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RESEARCH REPORT

An essential role for abscisic acid in the regulation of xylem fibre differentiation

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ABSTRACT

Division of the cambial cells and their subsequent differentiation into xylem and phloem drives radial expansion of the hypocotyl. Following the transition to reproductive growth, a phase change occurs in the *Arabidopsis* hypocotyl. During this second phase, the relative rate of xylem production is dramatically increased compared with that of phloem, and xylem fibres that contain thick secondary cell walls also form. Using two different genetic backgrounds and different environmental conditions, we identified a set of core transcriptional changes that is associated with the switch to the second phase of growth in the hypocotyl. Abscisic acid (ABA) signalling pathways are significantly over-represented in this set of core genes. Reverse genetic analysis demonstrated that mutants that are defective in ABA-biosynthesis enzymes exhibited significantly delayed fibre production without affecting the xylem:phloem ratio, and that these effects can be reversed by the application of ABA. The altered morphology is also reflected at the transcript level, with a reduced expression of marker genes that are associated with fibre formation in *aba1* mutants. Taken together, the data reveal an essential role for ABA in the regulation of fibre formation.

KEY WORDS: Hypocotyl, Xylem, Fibre, Secondary cell wall, Abscisic acid

INTRODUCTION

The primary plant body is produced by the activity of the root and shoot apical meristems, whilst the cambial meristem is responsible for secondary growth that results in radial expansion and produces most of the plant biomass (Campbell and Turner, 2017). The cambium produces vascular tissue in a bi-directional manner, with xylem developing on its inner side and phloem on its outer side. In secondary growth of the *Arabidopsis* hypocotyl, xylem development is characterized by two distinct phases. In the first phase, only xylem parenchyma and water-conducting vessels are produced (Chaffey et al., 2002). The second phase, referred to as phase II xylem, is triggered by flowering and is characterized by an increased rate of xylem development relative to phloem development, and the formation of lignified xylem fibres (Sibout et al., 2008). The xylem expansion phase thus provides structural support for the growing plant, and is remarkably similar in morphology to the wood produced by trees (Chaffey et al., 2002).

Exogenous application of gibberellin (GA) can stimulate premature xylem expansion and fibre formation in late-flowering mutants, whereas the inhibition of GA-biosynthesis has the opposite effect (Ragni et al., 2011). However, the identification of plants that have undergone xylem expansion but not yet begun to produce fibres suggests that the mechanisms leading to these processes are somewhat distinct (Ragni et al., 2011), with fibre formation typically initiating ∼2 weeks after flowering (Ikematsu et al., 2017). It has recently been shown that ERECTA (ER) acts redundantly with ER-LIKE1 (ERL1) to negatively regulate radial growth and fibre differentiation in the hypocotyl, acting downstream of GA following the hypocotyl phase change (Ikematsu et al., 2017; Ragni et al., 2011). GA and ER require the activity of the class I KNOX transcription factors BREVIPEDICELLUS (BP) and SHOOT MERISTEMLESS (STM) to trigger fibre differentiation (Liebsch et al., 2014; Xiong and Zhu, 2003). Despite these advances in our understanding of the hypocotyl phase change, we still do not know what the major trigger for fibre differentiation is, and what determines the delay between flowering and fibre differentiation. A role for abscisic acid (ABA) in vascular development was highlighted by a recent study which demonstrated an essential role of ABA in xylem formation and revealed how xylem formation is altered in response to drought stress (Ramachandran et al., 2018). In this study, we report that the phytohormone ABA is also required for the normal transition to the second phase of xylem development.

RESULTS AND DISCUSSION

Identifying changes in the transcriptome during the phase transition

In order to gain insight into what regulates the alterations in vascular development that occur during development of the hypocotyl, we sought to identify the gene expression changes that underlie xylem deposition pre- and postflowering. Consequently, Columbia-0 (Col-0) plants were held under short days for 6 weeks, before switching to long-day conditions to induce synchronized flowering (Vanholme et al., 2012). RNA was extracted from hypocotyls that were harvested 0, 1, 2, 4 and 10 days after the day-length switch (Fig. 1A) and gene expression levels were measured using microarray analysis. We identified a total of 939 genes that demonstrated a >twofold change in expression at a high significance level ($P<0.001$) between any two time points. A number of Gene Ontology (GO) terms relating to secondary cell wall biosynthesis, circadian rhythm and hormonal responses were enriched, consistent with the dataset capturing the major transcriptomic changes that occur in the hypocotyl following flowering (Fig. 1B).

Analysis of the microarray dataset is complicated by the reliance on switching the day length to induce flowering, which results in genes being differentially expressed simply as a consequence of the altered day length. We sought to create an alternative expression

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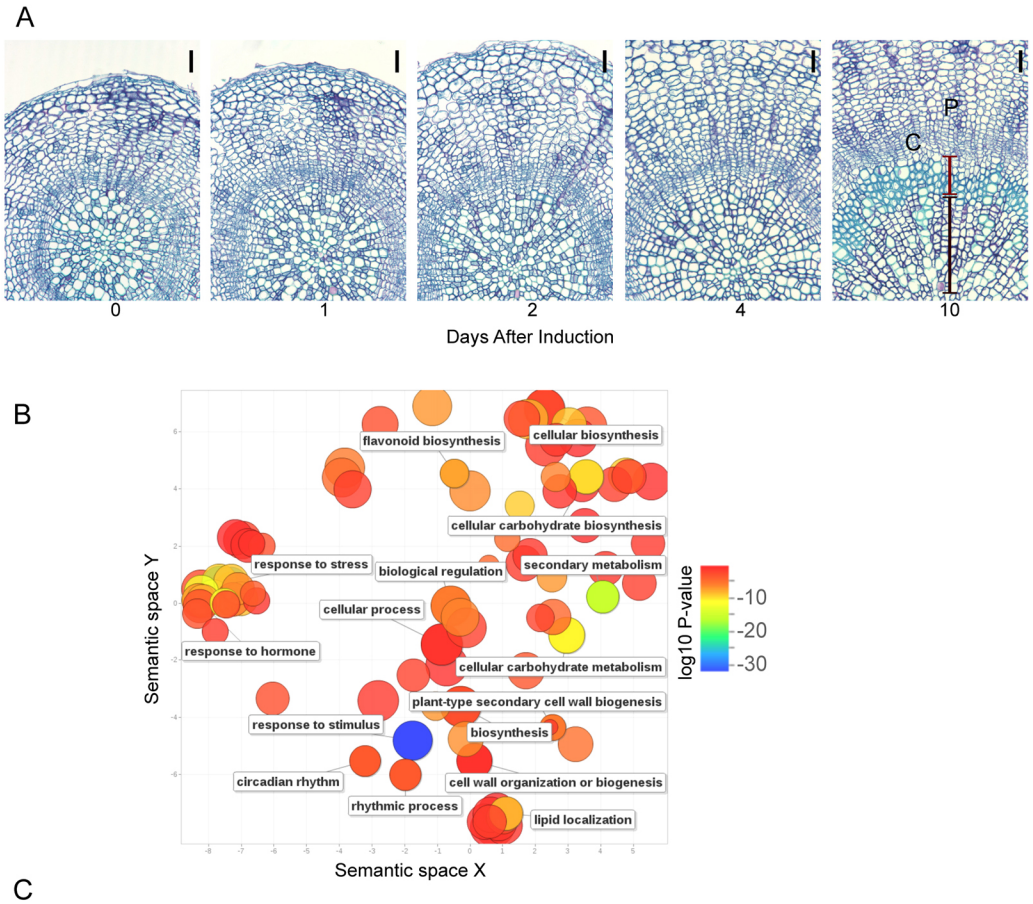

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GO term	Description	# Diff. Expressed	# Genome	p-value	FDR
GO:0009753	response to jasmonic acid stimulus	12	215	4.60E-07	2.40E-05
GO:0009737	response to abscisic acid stimulus	15	378	1.10E-06	3.90E-05
GO:0009751	response to salicylic acid stimulus	8	200	0.00035	0.0042

Fig. 1. An expression analysis of xylem expansion. (A) Representative cross-sections of five time points used for microarray analysis following a switch from short to long days to induce flowering and xylem expansion. Xylem phase I (black line), xylem phase II (maroon line), phloem (P) and cambium (C) are indicated. (B) A visual representation of the most significantly altered GO terms (represented by circles) in the microarray dataset. Selected GO terms of relevance to the current study have been labelled. GO terms closer together within the 2D space have more similarity to each other, with their colour denoting their significance (see key). (C) The over-represented hormonal GO terms (FDR<0.05) within the 316 genes differentially expressed in both expression datasets. Scale bars: 30 µm.

dataset by taking advantage of a previously described transgenic 35S::CO:GR *co-2 tt4* line, in which the CO flowering regulator is fused to a rat glucocorticoid receptor (GR) and placed under the control of a 35S promoter (Coupland, 1997). We grew 35S::CO:GR *co-2 tt4* plants under constant long-day conditions and applied dexamethasone at the 5 week stage to induce synchronized flowering across all plants without altering the day length. Examination of the hypocotyls following induction suggested that, similar to plants exposed to the day-length shift, there was little sign of phase II xylem at 4 days after induction, but abundant phase II xylem 8 days after induction (Fig. S1A). RNA was extracted from hypocotyls 0, 1, 2, 3, 4, 6 and 14 days after dexamethasone application. We also collected samples at days 0, 1, 3 and 4 from *co-2 tt4* mutant controls that do not flower during the time course (Fig. S1B). Gene expression was analyzed using RNA-seq to quantify changes in expression with a greater resolution and accuracy.

Replicates taken at each time point tended to cluster well with each other and, as expected, the samples taken 14 days after flowering induction are the most distinct (Fig. S2A). In total, 3922 genes were differentially expressed, in comparison with either day 0 or the control plants at the same time points. A GO analysis revealed that a variety of genes were altered during the time course (Fig. S2B), including carbohydrate metabolism and cell wall metabolism genes, consistent with the transcription changes known to occur during xylem expansion and fibre differentiation (Brown et al., 2005).

Although the microarray dataset was created using the Col-0 ecotype and the 35S::CO:GR *co-2 tt4* line used here is in an L*er* background that is reported to undergo a more pronounced xylem

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expansion than Col-0 (Ragni et al., 2011), we hypothesized that genes central to the process of the phase II xylem formation should be significantly altered in both datasets. Consequently, we compiled a list of genes that were differentially expressed in both the microarray and RNA-seq datasets. In total, 316 genes fit this criterion. A GO analysis of these 316 core xylem expansion genes revealed a strong over-representation of jasmonic acid (JA)- and ABA-related genes, as well as an over-representation of salicylic acid-related genes (Fig. 1C). These were the only three hormone classes that were significantly over-represented in the core gene list [false discovery rate (FDR)<0.05], with the GA-related class being notably absent. The complete list of 313 genes together with their expression patterns is shown in Table S2 and Fig. S3.

Hormonal regulation of phase II xylem development in hypocotyl

Because of the significant over-representation of JA and ABA genes in the expression datasets (Fig. 1C), we considered the possibility that both hormones play a role in xylem expansion. JA has been previously reported to control secondary growth in *Arabidopsis* (Sehr et al., 2010), so we explored a role for JA in the phase II xylem development by screening a number of transfer DNA (T-DNA) insertion mutants that disrupt several key genes known to be either positive or negative regulators of JA signalling (Fig. S4A). None of the JA-signalling mutants showed a significant disruption in the phase ratio (Fig. S4B) or the proportion of xylem in the hypocotyl (Fig. S4C), suggesting that either the hormone plays no direct role in xylem expansion or fibre differentiation or that there is significant functional redundancy for the genes we have analyzed.

To investigate the influence of ABA upon hypocotyl secondary growth, we identified plants with mutations in each of the three important enzymes of the ABA-biosynthesis pathway: ABA DEFICIENT 1 (ABA1) encoding zeaxanthin epoxidase, ABA DEFICIENT 2 (ABA2) encoding xanthoxin dehydrogenase and ABA DEFICIENT 3 (ABA3) encoding a molybdenum cofactor sulfurase (Xiong and Zhu, 2003). We grew *aba1*, *aba2-3* and *aba3-1* alongside Col-0 wild-type controls and carried out a short-to-long day switch at 6 weeks to induce flowering. At 3 weeks after flowering, we observed a significant reduction in the ratio of phase II to total xylem in all three mutants (Fig. 2A,B). Notably, ∼25% of *aba1* mutants had not produced xylem fibres at this stage. The phenotype was confirmed by phloroglucinol staining that confirmed the absence of fibre formation (Fig. 2E). Therefore the pathway that connects flowering to the onset of fibre production in the hypocotyl had been severely disrupted. In *aba2* and *aba3* mutants, progression to phase II always occurred, albeit with a significant delay. Consequently, when compared with wild type, the relative proportion of phase II xylem in *aba2* and *aba3* lines was reduced by 50% and 36%, respectively. The relative strength of these vascular phenotypes corresponded with the mean plant height of the mutants at 21 days postflowering (Fig. 2C) and suggests that the *aba1* mutant is the most severe of the three ABA biosynthesis

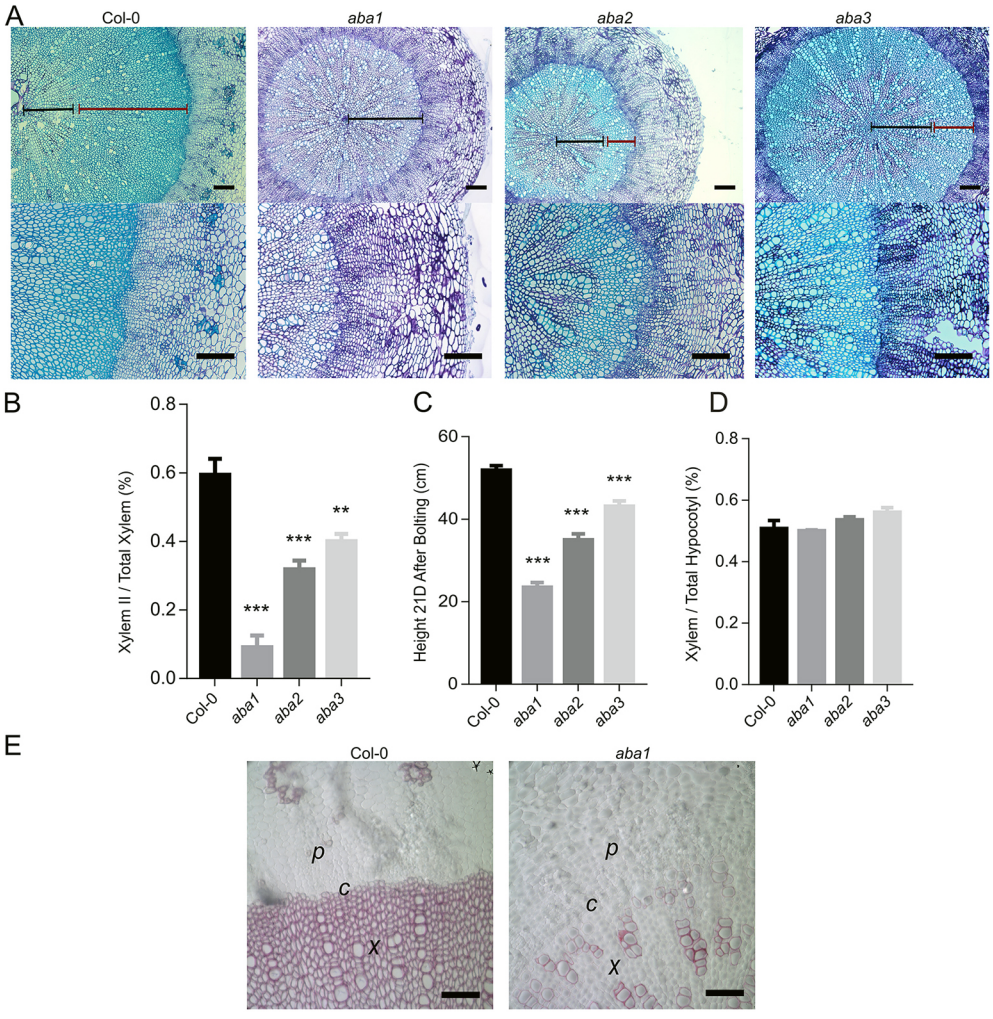

Fig. 2. ABA biosynthesis mutants have a disrupted phase transition. (A) Representative cross-sections of Col-0, *aba1*, *aba2* and *aba3* plants 21 days after the first signs of bolting. Xylem I (black line) and xylem II (maroon line) are indicated. Bottom panels are increased magnification images of top panels. (B) Ratio of size of xylem II as a proportion of the total xylem. (C) Height of all genotypes 21 days after bolting. (D) The percentage of the hypocotyl area occupied by xylem. Error bars on B-D indicate the s.e.m. At least five plants of each genotype were analyzed. The means in B-D were compared with wild type using a two-tailed *t*-test (***P*<0.01, ****P*<0.001; significance versus the Col-0 control). (E) Phloroglucinol staining of a Col-0 plant and an *aba1* mutant 21 days after flowering. The phloem (p), cambium (c) and xylem (x) are indicated. Scale bars: 100 µm in A; 50 µm in E.

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mutants. Whereas the ratio of xylem I/xylem II is altered in the mutant, the proportion of xylem relative to the total hypocotyl was not significantly different from the wild type (Fig. 2D). Thus, changes in the formation of xylem II are not a result of gross alteration in hypocotyl development, supporting the idea that the increase in cell division and fibre formation in phase II are genetically separable (Ragni et al., 2011).

To understand the temporal nature of the defects in *aba1*, samples were collected at weekly intervals following a switch from short to long days. Neither *aba1* mutants nor wild-type controls had developed fibres after 1 week under long-day conditions. Delays in the onset of phase II xylem differentiation in the *aba1* mutant were evidenced by the continued absence of fibres 2 weeks after induction (week 7 of the experiment), in contrast to the Col-0 controls, in which phase II xylem occupied 30% of the xylem area (Fig. 3A,B). Importantly, the difference in phase II growth could not be accounted for by differences in radial growth, as the percentage of the total hypocotyl occupied by xylem was identical in both *aba1* and wild-type plants at all time points (Fig. 3C). Although the *aba1* mutants had smaller rosettes throughout their growth (Fig. 3D), they produced an inflorescence more quickly after the day-length switch than wild type, as evidenced by their increased height compared with wild type at week 7 (Fig. 3E). This demonstrates that plant height is not a key determinant of phase II xylem development.

To ensure that the fibre differentiation phenotype of the *aba1* mutant was a consequence of decreased ABA content, exogenous ABA was applied to the hypocotyl of a subset of *aba1* plants throughout the course of the 9 week experiment. The increased height and rosette diameter of the treated *aba1* mutants demonstrated that the plants were able to take up the ABA (Fig. 3D,E). The delayed formation of the phase II xylem in *aba1* mutants was almost fully rescued by the application of exogenous ABA (Fig. 3A). Although phenotypic variation was observed at week 7, by week 8 the proportion of phase II xylem was identical to wild type in week 8 (Fig. 3B). As with the untreated *aba1* mutant, the xylem as a proportion of the total hypocotyl area was no different to wild type in ABA-treated *aba1* lines (Fig. 3C), confirming the specific role of ABA on fibre differentiation.

We examined the expression of all known ABA biosynthesis genes that are listed in TAIR in our expression dataset. Whereas the expression of *ABA1*, *ABA2* and *ABA3* did not change significantly, *NCED5* appears to increase almost tenfold in the induced samples, with no increase in the controls (Fig. S5). *NCED5* is reported to be a positive regulator of ABA biosynthesis (Frey et al., 2012), however

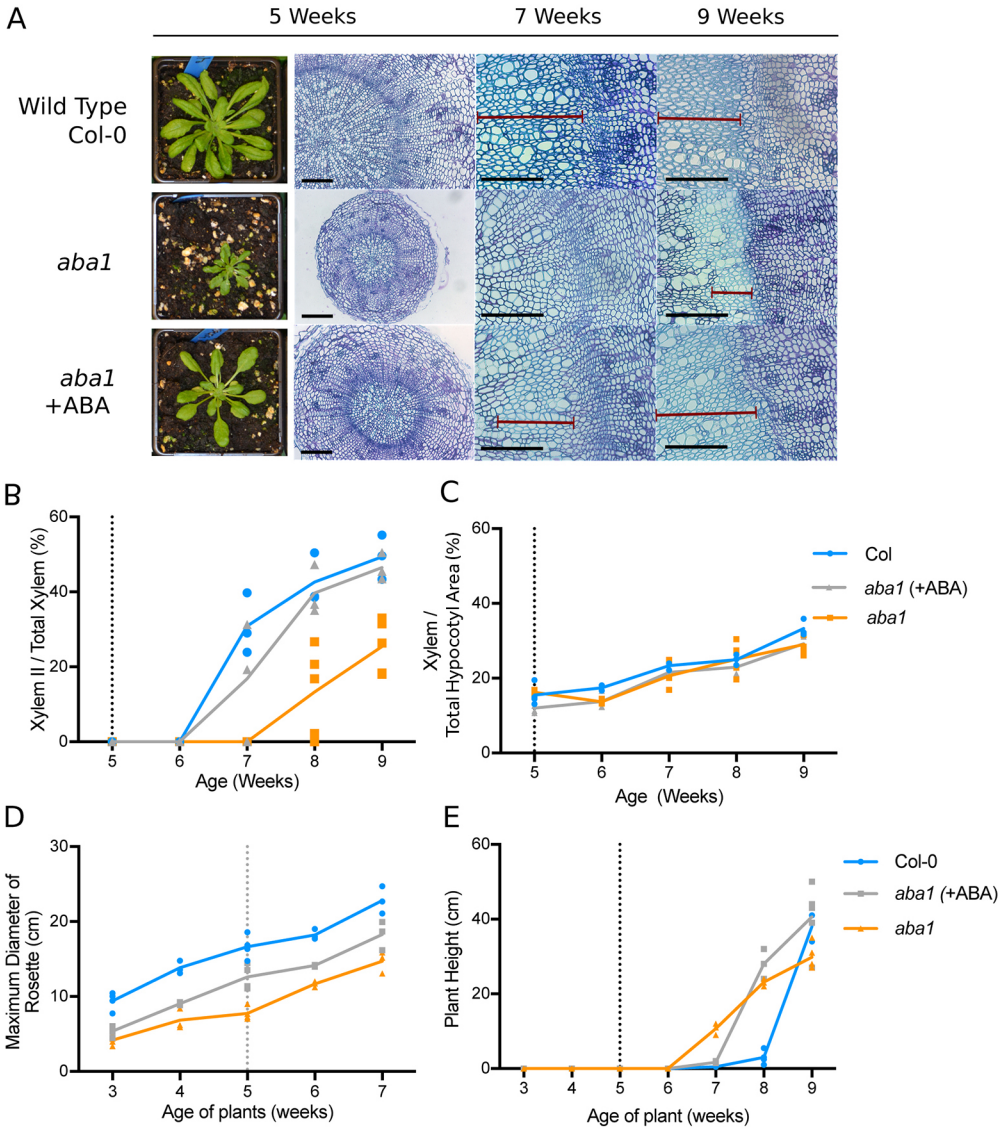

Fig. 3. Phenotype of *aba1* mutant plants following a switch to long days. (A) Representative plant photos at 5 weeks and hypocotyl cross-sections at 5, 7 and 9 weeks. Phase II xylem (maroon line) is indicated. (B) Ratio of xylem II as a percentage of the total xylem versus plant age. The *aba1* mutant was significantly different from Col-0 and *aba1* (+ABA) at weeks 7, 8 and 9. (C) Ratio of xylem as a percentage of the total hypocotyl area versus plant age. There were no significant differences at any stage. (D) Rosette diameter versus plant age. *aba1* (+ABA) was significantly different from Col-0 at all stages and from *aba1* at weeks 5-7. (E) Plant height versus plant age. *aba1* was significantly different from Col-0 at weeks 7-9 and from *aba1* (+ABA) at weeks 7 and 9. Individual data points are shown, the line connects the means at each time point. Results were analyzed using two-way ANOVA, differences were considered significant if $P<0.05$. Dotted lines indicate shift from short- to long-day growth conditions. Scale bars: 100 µm.

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as the site of ABA biosynthesis required for phase transition in the hypocotyl is currently unknown, this data must be interpreted with caution.

As a previous study has demonstrated that ABA can influence the morphology of poplar xylem (Arend and Fromm, 2013), we investigated whether this is also the case in *Arabidopsis*. The maceration of hypocotyl tissue revealed that the general morphology of fibres and vessels is the same as in wild type and that the average length of these cells is not affected by the loss of ABA (Fig. 4A,B). Therefore, in *Arabidopsis*, the role of ABA in xylem development is specifically to initiate fibre differentiation, but it does not affect any other aspect of the differentiation of the cell type.

ABA promotes the expression of genes associated with fibre differentiation

NAC SECONDARY WALL THICKENING PROMOTING FACTOR 1 (NST1) and SECONDARY WALL-ASSOCIATED NAC DOMAIN PROTEIN 1 (SND1) are a pair of functionally redundant NAC transcription factors that act as master regulators for secondary cell wall formation in xylem fibres (Mitsuda et al., 2007; Zhong et al., 2007). Another NAC transcription factor, SND2, is also implicated in the regulation of fibre secondary cell walls (Hussey et al., 2011; Taylor-Teeples et al., 2015). IRREGULAR XYLEM 3 (IRX3) is a subunit of the cellulose synthase complex (CSC) that makes the cellulose of secondary cell walls (Taylor et al., 1999), including that found in mature fibres. Consistent with an absence of fibres at 6 days post-transfer, no differences in expression of *IRX3*, *SND1*, *SND2* or *NST1* were observed 6 days after the switch from short to long days in Col-0, *aba1* or ABA-treated *aba1* hypocotyls (Fig. 3B). A significant induction of all four genes at day 12 was observed in wild-type hypocotyls, but not in those of *aba1* mutants (Fig. 4C). The ABA-treated *aba1* mutants demonstrated a significant induction of *IRX3*, *SND1* and *NST1* at day 12, although increases were smaller than those of wild-type

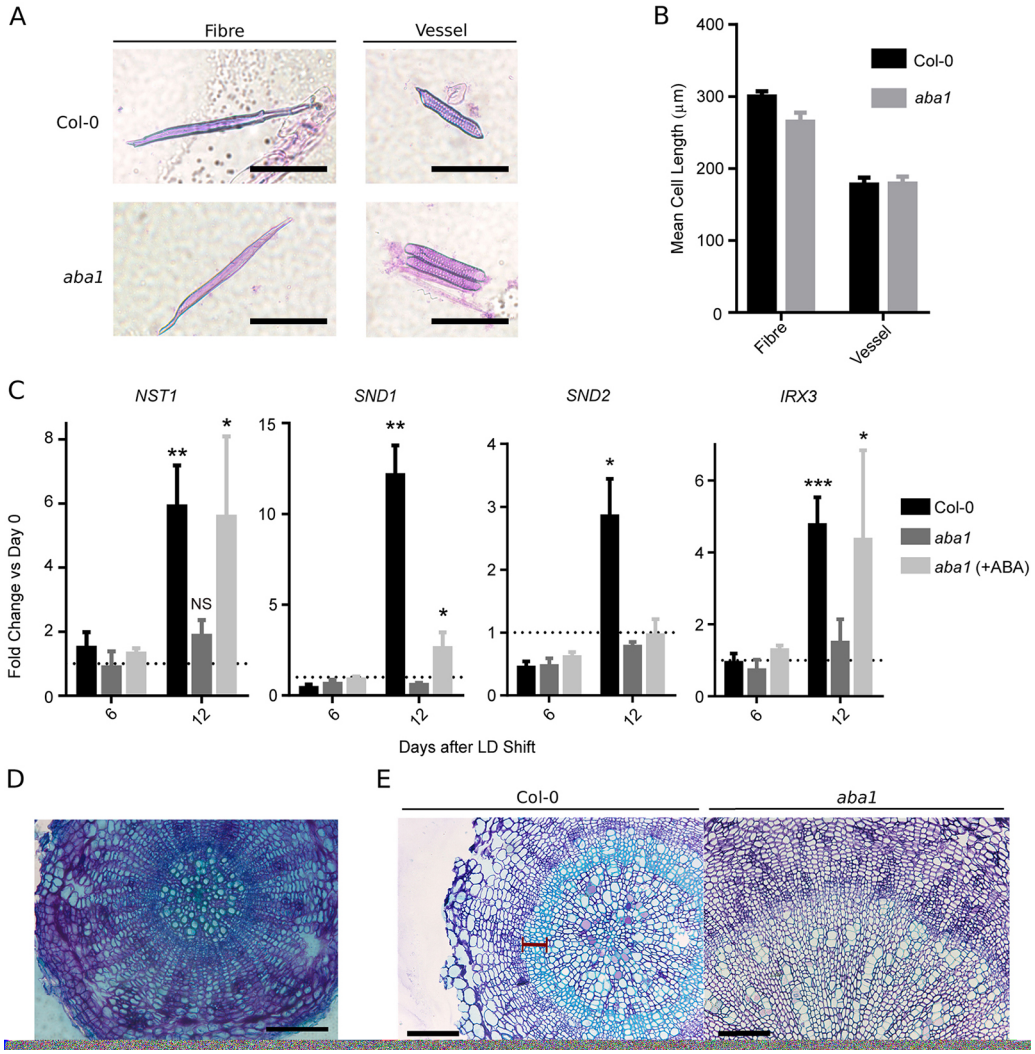

Fig. 4. Characterization of fibre differentiation in *aba1* mutants. (A) Representative images of fibre cells and xylem vessel from macerated Col-0 and *aba1* hypocotyl tissue. (B) The mean cell lengths±s.d. of fibre cells and xylem vessel taken from three biological replicates. More than 20 cells of each type were measured from each plant. Means were compared using a two-tailed *t*-test and no significant difference was detected. (C) qPCR expression analysis of four xylem fibre marker genes, 6 and 12 days after a day-length shift (day 0) to induce flowering. Three samples were collected for each time point and qPCR was carried out using technical duplicates, with elongation factor 1α as an endogenous control. Error bars indicate the s.e.m. Means were compared using a two-tailed *t*-test (*P<0.05, **P<0.01, ***P<0.001; significant difference from the day 0 expression level). NS, not significant. (D) A representative cross-section of a Col-0 plant treated with exogenous ABA throughout its lifecycle. (E) An *aba1* mutant that has not yet begun to produce fibres is shown alongside a thin Col-0 plant for comparison. Plants were grown under long-day conditions. Phase II xylem is indicated (maroon line). Scale bars: 100 µm.

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plants, reflecting the variable onset of phase II xylem deposition in the treated mutants at this time point (Fig. 3B). These results are consistent with a transcriptomic study of the *Arabidopsis* NAC family which reported that ABA can upregulate *SND1* (Jensen et al., 2010). In contrast, *BP* expression in *aba1* mutants 12 days after flowering barely changed (fold change of 1.11±0.13 versus Col-0; mean±s.e.m.; n=3). This suggests that the effect of ABA upon fibre formation does not occur through *BP*. Together, this data demonstrates that, during the phase change in the wild type, there is an increase in expression of key transcription factors and cell wall synthesis genes; however, these increases are delayed in *aba1* mutants.

Exogenous ABA cannot overcome the requirement for flowering in fibre differentiation

To investigate whether the stimulatory effect of ABA on fibre differentiation is also dependent upon floral transition, we applied exogenous ABA to wild-type Col-0 plants throughout their vegetative phase and harvested hypocotyls at the first sign of bolting. We found that the ABA-treated plants had not prematurely transitioned to phase II of xylem development (Fig. 4D) and, consequently, the hypocotyl is insensitive to the effect of ABA before floral transition.

To test whether the day-length switch was influencing the fibre phenotype, we grew Col-0 and *aba1* plants alongside each other under constant long days. Under these conditions, we found that *aba1* mutants had not yet progressed to phase II xylem 21 days after flowering (Fig. S6A,C). We also allowed some plants to grow until the end of their life cycle, signified by the drying of most siliques under well-watered conditions. Again, a significant reduction in the xylem phase ratio of the mutant was observed (Fig. S6B,C).

As all the *aba* biosynthesis mutants that we tested exhibited altered growth and stature, it is conceivable that the delay in phase II xylem formation may be a consequence of this altered growth. In order to test whether reduced fibre formation in *aba1* mutants might be a consequence of its reduced stature, we grew Col-0 plants at a high density to intentionally reduce their secondary growth. These small Col-0 plants were still capable of normal fibre production even when their hypocotyls are much thinner than the *aba1* mutants described above (Fig. 4E), demonstrating that the late fibre differentiation of *aba1* hypocotyls is not a result of their smaller diameter compared with Col-0. Furthermore, the *aba1* plants actually bolt earlier than the controls, even though they exhibit delayed phase II xylem formation, meaning that the delayed phase transition is not a result of the thinner inflorescence stem. This would be consistent with several mutants in which the plants are very small, but they are still able to transition to phase II xylem formation (Etchells et al., 2012). Taken together, the data all demonstrate a specific role for ABA in regulating the formation of fibres during the transition to phase II xylem formation in the hypocotyl.

MATERIALS AND METHODS

Plant growth conditions and materials

Mutant seeds were obtained from the National *Arabidopsis* Stock Center (www.arabidopsis.info): *aba1* (SALK_059469C), *aba2-3* (N3834), *aba3-1* (N157), *myc2-2* (SALK_083483), *myc3* (SALK_012763), *jaz9-1* (SALK_004872), *coi1* (SALK_045434) and *jaz10-1* (SAIL_92_D08). The presence of a T-DNA insertion within the relevant lines was confirmed using a single PCR reaction containing a T-DNA-binding left border primer and two gene-specific primers. Seeds were sterilized in 70% ethanol for 5 min, washed in sterile water three times and stratified at 4°C in the dark for 2 days before being germinated on 0.5 Murashige and Skoog medium with 1% agar. After 1 week, the seedlings were transferred to individual pots containing Levington F2 compost mixed with vermiculite and perlite (10:1:1). Plants were grown in controlled environment chambers at 22°C under either short-day (8 h light) or long-day (16 h light) conditions, depending on the experiment. In order to induce synchronized flowering across the full population, plants were first grown under short days for 5 or 6 weeks before being switched to long-day conditions (Vanholme et al., 2012). We quantified the phase transition by measuring the average length of each phase from five evenly distributed points around the hypocotyl and calculating the relative amount of xylem as a proportion of the total hypocotyl, as has been used in previous studies (Liebsch et al., 2014; Ragni et al., 2011; Xiong and Zhu, 2003).

Experimental treatments

A Pasteur pipette was used to apply 100 µl of 100 µM ABA solution to plant hypocotyls twice a week for the duration of the experiment. Plants receiving ABA treatment were grown in separate trays from those receiving mock treatments of water to ensure that the control plants received no exogenous ABA.

RNA extraction and expression analysis

For the microarray analysis, Col-0 plants were grown under short-day conditions for 6 weeks and switched to long days to induce flowering. RNA was extracted in triplicate from the hypocotyls of plants 0, 1, 2, 4 and 10 days following the day-length switch, with eight individual plants pooled per replicate. Samples at each time point were taken at midday. Hypocotyl tissue was frozen in liquid nitrogen and ground using a mortar and pestle. RNA was extracted using TRIzol reagent (Life Technologies), as per the manufacturer's instructions. Biotinylated cDNA samples from each of the three replicates for each hypocotyl stage were subsequently synthesized and hybridized to Affymetrix ATH1 22k transcriptomic arrays. GO analysis was performed using AgriGO (Du et al., 2010) and GO terms with an FDR<0.05 were visualized with REVIGO (Supek et al., 2011).

For the RNA-seq expression experiment, 35S::CO:GR *co-2 tt4* plants (Coupland, 1997) were grown individually under long-day conditions alongside *co-2 tt4* control mutants. At 6 weeks of age, both the 35S::CO:GR *co-2 tt4* and *co-2 tt4* control plants were watered with 25 ml of a 10 µM dexamethasone solution to induce flowering. Hypocotyl tissue was harvested and frozen in liquid nitrogen 0, 1, 2, 3, 4, 6 and 14 days after induction for the 35S::CO:GR *co-2 tt4* plants and on days 0, 1, 3 and 4 for the *co-2 tt4* controls. Samples were collected at midday in triplicate, with five pooled plants per replicate. RNA was extracted and cDNA synthesized as described above. Quality and integrity of total RNA samples were assessed using a 2100 Bioanalyzer or a 2200 TapeStation (Agilent Technologies) according to the manufacturer's instructions. RNA-seq libraries were generated using the TruSeq Stranded mRNA assay (Illumina) according to the manufacturer's protocol. Adapter indices were used to multiplex libraries, which were pooled before cluster generation using a cBot instrument (Illumina). The loaded flow-cell was then paired end sequenced (101+101 cycles, plus indices) on an Illumina HiSeq2500 instrument. Demultiplexing of the output data (allowing one mismatch) and bcl2fastq conversion was performed with CASAVA 1.8.3. Each sample generated between 6 million and 19 million 100 bp paired-end reads per sample that were successfully mapped onto the *Arabidopsis* genome.

Post-sequencing libraries for the next-generation sequencing experiment were assessed for read quality and adapter contamination using FastQC (version 0.11.3) before reads were aligned to the *Arabidopsis thaliana* genome using the STAR read aligner (Dobin et al., 2013). Read counts for each gene were normalized using the edgeR (Robinson et al., 2010) module in the R programming language and significantly different genes between treatment and control samples were identified using a quasi-likelihood F-test, also using edgeR. As this was a time-course experiment, genes were only listed as significant if they were significantly different between control and treated samples throughout the length of the experiment, and were not significantly different at day 0.

For real-time qPCR analysis, hypocotyl tissue was collected in triplicate, with six individual plants per replicate. RNA was extracted and cDNA synthesized as described above. Target-specific primers were designed

using Primer-BLAST (Ye et al., 2012) and Primer3 (Untergasser et al., 2012). All primer sequences used in this study are listed in Table S1. qPCR reactions were carried out using two technical replicates per biological replicate on an ABI Prism 7000 machine (Thermo Fisher Scientific) using the Sensifast HI-ROX SYBR kit (Bioline). Melt-curve analysis was used to confirm the presence of a single amplification peak. Relative expression levels were calculated using the delta-Ct method described by Livak and Schmittgen (2001). Expression of test genes was normalized to the expression of elongation factor 1α (At5g60390).

Histological analysis and maceration of hypocotyl tissue

Plant tissue was harvested at the relevant time points and fixed immediately in a formaldehyde-acetic acid solution (3.8% formaldehyde, 5% acetic acid, 50% ethanol, 41.2% water v/v) for at least 24 h. The samples were dehydrated in a graded series of ethanol (70%, 80%, 90% and 100%) for 1 h each. The dehydrated samples were infiltrated with JB-4 infiltration solution (Polysciences) and embedded in plastic moulds, according to the manufacturer's instructions. Dry embedded blocks were sectioned using a Reichert Jung Model 1140/Autocut microtome at a thickness of 4-10 µm. Sections were stained with 0.05% Toluidine Blue or 3% phloroglucinol-HCl (Sigma-Aldrich), visualized on a Leica 5500 microscope and photographed using a Spot RT3 camera (SPOT Imaging). Area and length measurements were obtained using ImageJ and statistical analysis was performed in GraphPad Prism 7.

Hypocotyl tissue was macerated by incubating samples for 6 h in a 3% H_2O_2/50% acetic acid solution at 90°C. Na_2CO_3 was added to neutralize the acid and cells were dispersed by rapidly pipetting up and down with a Pasteur pipette. Droplets of the homogenized solution were stained with 0.05% Toluidine Blue.

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Competing interests

The authors declare no competing or financial interests.

Author contributions

Conceptualization: J.P.E., S.R.T., L.C.; Formal analysis: M.C., M.K.; Investigation: L.C., J.P.E.; Writing - original draft: L.C.; Writing - review & editing: L.C., M.C., M.K., J.P.E., S.R.T.; Supervision: S.R.T.; Project administration: S.R.T.; Funding acquisition: S.R.T.

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Data availability

Data from the RNA-seq and microarray analysis data and have been deposited in GEO under accession numbers GSE114782 and GSE114638, respectively.

Supplementary information

Supplementary information available online at
http://dev.biologists.org/lookup/doi/10.1242/dev.161992.supplemental

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