but not by spermine, spermindine, and putrescine. The *acl5-1* mutant was grown for 7 d in liquid medium supplemented with norspermine (NSPM), putrescine (PUT), spermindine (SPD), spermine (SPM), norspermine and 2,4-D (NSPM+2,4-D), putrescine and

2,4-D (PUT+2,4-D), spermindine and 2,4-D (SPD+2,4-D) or spermine and 2,4-D (SPM+2,4-D), and xylem vessels in cotyledons were analyzed under a microscope. The concentration of 2,4-D is 3 μM and the concentration of polyamines is 10 μM. Scale bar represents 100 μm.