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ACAULIS5 controls *Arabidopsis* xylem specification through the prevention of premature cell death

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Cell size and secondary cell wall patterning are crucial for the proper functioning of xylem vessel elements in the vascular tissues of plants. Through detailed anatomical characterization of *Arabidopsis thaliana* hypocotyls, we observed that mutations in the putative spermine biosynthetic gene *ACL5* severely affected xylem specification: the xylem vessel elements of the *acl5* mutant were small and mainly of the spiral type, and the normally predominant pitted vessels as well as the xylem fibers were completely missing. The cell-specific expression of *ACL5* in the early developing vessel elements, as detected by in situ hybridization and reporter gene analyses, suggested that the observed xylem vessel defects were caused directly by the *acl5* mutation. Exogenous spermine prolonged xylem element differentiation and stimulated cell expansion and cell wall elaboration in xylogenic cell cultures of *Zinnia elegans*, suggesting that ACL5 prevents premature death of the developing vessel elements to allow complete expansion and secondary cell wall patterning. This was further supported by our observations that the vessel elements of *acl5* seemed to initiate the cell death program too early and that the xylem defects associated with *acl5* could be largely phenocopied by induction of premature, diphtheria toxin-mediated cell death in the *ACL5*-expressing vessel elements. We therefore provide, for the first time, mechanistic evidence for the function of ACL5 in xylem specification through its action on the duration of xylem element differentiation.

KEY WORDS: ACL5, *Arabidopsis*, Cell death, Secondary cell wall, Tracheary element, Xylem specification

INTRODUCTION

Organ differentiation in eukaryotes typically requires the proper coordination of several different developmental processes. For example, the shift from vegetative to reproductive development in *Arabidopsis thaliana* involves a significant increase in the internode length of the inflorescence stem, which has to be coordinated with building of a functional vascular system, including the elongated structures of xylem. During the active elongation phase of the inflorescence stem, primary 'protoxylem' vessel elements differentiate and develop spiral or annular secondary cell wall thickenings that allow longitudinal expansion of the cells and, therefore, elongation of the stem. When internode elongation ceases, primary 'metaxylem' vessel elements with the more elaborate type of reticulate or pitted secondary cell wall thickenings differentiate. The primary vascular development is followed by the formation of vascular cambium and secondary growth, which, in addition to vessel differentiation, involves the formation of xylem fibers.

Plant hormones are involved in the control of all the different stages of xylem development. Physiological and pharmacological studies have demonstrated the important role of auxins and cytokinins in controlling the activity of the vascular cambium and the initiation of xylem development, while brassinosteroids, ethylene and gibberellins are important in the modulation of the cambial activity and the control of xylem differentiation (reviewed by Ye, 2002). Mutations in the various components of hormone synthesis, transport or signal transduction in *Arabidopsis* have largely confirmed the action of the various hormones and demonstrated the involvement of additional compounds, such as sterols (reviewed by Fukuda, 2004). Mutations affecting hormone transport and/or signaling provide evidence for the role of auxins in the initiation of the vascular meristem and the maintenance of vascular continuity (Gälweiler et al., 1998; Hardtke and Berleth, 1998; Hobbie et al., 2000), the role of cytokinins in phloem specification (Mähönen et al., 2000) and the inhibition of protoxylem differentiation (Mähönen et al., 2006), and the role of brassinosteroids in promoting xylem differentiation (Caño-Delgado et al., 2004). However, *Arabidopsis* mutants have demonstrated that there are as yet unknown signals that regulate xylem development (Koizumi et al., 2000; Parker et al., 2003). In addition, the signals controlling the maturation of xylem elements remain largely unknown.

Polyamines (PAs) are low molecular weight cationic molecules, the synthesis of which is initiated by decarboxylation of arginine or ornithine to produce putrescine, and sequential addition of two aminopropyl groups to putrescine through the activity of the aminopropyltransferases spermidine synthase and spermine synthase, to produce the triamine spermidine and the tetraamine spermine, respectively (Ikeguchi et al., 2006). *Arabidopsis* has two putative spermidine synthases (SPDS1 and SPDS2) and two putative spermine synthases (SPMS and ACL5) (Imai et al., 2004b; Panicot et al., 2002). Bacterially produced ACL5 was recently related to synthesis of thermospermine, which is an isomer of spermine (Knott et al., 2007), but this remains to be validated in planta. PAs have been shown to be involved in a variety of processes, such as cell proliferation and defense against both abiotic and biotic stresses, but they are also associated with the normal development of plants (Kumar et al., 1997; Walden et al., 1997). It

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has also been proposed that they participate in the control of vascular development based on their effect on cell division, interaction with other hormones and H_2O_2 produced during PA catabolism that could potentially affect processes such as vascular cambial activity, cell differentiation and cell death (Bais and Ravishankar, 2002; Møller and McPherson, 1998; Sebela et al., 2001). Indirect support for this suggestion was provided by spermidine- and spermine-deficient transgenic plants, which exhibited a stunted phenotype (Kumar et al., 1996). In addition, an *Arabidopsis* mutant in the polyamine biosynthesis-related S-adenosylmethionine decarboxylase, with slightly reduced spermidine and spermine levels, was found to be stunted and to exhibit severely altered vascular development (Ge et al., 2006). However, a more direct involvement was demonstrated only recently, with the identification of *ACAULIS 5* (*ACL5*) as a putative spermine synthase (Hanzawa et al., 2000). The *Arabidopsis acaulis* mutants were isolated on the basis of their severely impaired internodal elongation after the transition from the vegetative to the reproductive stage (Akamatsu et al., 1999), and *acl5* was found to display overproliferation of xylem elements (Hanzawa et al., 1997). Furthermore, the *thickvein* mutant, harboring another loss-of-function allele of *ACL5*, was shown to display thicker veins and an increased number of vascular cells in the inflorescence stems (Clay and Nelson, 2005). Interestingly, changes to vascular development seem rather specific to the *acl5* mutant, as the lack of spermidine synthesis in *Arabidopsis* is embryo lethal (Imai et al., 2004b), whereas mutations in *SPMS*, which encodes the major spermine synthase, do not affect plant development (Imai et al., 2004a). Recently, a mutation that allows higher production of a bHLH transcription factor, SAC51, was shown to suppress all the defects associated with the loss of ACL5 function (Imai et al., 2006), but as the suppressor mutant of bHLH was dominant it is not entirely clear whether its function is directly related to PA signaling. Therefore, the underlying mechanism for the action of PAs in plant growth and development remains unclear.

ACL5 is specifically expressed in the procambial and/or the provascular tissues during primary growth of the root (Birnbaum et al., 2003; Clay and Nelson, 2005), but it does not seem to have any major function at this stage (Clay and Nelson, 2005). To elucidate the function of ACL5 during vascular development, we therefore focused in the current study on the vascular tissues of the hypocotyl, which display extensive secondary growth during prolonged growth period. We demonstrate here that, in the hypocotyl as well as in the inflorescence stem, *ACL5* is expressed not just broadly with respect to vasculature, as shown earlier (Clay and Nelson, 2005), but specifically in the xylem vessel elements at a strictly defined developmental stage, suggesting direct involvement of ACL5 in xylem vessel differentiation. Furthermore, we show that the *acl5* mutant displays severe overall inhibition of the secondary growth of the vascular tissues, dramatic alteration in the morphology of the vessel elements and complete lack of xylem fibers. Finally, we propose a mechanistic model for the function of ACL5 in xylem specification, based on experiments carried out in transgenic plants expressing a *DT-A* toxin gene under the control of the *ACL5* promoter and in the *Zinnia elegans* tracheary element differentiation system.

MATERIALS AND METHODS

Plant material and growth conditions

The analyses of the *Arabidopsis thaliana acl5* mutant were conducted using the mutant allele *acl5-4* (in L*er* background) or the SALK_028736 line, when comparisons were made with transgenic Pro_{ACL5}*:DT-A*, Pro_{DR5}*:GUS*, or Pro_{XCP2}*:GUS* lines, which were all in the *Arabidopsis* Col-0 background. Soil-grown plants were kept in a walk-in climate chamber (Kryo Service, Helsinki, Finland) under long-day conditions (18 hours light/6 hours darkness, 70% humidity, 21°C/18°C). The plants grown in vitro were kept, without selection, on MS plates (Duchefa) containing 10 g/l sucrose, in growth rooms under long-day conditions (18 hours light/6 hours darkness, 21°C/18°C).

Construction of transgenic Pro_{ACL5}*:DT-A* and Pro_{ACL5}*:GUS* plants

2.48 kb of the ACL5 (At5g19530) sequence, upstream from the start codon, was amplified from Col-0 genomic DNA using the primers 5′-GGGGACAAGTTTGTACAAAAAAGCAGGCTCCACCATCGAATGGTATGC-3′ and 5′-GGGGACCACTTTGTACAAGAAAGCTGGGTATCCAAGTTGAGGAGAAGAT-3′, which include recognition sequences for the Gateway recombination system (Invitrogen). Amplification conditions followed the manufacturer's instructions, and the promoter fragment was sequenced after the first cloning step in pDONR201.

To create Pro_{ACL5}*:DT-A* plants, the 3′UTR of the ACL5 gene was amplified from Col-0 genomic DNA with the primers 5′-CACAGTCGACAGACGAACCGGTTTCAGTTTC-3′ and 5′-CACAGAATTCAGATTTGGTGTGGAGAAATAAG-3′, which include restriction sites for *Sal*I and *Eco*RI, respectively (underlined). This was subsequently cloned into pBluescript SK II to produce pACL-3′UTR/SK. The sequence of the diphtheria toxin A chain (DT-A) was amplified from pEW3 (Nilsson et al., 1998) with primers 5′-GAGTCGACATGGATCCTGATGATGTTGTTG-3′ and 5′-CCACGTCCAGACGTCGAC-3′, including restriction sites for *Sal*I, and was cloned into pACL-3′UTR/SK. Orientation and sequence fidelity were checked by sequencing. The DTA-ACL5 3′UTR cassette was transferred, as a *Kpn*I/*Sac*I restriction fragment, to the binary vector pMDC205 (Curtis and Grossniklaus, 2003), the GFP-coding sequence of which was simultaneously removed to create the construct DTA-utr/pMDC205. Finally, the 2.48 kb ACL5 upstream sequence was recombined into DTA-utr/pMDC205 using the Gateway LR reaction (Invitrogen), and the resulting vector was transformed into Col-0 plants by the method of Clough and Bent (Clough and Bent, 1998). Transformants were selected on MS medium containing 20 g/l sucrose and 50 μg/ml hygromycin. Homozygous and heterozygous plants were identified by PCR and segregation analysis.

To create the Pro_{ACL5}*:GUS* plants, the 2.48 kb ACL5 promoter fragment was cloned into pK2GWFS7.0 (Karimi et al., 2002). This vector was transformed into Col-0 plants using the method described by Clough and Bent (Clough and Bent, 1998). Transformants were selected on MS medium containing 20 g/l sucrose and 50 μg/ml kanamycin. A non-segregating line was crossed to each of the *acl5* mutant and a heterozygous Pro_{ACL5}*:DT-A* line 4. The resultant progenies were screened for homozygosity of either the *acl5* mutation or the Pro_{ACL5}*:DT-A* transgene in F2 by the seedling phenotype and for the Pro_{ACL5}*:GUS* transgene in F3 by antibiotic resistance.

Microscopic analyses

Histochemical β-glucuronidase (GUS) activity assays were performed for whole seedlings grown in vitro or tissue pieces that were excised from plants grown in soil for indicates times. The chromogenic substrate 5-bromo-4-chloro-3-indoxyl-beta-D-glucuronide cyclohexylammonium (Gold Biotechnology) was used, according to the manufacturer's instructions, to detect GUS activity. The GUS-stained whole seedlings were examined directly by microscopy. The excised tissue pieces were mounted after GUS staining in LR white resin (TAAB Laboratories) containing 10% PEG400 and sectioned at 25 μm. If needed, the sections were counterstained with 0.05% Ruthenium Red.

Localization of the *ACL5* mRNA was determined, using 8 μm sections embedded in paraplast, by in situ hybridization, as described by Jackson (Jackson, 1991). Briefly, antisense and sense riboprobes were generated from the complete cDNA of *ACL5* isolated from the PRL1 gene library (Newman et al., 1994), using SP6 and T7 RNA polymerases, respectively, and then hydrolyzed by carbonate hydrolysis into 100-200 bp fragments. Probes were labeled with digoxigenin and immunodetected with an alkaline phosphatase-conjugated antidigoxigenin antibody. Alkaline phosphatase was detected using the BCIP-NBT procedure. Photographs were taken under the bright field of a Nikon Eclipse microscope.

For anatomical characterization, hypocotyls were collected from plants that were grown in soil for indicates times, fixed in FAA (5% formaldehyde, 10% acetic acid, 50% ethanol) and embedded in LR white resin (TAAB Laboratories). Transverse and longitudinal sections were taken and stained with 0.05% Toluidine Blue (Merck).

For analysis of the individual xylem elements, hypocotyls were macerated from soil-grown 2-month-old plants that were stimulated for extensive secondary growth by continuous decapitation of the inflorescence stems. The tissues were incubated at 95°C for 4 hours in 3% H_2O_2/50% acetic acid, gently washed twice with distilled water and neutralized by the addition of solid Na_2CO_3 to the last wash. Samples were then disaggregated mechanically and stored at room temperature. The cell suspensions were stained with 0.01% CelluFluor (Polysciences) and examined under UV illumination with a light microscope. For each genotype, three plants were analyzed and 50-300 vessel elements per plant were scored for length, width and the pattern of secondary cell wall thickenings.

Light microscopy images were taken using a Zeiss Axioplan II microscope equipped with Zeiss AxioCam CCD camera (Zeiss, Oberkochen, Germany). Xylem vessel elements were measured using the microscope images and Zeiss Axiovision 3.1 software.

Electron microscopy images were taken of hypocotyls embedded in Spurr resin (Sigma) according to Rensing (Rensing, 2002), and examined in a Hitachi H-7000 transmission electron microscope (Hitachi, Tokyo, Japan).

Confocal images of vessels were taken using a Leica TCS SP2 microscope (Leica Microsystems, Wetzlar, Germany) with an excitation wavelength of 568 nm (helium-neon laser) and an emission wavelength of 585 nm. Projections of the confocal data were exported using TCS software.

Zinnia elegans xylogenic cell cultures and pharmacological treatments

The first pair of leaves from 14-day-old seedlings of *Zinnia elegans* cv Envy (Hem Zaden BV, Venhuizen, Holland) were used to isolate mesophyll cells for xylogenic cell suspension cultures according to the method of Fukuda and Komamine (Fukuda and Komamine, 1980). Cells were cultured in an induction medium containing 0.1 mg/l α-naphthaleneacetic acid and 0.2 mg/l benzyladenine (Sigma-Aldrich).

For the pharmacological treatments, 1 ml of the differentiating cell culture was treated, in 12-well plates, with various amounts of spermine (Sigma-Aldrich), ranging from 0 to 200 μM final concentration, and monitored for 7 days. Quantification of the tracheary element (TE) differentiation efficiency, as well as the width, length and type of TEs, were recorded using microscope images for 50 cells from each replicate cell culture.

RESULTS

Cell-specific expression of *ACL5* in the xylem vessel elements

The expression of *ACL5* was earlier localized into the procambial and/or provascular tissues of *Arabidopsis* plants (Birnbaum et al., 2003; Clay and Nelson, 2005). We detected with high-resolution analyses using in situ hybridization and resin-embedded sections of Pro_{ACL5}*:GUS* plants that, in all parts of the plant, *ACL5* was expressed in the vascular tissues (Fig. 1). More specifically, *ACL5* expression was confined to xylem vessel elements in the vascular bundles of the inflorescence stems (Fig. 1C,E,F), in the junction between the silique and the pedicel (Fig. 1A) and in the hypocotyl (Fig. 1H). Expression was apparent immediately after expansion of the vessel elements, but before the onset of the secondary cell wall deposition. Protoxylem cells did not express *ACL5* but sometimes parenchymatic cells next to the protoxylem elements showed expression in the inflorescence stem (Fig. 1F) and the roots (data not shown). In line with the earlier reports, we also found expression of Pro_{ACL5}*:GUS* in the procambial and/or provascular tissues of young hypocotyls (Fig. 1G). To assess the function of ACL5 during vascular development, we chose to focus on the hypocotyls, where large numbers of the *ACL5*-expressing vessels are continuously being formed.

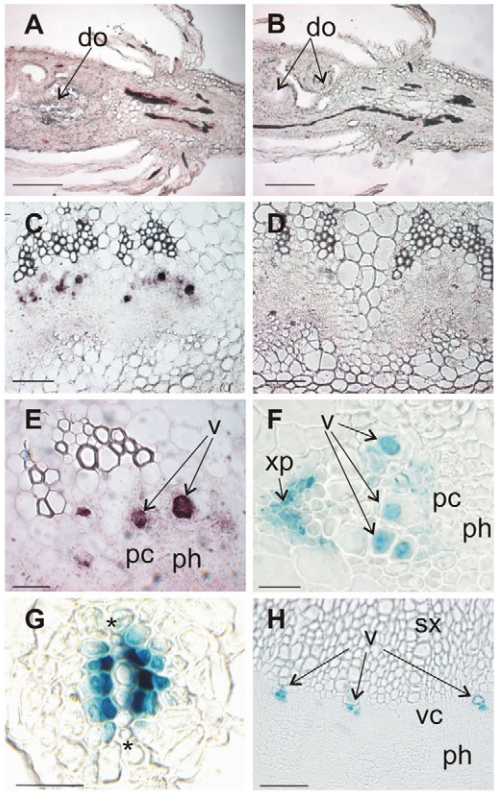

Fig. 1. *ACL5* is specifically expressed in the xylem vessel elements. (**A-E**) In situ hybridization of *ACL5*. Sections were taken from the junction between the silique and the pedicel (A,B) and the basal part of the inflorescence stem (C-E), and analyzed using antisense (A,C,E) or sense (B,D) probes for the *ACL5* gene. (A,B) Longitudinal sections; (C-E) an area of the stem with the vascular bundles in the transverse plane. The sections hybridized with the sense probe showed sometimes dark coloration of the vascular tissues (B) but not the positive purple precipitate derived from the chromogenic substrate (A,C,E). (**F-H**) Histochemical β-glucuronidase (GUS) staining of transgenic Pro_{ACL5}*:GUS* seedlings. Transverse sections were taken from the inflorescence stem (one vascular bundle shown in F), the hypocotyl of a 4-day-old seedling (G) and the hypocotyl of a 1.5-month-old seedling (H). do, developing ovary; pc, procambium; ph, phloem; sx, secondary xylem; v, vessel element; vc, vascular cambium; xp, xylem parenchyma. Asterisks indicate the protoxylem poles (G). Scale bars: 20 μm in E-G; 50 μm in C,D,H; 100 μm in A,B.

Loss of ACL5 function alters morphology of xylem vessel elements

Consistent with the expression profile of *ACL5*, its loss of function caused severe defects in the vasculature of the hypocotyl. Seven-day-old *acl5* seedlings looked normal and the diameter of the stele was indistinguishable from that of the wild type (Fig. 2A,B) although slight changes, such as asymmetry of the stele and of the whole hypocotyl, could be discerned. As with the overproliferation of xylem vessels in the inflorescence stems and leaf veins reported previously (Clay and Nelson, 2005; Hanzawa et al., 1997), the hypocotyls of 13-day-old *acl5* seedlings had more xylem vessels than the wild type (Fig. 2E,F). However, during prolonged growth of the plants, *acl5* did not display further secondary growth, and the hypocotyls of 35-day-old *acl5* seedlings were significantly thinner than those of the wild type (Fig. 2G,H). The lack of secondary growth was accompanied by complete lack of xylem fibers in the

acl5 hypocotyls (Fig. 2H). In addition to the weak secondary growth of the hypocotyl, defects were visible in the secondary cell wall patterning of the *acl5* mutant. Instead of having clearly defined spiral-type protoxylem and pitted-type metaxylem vessels, as in the wild type (Fig. 2C), *acl5* seemed to form spiral-type vessels that were slightly reticulated, i.e. that had a few interconnecting strands between the spiral whorls (Fig. 2D).

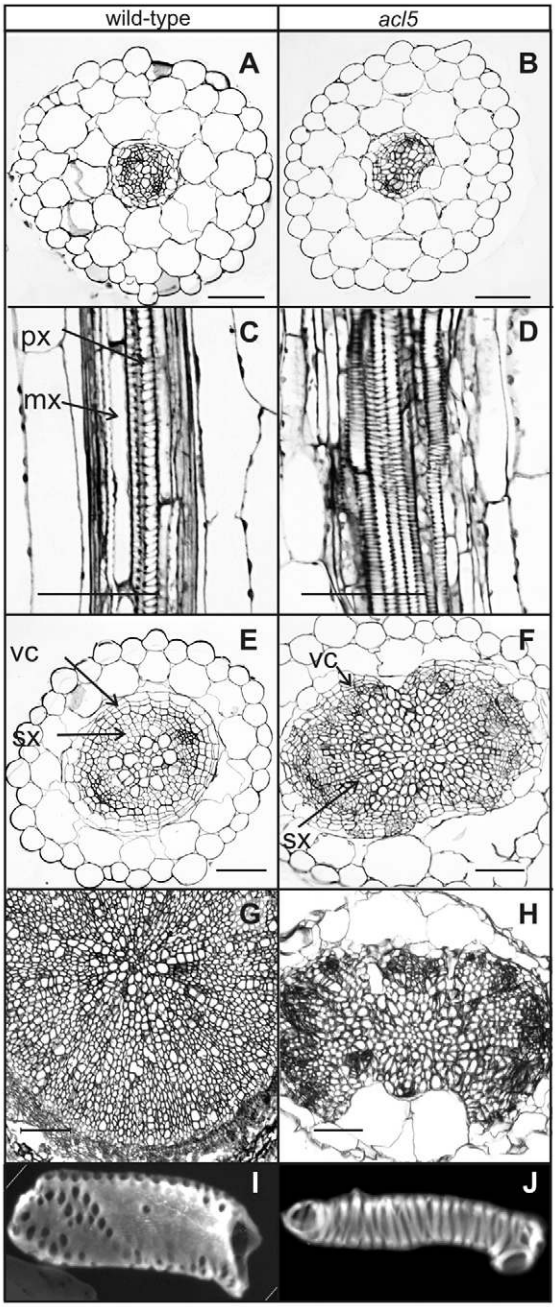

Fig. 2. Xylem development is severely distorted in the *acl5* mutant. (**A-H**) General anatomy of *acl5* and wild-type hypocotyls was examined by light microscopy in transverse sections (A,B,E-H) and in longitudinal sections (C,D) from seedlings grown for seven (A-D), 13 (E,F) or 35 days (G,H). (**I**) A confocal image of a representative vessel element from wild type. (**J**) A confocal image of a representative vessel element from *acl5*. mx, metaxylem; px, protoxylem; sx, secondary xylem; vc, vascular cambium. Scale bars: 50 μm in A-F,H; 100 μm in G.

In addition, electron micrographs of 3-week-old hypocotyls showed the presence of numerous vessels with altered patterns of secondary cell wall deposition and the lack of xylem fibers in *acl5* (Fig. 3E). Interestingly, the pattern of vessel maturation seemed to differ as well. Normal maturation of vessel elements involves extensive deposition of secondary cell wall material, which is terminated by the collapse of the central vacuole and the release of the vacuolar contents into the cytoplasm, leading into rapid cell death and concomitant autolysis of the vessel elements. Maturation can proceed after the death of the vessel elements, even though majority of the secondary cell wall material is normally deposited before the collapse of the vacuole. In the wild type, it was always possible to distinguish a few maturing vessel elements with an intact central vacuole and ongoing secondary cell wall deposition (Fig. 3B-D). However, we could not find any vessel elements in *acl5* hypocotyls that had distinguishable secondary cell walls and that still had intact central vacuole (Fig. 3F-H). Therefore, the maturing vessel elements observed in *acl5* must have surpassed the vacuolar collapse, and it seems that the vacuole collapses in *acl5* too early in relation to the progress of the cell wall deposition.

To get a more in depth insight into the morphological changes of the xylem elements, hypocotyls of wild-type and *acl5* plants were macerated in an alkaline medium, and the size and secondary cell wall architecture of the individual xylem elements that remained intact after the procedure were examined. Hypocotyls were collected from two-month-old plants, which normally at this age exhibit extensive secondary growth. Marked differences were observed in the morphology of vessel elements that were classified as annular, spiral, reticulate or pitted according to their secondary cell wall pattern (Esau, 1977). The vessel elements of *acl5* were mainly spiral, while only a very small portion of the vessel elements were of this type in the wild-type (Fig. 4A, Fig. 2J). Most strikingly, the pitted elements, that were dominant in the wild type during secondary growth, were completely missing in *acl5* (Fig. 4A, Fig. 2I). The length and the width of the vessel elements were also reduced in *acl5* (Fig. 4B,C). In addition, we were able to verify the absence of xylem fibers in the macerates of all *acl5* plants examined (see Fig. 7E). Thus, our microscopic analyses showed that the absence of ACL5 function has a profound effect on xylem development. In particular, the pattern of xylem maturation was altered in a dramatic way, which raises the issue of whether it is causally related to the observed alterations in xylem specification.

Vessel cell death is activated in *acl5* before the onset of secondary cell wall formation

To further examine the function ACL5 in xylem specification and maturation, *acl5* was crossed to two different marker lines that are indicative of vascular development. Pro_{DR5}*:GUS* is a well-established marker for endogenous auxin levels (Ulmasov et al., 1997), normally showing highest expression in the root tips and no expression in the hypocotyls of young *Arabidopsis* seedlings (Fig. 5A). However, when expressed in the *acl5* background, Pro_{DR5}*:GUS* showed weak activity in the developing xylem vessel elements of the hypocotyls (Fig. 5B). As auxin is effective in stimulation of cambial activity, increase in the expression of an auxin marker is in line with the observed increase in the cambial activity and vessel differentiation during secondary growth of the young *acl5* seedlings.

Pro_{XCP2}*:GUS* is a marker for cell death in the xylem elements of *Arabidopsis* seedlings (Funk et al., 2002). The *XCP2* promoter is isolated from a gene encoding a xylem-specific cysteine protease that is believed to function as an effector protease during autolysis

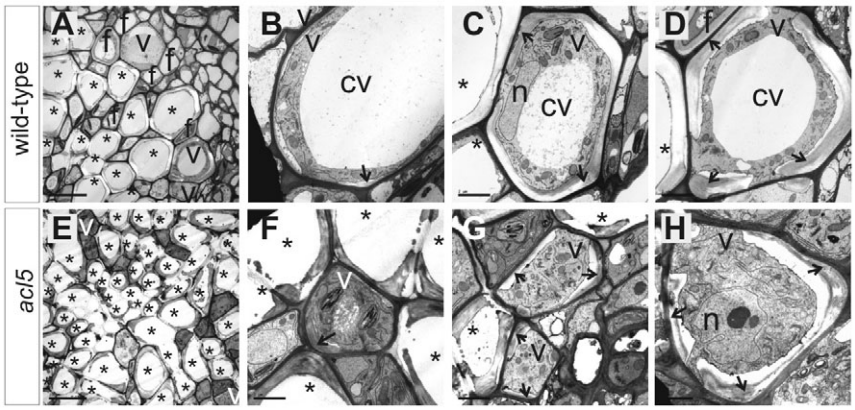

Fig. 3. The vacuole collapses early during vessel maturation in *acl5*. (**A-D**) Xylem anatomy and cell morphology of the wild-type. (**E-H**) Xylem anatomy and cell morphology of *acl5*. An overview of the xylem tissues (A,E) reveals the absence of fiber differentiation in *acl5*. Individual vessel elements are shown during early maturation (B,F), moderate maturation (C,G) and late maturation (D,H). All panels represent electron microscopy images of transverse sections from the hypocotyls of 3-week-old plants. The central vacuole is absent in maturing (i.e. secondary cell wall depositing) vessel elements of *acl5*, even at the earliest stage of maturation (F). f, fiber; cv, central vacuole; n, nucleus; v, a vessel element that is either living or undergoing cell death. The arrows indicate presence of secondary cell walls. The asterisks indicate dead, autolyzed vessel elements. Scale bars: 10 μm in A,E; 2 μm in B-D,F-H.

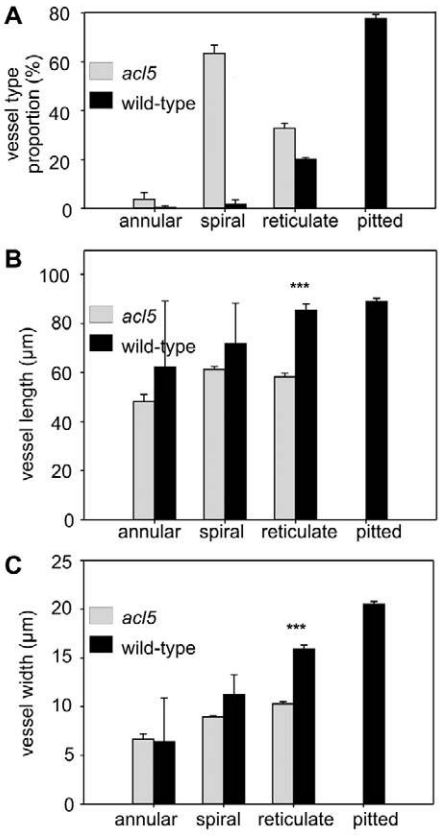

Fig. 4. Xylem vessel elements of the *acl5* mutant are small and simple in structure. Two-month-old hypocotyls of *acl5* and wild-type plants were macerated and their vessel element morphology was investigated using light microscopy. (**A**) Proportion of the different types of vessel elements. (**B**) Length of the vessel elements. (**C**) Width of the vessel elements. More than 200 cells were examined from three plants of each genotype. Data were compared (B,C) using a Welch corrected *t*-test (***P<0.005; *acl5* versus wild-type), and presented as average±s.e.m.

of the cell contents (Zhao et al., 2000). In the wild-type background, Pro_{XCP2}*:GUS* was expressed in the maturing xylem vessels of the hypocotyls, but not in the immature vessel elements without secondary cell wall thickenings (Fig. 5C). A slightly different pattern was observed in the *acl5* background; Pro_{XCP2}*:GUS* was expressed not only in the maturing vessels but also at an earlier developmental stage in the immature vessel elements (Fig. 5D). Therefore, the results are in accordance with the results of the electron microscopy analysis, suggesting premature onset of the cell death program in relation to the formation of the secondary cell walls in the vessel elements of *acl5*.

Spermine prolongs differentiation and increases the size of xylem vessels in *Zinnia elegans* cell cultures

To investigate the function of polyamines in xylem maturation and cell wall patterning in a simplified system, we examined the effect of spermine in the in vitro *Zinnia elegans* xylogenic system. This system allows direct transdifferentiation of freshly isolated mesophyll cells of *Zinnia* into structures similar to xylem vessels, commonly called tracheary elements (TEs), within a time frame of 72 to 96 hours and in a semi-synchronous manner (Fukuda and Komamine, 1980). Concentrations of exogenous spermine were physiologically relevant as the endogenous levels of spermine were at a maximum ~50 μM in the *Zinnia* cells (data not shown).

Exogenous spermine delayed the time of TE appearance and reduced the rate of TE differentiation in a dose-dependent manner (Fig. 6A). Spermine also altered TE type by stimulating differentiation of the more elaborate, metaxylem-type TEs (characterized by their reticulated or pitted secondary cell walls). While control TE cultures contained about 25% spiral and 75% reticulated cells, the addition of 50 μM spermine caused the appearance of pitted cells amounting to about 10% of the total (Fig. 6B). This trend was enhanced with higher concentrations of spermine: at 100 μM spermine, the proportion of reticulated and pitted cells was roughly equivalent (Fig. 6B). The same was true for 200 μM spermine (Fig. 6B,E), although very few TEs differentiated under these conditions (Fig. 6A). Concentrations above 200 μM

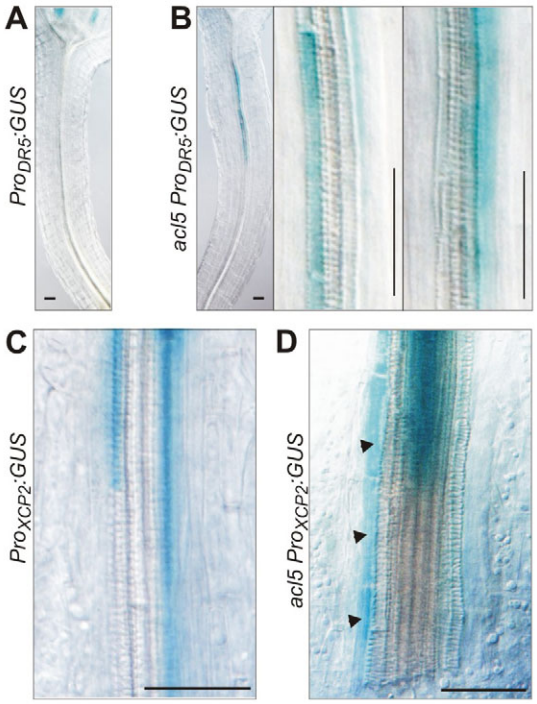

Fig. 5. Expression of an auxin and a cell death marker is altered in *acl5*. (**A**,**B**) Histochemical GUS staining of 7-day-old *Pro$_{DR5}$:GUS* (A) and *acl5 Pro$_{DR5}$:GUS* (B) seedlings grown in vitro. *Pro$_{DR5}$:GUS* expression is present in the developing vessel elements of *acl5* hypocotyl but not in the wild type. (**C**,**D**) Histochemical GUS staining in 7-day-old *Pro$_{XCP2}$:GUS* (C) and in *acl5 Pro$_{XCP2}$:GUS* (D) seedlings grown in vitro. *Pro$_{XCP2}$:GUS* activity in the immature vessel elements (arrowheads) of *acl5* is indicative of an early onset of the vessel cell death program. Scale bar: 50 μm.

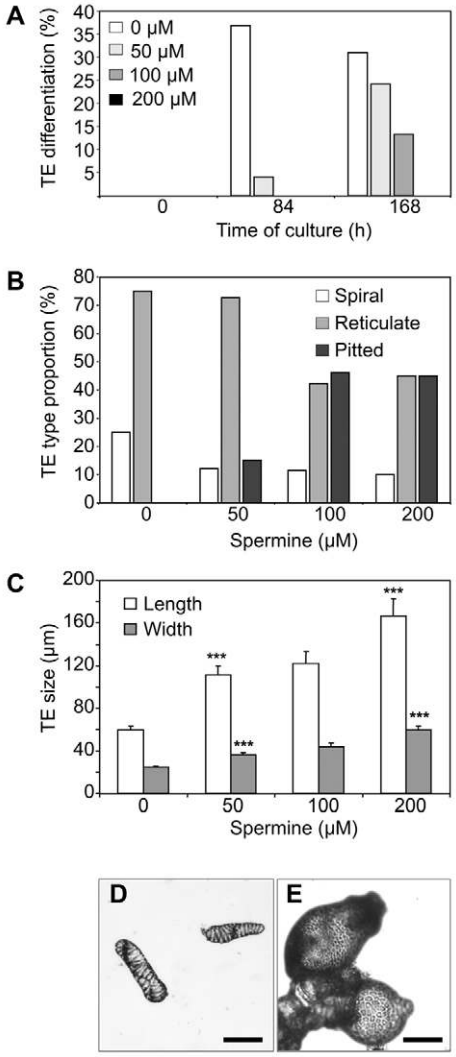

Fig. 6. Exogenous spermine modifies tracheary element differentiation in *Zinnia elegans* xylogenic cell cultures. (**A**) Tracheary element (TE) differentiation efficiency, expressed as the number of TEs as a percentage of all cells, in response to 50-200 μM spermine 84 and 168 hours after the initiation of the cell culture. (**B**) Proportion of the different types of TEs at 168 hours in response to 50-200 μM spermine. (**C**) The size of the TEs (±s.e.m.) after 168 hours in response to 50-200 μM spermine. Statistics are presented (C) for each treatment compared with the previous treatment, using a Kruskal-Wallis test (***P<0.005). (**D**) Typical TEs after 168 hours without the addition of spermine. (**E**) Typical TEs 168 hours after the addition of 200 μM spermine. Scale bar: 50 μm in D,E.

were lethal for the cell culture. The length and the width of the TEs were also strongly affected, with a dose-dependent increase of over threefold for the width and twofold for the length, compared with the control values (Fig. 6C). In summary, these results show that spermine: (1) delays the time of TE appearance, (2) increases TE size and (3) favors pitted-type TE differentiation. It was recently reported that, instead of spermine synthase, *ACL5* might encode an enzyme for synthesis of thermospermine, which is a more rare tetraamine (Knott et al., 2007). Our conclusions on the function of ACL5 in vessel specification, which is analogous to what we observed here for spermine, indicate that if thermospermine is the product of ACL5 activity it has at least to some extent, if not completely, equivalent role to spermine in the xylem vessels.

Premature vessel death mimics the effect of loss of ACL5 function

The evidence presented above is compatible with a model in which ACL5 prevents premature cell death in developing xylem vessel elements in order to allow proper xylem specification and especially formation of the elaborate type of vessels. One prediction based on this model is that the induction of premature death of the *ACL5*-expressing subset of cells should render a phenotype similar, in terms of xylem differentiation, to the one caused by the loss of ACL5 function. To test this, we constructed transgenic *Arabidopsis* plants expressing the diphtheria toxin A chain (*DT-A*) under the control of the *ACL5* promoter. *DT-A* has been previously used in plants as a tool to ablate specific cell types by directing the production of this cell-autonomous toxin to those target cells (Nilsson et al., 1998).

The general phenotype of *Pro$_{ACL5}$:DT-A* plants resembled that of the *acl5* mutant, with the severity depending on the gene dose (Fig. 7). Heterozygous plants were more slender and slightly smaller than wild-type plants, while homozygous plants had a greatly reduced rosette leaf size and stem length, resembling an extreme *acl5* phenotype (Fig. 7A). It was evident that the expression of *DT-A* from the *ACL5* promoter delayed the onset of xylem differentiation by a few days in in vitro grown seedlings, but 6 days after germination a broad cambium was already present and numerous vessels were

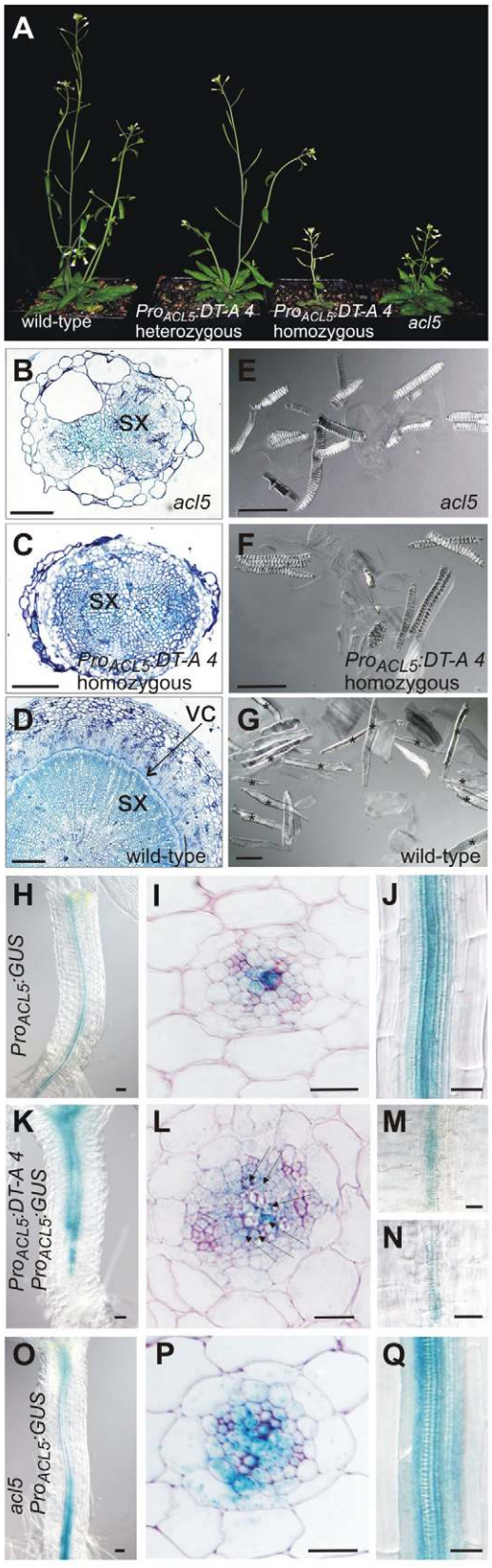

Fig. 7. Expression of *Pro$_{ACL5}$:DT-A* alters plant growth and xylem development. (**A**) The general phenotype of 1-month-old wild-type, *Pro$_{ACL5}$:DT-A* heterozygous line 4, *Pro$_{ACL5}$:DT-A* homozygous line 4 and *acl5* seedlings. (**B-D**) Resin-embedded transverse sections of 2-month-old *acl5* (B), *Pro$_{ACL5}$:DT-A* homozygous line 4 (C) and wild-type (D) hypocotyls stained with Toluidine Blue. (**E-G**) Appearance of xylem elements after maceration of the hypocotyls of 2-month-old *acl5* (E), *Pro$_{ACL5}$:DT-A* homozygous line 4 (F) and wild type (G). Asterisks indicate the presence of xylem fibers in the wild-type (G). (**H-Q**) Expression of *Pro$_{ACL5}$:GUS* in wild-type (H-J), *Pro$_{ACL5}$:DT-A* homozygous line 4 (K-N) and *acl5* seedlings (O-Q). Histochemical GUS staining is shown for hypocotyls of whole mounts (H,J,K,M,N,O,Q) and transverse sections of resin-embedded hypocotyls (I,L,P). Xylem differentiation was delayed in *Pro$_{ACL5}$:DT-A* seedlings, and comparisons were therefore made between 3-day-old wild-type and *acl5* and 6-day-old *Pro$_{ACL5}$:DT-A* in vitro grown seedlings. The arrows indicate expression of *Pro$_{ACL5}$:GUS* and therefore DT-A toxin production in the incipient vessel elements (with first signs of secondary cell wall deposition in cell corners) of the *Pro$_{ACL5}$:DT-A* seedlings (L). sx, secondary xylem; vc, vascular cambium. Scale bars: 20 μm in I,J,L,M,N,P,Q; 50 μm in E,F,G,H,K,O; 100 μm in B,C; 200 μm in D.

being produced in the hypocotyl (Fig. 7L). This pattern was similar to the one observed in *acl5*, even though minor differences in the general anatomy of these two genotypes could be discerned (Fig, 7L,P). Further progression of the secondary growth was also similar to *acl5*: transverse sections of 2-month-old hypocotyls revealed that the secondary growth was severely reduced in the homozygous *Pro$_{ACL5}$:DT-A* hypocotyls compared with the wild type (Fig. 7B-D). Comparison of the xylem cell types present in hypocotyls of two independent transgenic lines, after maceration, confirmed the similarity between the *Pro$_{ACL5}$:DT-A* plants and the *acl5* mutant in terms of xylem specification. The reticulate-type vessel elements were predominant in the homozygous *Pro$_{ACL5}$:DT-A* lines, the pitted-type vessels were almost completely missing (Fig. 7F, Fig. 8A) and the vessel elements were significantly shorter and thinner than in the wild-type (Fig. 8B). Mature xylem fibers were not encountered in the macerates of the *Pro$_{ACL5}$:DT-A* or *acl5* hypocotyls (Fig. 7E,F), whereas this was the predominant type of xylem element produced in the wild-type hypocotyl at this stage (Fig. 7G). In conclusion, similarity in xylem specification and cell morphology of the *Pro$_{ACL5}$:DT-A* plants to that observed in *acl5* supports the proposed role of ACL5 in preventing premature death of the vessel elements.

To confirm that the observed alterations in vessel morphology of the *Pro$_{ACL5}$:DT-A* lines were caused directly by DT-A toxin production and not, for example, by induction of ectopic non-DT-A-expressing xylem elements resulting from early ablation of the xylem elements, we analyzed expression of the *Pro$_{ACL5}$:GUS* marker in the homozygous *Pro$_{ACL5}$:DT-A* line 4. As expression of both *GUS* and *DT-A* are driven by the *ACL5* promoter, it can be assumed that GUS activity reveals the sites of toxin production in the *Pro$_{ACL5}$:DT-A Pro$_{ACL5}$:GUS* plants. Whole-mount staining of hypocotyls revealed that GUS activity was limited to the vasculature of these plants (Fig. 7K). Higher resolution images allowed definition of this activity into developing xylem with highest activity in the incipient vessel elements (Fig. 7L,M) and sometimes even in the maturing vessel elements (Fig. 7N). The expression domain of *Pro$_{ACL5}$:GUS* was broader both in *Pro$_{ACL5}$:DT-A* (Fig. 7K-N) and *acl5* seedlings (Fig. 7O-Q) compared with the wild type (Fig. 7H-J), which is most probably related to the initial increase in the cambial activity and overproliferation of vessel elements in the young *Pro$_{ACL5}$:DT-A* and *acl5* seedlings. A similar increase in the expression of *ACL5* in the *acl5* background was earlier suggested to

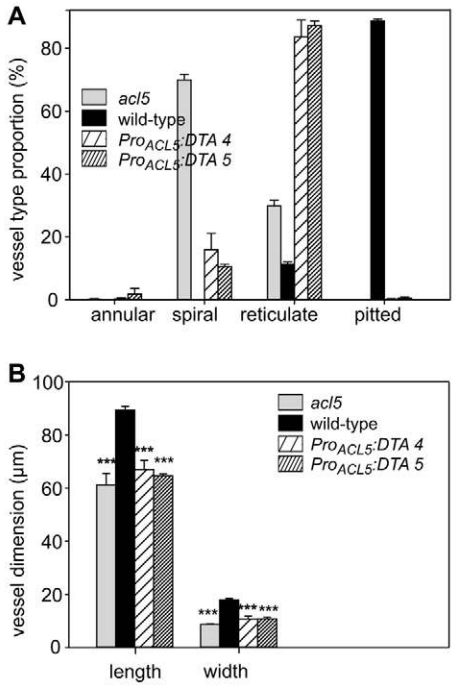

Fig. 8. *Pro$_{ACL5}$:DT-A* expressing plants show *acl5*-like xylem specification. (**A**) Proportion of the different types of the vessel elements in the hypocotyls of wild-type, *acl5* and *Pro$_{ACL5}$:DT-A* homozygous lines 4 and 5. (**B**) The length and width of individual xylem vessel elements in the hypocotyls of the different genotypes. More than 200 cells were scored from macerated hypocotyls of three 2-month-old plants for each genotype. Data are presented as average±s.e.m. ***P<0.005, as compared with the wild type using a Kruskal-Wallis test.

indicate negative-feedback regulation of *ACL5* expression (Clay and Nelson, 2005; Hanzawa et al., 2000). Together, our results using the *Pro$_{ACL5}$:GUS* marker support: (1) the production of DT-A toxin in the xylem vessel elements of the *Pro$_{ACL5}$:DT-A* plants at a stage which allows further maturation of the cells and (2) that the toxin-induced premature death of the vessels is causally related to the altered xylem specification of *Pro$_{ACL5}$:DT-A* plants.

DISCUSSION

ACL5 prevents premature death of the xylem vessel elements

Our work establishes that ACL5 has a prominent role in the correct specification of xylem cells. This is supported by at least three observations: (1) the xylem-specific expression of *ACL5* (Fig. 1); (2) the predominance of the spiral-type vessels and the lack of pitted vessels as well as fibers in the *acl5* mutant (Figs 2-4); and (3) the misregulation of vascular-related markers in the *acl5* mutant (Fig. 5). The earliest xylem defects of *acl5* colocalise with the *ACL5*-expressing subset of cells, and we therefore conclude that xylem specification is the primary function of ACL5. Activity of ACL5 as a polyamine biosynthetic enzyme indicates a completely novel role for polyamines in control of xylem specification by preventing premature death of the xylem vessel elements. This conclusion is substantiated by our observations of the early onset of the cell death program in the vessel elements of *acl5* (Fig. 3, Fig. 5D) and by the fact that the xylem vessel defect of *acl5* could be phenocopied by the induction of premature DT-A toxin-mediated cell death in the *ACL5*-expressing vessels (Figs 7 and 8).

That polyamines protect against premature cell death and senescence is not surprising. A large number of studies demonstrate the role of polyamines in protection from apoptotic cell death in animals, even though the opposite function has also frequently been reported (Seiler and Raul, 2005). In plants, it has been suggested that polyamines retard senescence by maintaining membrane stability and by reducing the quantity and effects of free radicals (Bais and Ravishankar, 2002). In addition, putrescine, spermidine and in particular spermine have been shown to block tonoplast cation channels (Brüggemann et al., 1998); this is believed to block ion leakage from the vacuoles and contribute to the regulation of osmotic potential in the cell and provide protection against salt stress (Yamaguchi et al., 2006; Yamaguchi et al., 2007). On the basis of our results, we cannot predict the exact molecular mechanism of the ACL5-mediated protection against cell death, but the fact that alterations in ion fluxes, especially across the tonoplast, are known to be central in the control of xylem cell death (Kuriyama, 1999) makes it tempting to speculate that ACL5 may function through regulation of tonoplast channels in the xylem elements. An analogous mechanism was proposed for polyamines in the regulation of mitochondrial integrity, which is central to apoptotic cell death in animals (Tassani et al., 1995). In this context, it is interesting to note that spermine has been shown to inhibit mitochondrial membrane permeability in oats (Curtis and Wolpert, 2002).

Premature vessel cell death in *acl5* alters xylem specification

The premature vessel death in *acl5* and in the transgenic *Pro$_{ACL5}$:DT-A* plants is accompanied by smaller sized vessels and the formation of the simpler spiral- and reticulate-type secondary cell walls instead of the more elaborate pitted-type secondary cell walls that usually dominate (Figs 4 and 8). Consistent with these results, an increase in the duration of differentiation in the *Zinnia* xylogenic cultures induced the formation of large tracheary elements and a shift towards the more elaborate type of secondary cell walls (Fig. 6). Our results therefore support the importance of the duration of vessel differentiation in determining vessel specification and especially the formation of the pitted-type vessels. It is generally believed that longer duration of secondary cell wall formation allows increased deposition of the secondary cell wall material (Barnett, 1981). It is therefore possible that ACL5 controls xylem specification by extending the secondary cell wall deposition phase of the vessels to allow formation of the most elaborate types of vessels that also require the most extensive secondary cell wall deposition. However, it is also possible that, instead of the duration of vessel maturation, ACL5 mediates an increase in the duration of vessel expansion, resulting in an increase in the size of the vessel elements, which in turn determines the complexity of the secondary cell wall patterning, as suggested by Roberts and Haigler (Roberts and Haigle, 1994).

The molecular control of xylem cell specification is poorly understood. The NAC family transcription factors VND7 and VND6 have been suggested to control the differentiation of the protoxylem and metaxylem elements, respectively (Kubo et al., 2005). We found increased expression of *VND6* and especially *VND7* in *acl5*, suggesting that defects in xylem specification are not due to lack of expression of either of these genes (data not shown). Galactoglucomannans (Benová-Kákosová et al., 2006) and an arabinogalactan protein (Dahiya et al., 2006) have been related to control of protoxylem versus metaxylem type vessel elements, but no information exists about their mode of function. Recently,

reduced cytokinin signaling was shown to result in high abundance of protoxylem vessels in *Arabidopsis* roots (Mähönen et al., 2006). However, the predominance of protoxylem-type vessels in *acl5* does not seem to be due to impaired cytokinin signaling as *acl5* roots were actually shown to display increased cytokinin sensitivity (Clay and Nelson, 2005).

Although earlier studies were correct in concluding that *acl5* mutants exhibit enhanced vascular development with respect to the number of vessels in the inflorescence stems and leaves (Clay and Nelson, 2005; Hanzawa et al., 1997), this proliferation does not lead to enhanced overall size of the vasculature but rather to a dramatic decrease in the width of the vasculature and the whole stem in both the inflorescence stem (data not shown) and in the hypocotyl primarily because of the complete lack of fibers (Fig. 2). There could be several reasons for the lack of fibers, but the occasional presence of (immature) fibers in Pro_{ACL5}*:DT-A* plants suggests that fiber development is somehow dependent on the correct specification of vessels, and that differentiation of the pitted-type vessels (also occasionally observed in the Pro_{ACL5}*:DT-A* plants) is required for fiber differentiation. There are, to our knowledge, no other mutants that have the interfascicular fibers (see Hanzawa et al., 1997) but that completely lack the xylary fibers, and ACL5 therefore seems to control fiber development through a completely novel mechanism.

Our results, when considered together, suggest that ACL5 is required for correct xylem specification through regulation of the lifetime of the xylem elements. The shorter lifetime of the xylem vessels in the *acl5* mutant results in the development of only the simple type of xylem vessels and the complete lack of xylem fibers.

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