First posted online on 24 October 2018 as 10.1242/dev.161992
Access the most recent version at http://dev.biologists.org/lookup/doi/10.1242/dev.161992

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

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Key words: hypocotyl, xylem, fibre, secondary cell wall, abscisic acid

Summary statement:

Using a combination of expression analysis and reverse genetics, we demonstrate that the hormone ABA plays an essential role in regulating the differentiation of fibres during xylem formation in Arabidopsis.

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 to that of phloem and xylem fibres containing thick secondary cell walls also form. Using two different genetic backgrounds and different environmental conditions, we identified a set of core transcriptional changes associated with the switch to the second phase of growth in the hypocotyl. ABA signalling pathways were significantly over-represented in this set of core genes. Reverse genetic analysis demonstrated that mutants defective in ABA-biosynthesis enzymes exhibited significantly delayed fibre production without affecting the xylem:phloem ratio and 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 associated with fibre formation in *aba1* mutants. Taken together, the data reveals an essential role for ABA in the regulation of fibre formation.

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 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 characterised 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 characterised by an increased rate of xylem development relative to phloem 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 GA can stimulate premature xylem expansion and fibre formation in late-flowering mutants, whilst 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 about 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 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 abscisic acid (ABA) is also required for the normal transition to the second phase of xylem development.

Materials & Methods

Plant Growth Conditions and Materials

Mutant seeds - *aba1* (SALK_059469C), *aba2-3, aba3-1*, *myc2-2* (SALK_083483), *myc3* (SALK_012763), *jaz9-1* (SALK_004872), *coi1* (SALK_045434) and *jaz10-1* (SAIL_92_D08) - were obtained from the National Arabidopsis Stock Center (www.arabidopsis.info). 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 minutes, washed in sterile water three times and stratified at 4^{o}C in the dark for 2 days before being germinated on ½ MS media 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^{o}C under either short (8hr light) or long days (16hr light), depending on the experiment. In order to induce synchronized flowering across the full population, plants were first grown under short days (8hr light period) for 5 or 6 weeks before being switched to long day conditions (16hr light period) (Vanholme et al., 2012). We quantified the phase transition by measuring the average length of each phase from 5 evenly distributed points around the hypocotyl and calculating the relative amount of xylem as a proportion of the total hypocotyl, as is used in previous studies (Liebsch et al., 2014; Ragni et al., 2011; Xiong and Zhu, 2003).

Experimental Treatments

100 µl of 100 µM ABA solution was applied to plant hypocotyls using a Pasteur pipette twice a week for the duration of the experiment. Plants receiving ABA treatments were grown in

separate trays to 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 triplicates from the hypocotyls of plants 0, 1, 2, 4 and 10 days following the day length switch, with 8 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 synthesised and hybridized to Affymetrix ATH1 22k transcriptomic arrays. Gene ontology analysis was performed using AgriGO (Du et al., 2010) and GO terms with a 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, the both the 35S::CO:GR *co-2 tt4 and co-2 tt4* mutants 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 days 0, 1, 3 and 4 for the *co-2 tt4* controls. Samples were collected at mid-day in triplicate, with 5 pooled plants per replicate. RNA was extracted and cDNA synthesised 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 sequencing (RNA-seq) libraries were generated using the TruSeq® Stranded mRNA assay (Illumina, Inc.) according to the manufacturer's protocol. Adapter indices were used to multiplex libraries, which were pooled prior to cluster generation using a cBot instrument. 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 BCL-to-Fastq conversion was performed with CASAVA 1.8.3. Each sample generated between 6-19 million 100bp paired end reads per sample that were successfully mapped onto the Arabidopsis genome.

Post-sequencing libraries for the NGS experiment were assessed for read quality and adapter contamination using FastQC (version 0.11.3) before reads were aligned to the *A. thaliana* genome using the STAR read aligner (Dobin et al., 2013). Read counts for each gene were normalised using the edgeR (Robinson et al., 2010) module in the R programming language and significantly different genes between treatment and control

samples 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 6 individual plants per replicate. RNA was extracted and cDNA synthesised 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 2 technical replicates per biological replicate on an ABI Prism 7000 machine (Warrington, UK) using the Bioline (London, UK) Sensifast HI-ROX SYBR kit. 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 normalised 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 (FAA) solution (3.8% formaldehyde, 5% acetic acid, 50% ethanol, 41.2% water v/v) for at least 24h. The samples were dehydrated in a graded series of ethanol (70, 80, 90 and 100%) for 1 hour each. The dehydrated samples were infiltrated with JB-4 infiltration solution (Polysciences, Philadelphia, USA) and embedded in plastic moulds according to the manufacturer's instructions. Dry embedded blocks were sectioned using a Reichert Jung (Cambridge, UK) Model 1140/Autocut microtome at a thickness of 4-10µm. Sections were stained with 0.05% toluidine blue or 3% phloroglucinol-HCl (Sigma-Aldrich, Irvine, UK) and visualized on a Leica 5500 microscope and photographed using a Spot RT3 camera (Diagnostic instrument). 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 hours in a 3% H2O2/50% acetic acid solution at 90^{o}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.

Results and discussion.

Identifying changes in the transcriptome during the phase transition

In order to gains insight into the process that regulates the alterations in vascular development that occur during development of the hypocotyl, we sought to identify gene

expression changes underlying xylem deposition pre- and post-flowering. Consequently, Col-0 plants were held under short days (SD) for 6 weeks, before switching to long day (LD) conditions to induce synchronized flowering (Vanholme et al., 2012). RNA was extracted from hypocotyls harvested 0, 1, 2, 4 and 10 days after the day-length switch (Fig 1A) and gene expression levels measured using microarray analysis. We identified a total of 939 genes that demonstrated a > 2 fold change in expression at a high significance level ($p<0.001$) between any two time points. A number of 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 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 synchronised flowering across all plants without altering the day length. Examination of the hypocotyls following induction suggested that similar to the daylength 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 analysed using RNA-sequencing 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 to 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 expansion than Col-0 (Ragni et al., 2011), we hypothesised 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 RNAseq 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 abscisic acid (ABA)-related genes, as well as an over-representation of salicylic acid-related genes (Fig 1C). These were the only three hormone classes significantly over-represented in the core gene list (FDR < 0.05), with the GA-related class being notably absent. The complete list of 313 genes together with their expression patterns is shown supplementary data file S1 and figure S3.

Hormonal regulation of phase II xylem development of in hypocotyl

Due to 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 previously been 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 T-DNA insertion mutants that disrupt several key genes known to be either positive or negative regulator 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 analysed.

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* (SALK_059469), *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 to 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 post-flowering (Fig 2C) and suggests that the *aba1* mutant is the most severe of the three ABA biosynthesis mutants. While the ratio of xylem I/xylem ll 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 increases to 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 LDs. 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 since the percentage of the total hypocotyl occupied by xylem was identical in both *aba1* and wild type plants at all time-points (Fig 3C). While the *aba1* mutants had smaller rosette 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 to wild type at week 7 (Fig 3E). This demonstrates that plant height is not 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. While the expression of *ABA1*, *ABA2* and *ABA3* did not change significantly, *NCED5* appears to increase almost 10 fold 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 since 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 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 SCWs (Taylor et al., 1999) including those 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 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 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 that 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; 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 prior to 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 till 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).

Since all the *aba* biosynthesis mutants that we tested all exhibit 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* mutant 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 to 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 e.g. (Etchells et al., 2012). Taken together the data all demonstrates a specific role for ABA in regulating the formation of fibres during the transition to phase II xylem formation in the hypocotyl.

Competing interests

No competing interests declared.

Funding

The work was funded through The Biotechnology and Biological Science Research Council (BB/J014478/1, BB/P01013X/1, BB/H019928) and The Leverhulme Trust grant (RPG-2014-309).

Data Availability

Data from the RNAseq and microarray analysis (GSE114638) data and have been deposited and are available at: https://www.ncbi.nlm.nih.gov/geo/query/acc.cgi?acc=GSE114638 (microarray) or https://www.ncbi.nlm.nih.gov/geo/query/acc.cgi?acc=GSE114782 (RNAseq).

References

Arend, M. and Fromm, J. (2013). Concomitant analysis of cambial abscisic acid and cambial growth activity in poplar. *Trees-Struct Funct* **27**, 1271-1276.

Brown, D. M., Zeef, L. A. H., Ellis, J., Goodacre, R. and Turner, S. R. (2005). Identification of novel genes in Arabidopsis involved in secondary cell wall formation using expression profiling and reverse genetics. *Plant Cell* **17**, 2281-2295.

Campbell, L. and Turner, S. (2017). Regulation of vascular cell division. *J. Exp. Bot.* **68**, 27-43.

Chaffey, N., Cholewa, E., Regan, S. and Sundberg, B. (2002). Secondary xylem development in Arabidopsis: a model for wood formation. *Physiol. Plant.* **114**, 594-600.

Coupland, G. (1997). Regulation of flowering by photoperiod in Arabidopsis. *Plant Cell and Environment* **20**, 785-789.

Dobin, A., Davis, C. A., Schlesinger, F., Drenkow, J., Zaleski, C., Jha, S., Batut, P., Chaisson, M. and Gingeras, T. R. (2013). STAR: ultrafast universal RNA-seq aligner. *Bioinformatics* **29**, 15-21.

Du, Z., Zhou, X., Ling, Y., Zhang, Z. H. and Su, Z. (2010). agriGO: a GO analysis toolkit for the agricultural community. *Nucleic Acids Res.* **38**, W64-W70.

Etchells, J. P., Provost, C. M. and Turner, S. R. (2012). Plant Vascular Cell Division Is Maintained by an Interaction between PXY and Ethylene Signalling. *PLoS Genet.* **8**, e1002997-e1002997.

Frey, A., Effroy, D., Lefebvre, V., Seo, M., Perreau, F., Berger, A., Sechet, J., To, A., North, H. M. and Marion-Poll, A. (2012). Epoxycarotenoid cleavage by NCED5 fine-tunes ABA accumulation and affects seed dormancy and drought tolerance with other NCED family members. *Plant J.* **70**, 501-512.

Hussey, S. G., Mizrachi, E., Spokevicius, A. V., Bossinger, G., Berger, D. K. and Myburg, A. A. (2011). SND2, a NAC transcription factor gene, regulates genes involved in secondary cell wall development in Arabidopsis fibres and increases fibre cell area in Eucalyptus. *BMC Plant Biol.* **11**.

Ikematsu, S., Tasaka, M., Torii, K. U. and Uchida, N. (2017). ERECTA-family receptor kinase genes redundantly prevent premature progression of secondary growth in the Arabidopsis hypocotyl. *New Phytol.* **213**, 1697-1709.

Jensen, M. K., Kjaersgaard, T., Nielsen, M. M., Galberg, P., Petersen, K., O'Shea, C. and Skriver, K. (2010). The Arabidopsis thaliana NAC transcription factor family: structure-function relationships and determinants of ANAC019 stress signalling. *Biochem. J.* **426**, 183-196.

Liebsch, D., Sunaryo, W., Holmlund, M., Norberg, M., Zhang, J., Hall, H. C., Helizon, H., Jin, X., Helariutta, Y., Nilsson, O., et al. (2014). Class I KNOX transcription factors promote differentiation of cambial derivatives into xylem fibers in the Arabidopsis hypocotyl. *Development* **141**, 4311-4319.

Livak, K. J. and Schmittgen, T. D. (2001). Analysis of relative gene expression data using real-time quantitative PCR and the 2(T)(-Delta Delta C) method. *Methods* **25**, 402-408.

Mitsuda, N., Iwase, A., Yamamoto, H., Yoshida, M., Seki, M., Shinozaki, K. and Ohme-Takagi, M. (2007). NAC transcription factors, NST1 and NST3, are key regulators of the formation of secondary walls in woody tissues of Arabidopsis. *Plant Cell* **19**, 270-280.

Ragni, L., Nieminen, K., Pacheco-Villalobos, D., Sibout, R., Schwechheimer, C. and Hardtke, C. S. (2011). Mobile gibberellin directly stimulates Arabidopsis hypocotyl xylem expansion. *The Plant cell* **23**, 1322-1336.

Ramachandran, P., Wang, G. D., Augstein, F., Annelie, J. D. and Carlsbecker, A. (2018). Continuous root xylem formation and vascular acclimation to water deficit involves endodermal ABA signalling via miR165. *Development* **145**.

Robinson, M. D., McCarthy, D. J. and Smyth, G. K. (2010). edgeR: a Bioconductor package for differential expression analysis of digital gene expression data. *Bioinformatics* **26**, 139-140.

Sehr, E. M., Agusti, J., Lehner, R., Farmer, E. E., Schwarz, M. and Greb, T. (2010). Analysis of secondary growth in the Arabidopsis shoot reveals a positive role of jasmonate signalling in cambium formation. *Plant J.* **63**, 811-822.

Sibout, R., Plantegenet, S. and Hardtke, C. S. (2008). Flowering as a condition for xylem expansion in Arabidopsis hypocotyl and root. *Curr. Biol.* **18**, 458-463.

Supek, F., Bosnjak, M., Skunca, N. and Smuc, T. (2011). REVIGO Summarizes and Visualizes Long Lists of Gene Ontology Terms. *Plos One* **6**.

Taylor, N. G., Scheible, W. R., Cutler, S., Somerville, C. R. and Turner, S. R. (1999). The irregular xylem3 locus of arabidopsis encodes a cellulose synthase required for secondary cell wall synthesis. *Plant Cell* **11**, 769-779.

Taylor-Teeples, M., Lin, L., de Lucas, M., Turco, G., Toal, T. W., Gaudinier, A., Young, N. F., Trabucco, G. M., Veling, M. T., Lamothe, R., et al. (2015). An Arabidopsis gene regulatory network for secondary cell wall synthesis. *Nature* **517**, 571-U307.

Untergasser, A., Cutcutache, I., Koressaar, T., Ye, J., Faircloth, B. C., Remm, M. and Rozen, S. G. (2012). Primer3-new capabilities and interfaces. *Nucleic Acids Res.* **40**.

Vanholme, R., Storme, V., Vanholme, B., Sundin, L., Christensen, J. H., Goeminne, G., Halpin, C., Rohde, A., Morreel, K. and Boerjan, W. (2012). A Systems Biology View of Responses to Lignin Biosynthesis Perturbations in Arabidopsis. *Plant Cell* **24**, 3506-3529.

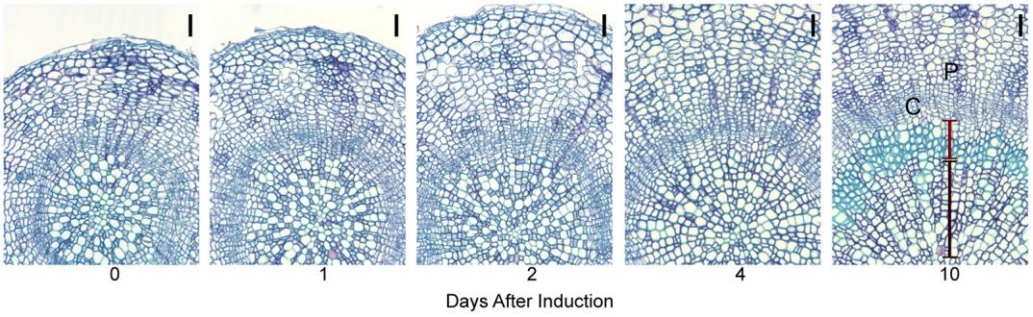
Xiong, L. M. and Zhu, J. K. (2003). Regulation of abscisic acid biosynthesis. *Plant Physiol.* **133**, 29-36.

Ye, J., Coulouris, G., Zaretskaya, I., Cutcutache, I., Rozen, S. and Madden, T. L. (2012). Primer-BLAST: A tool to design target-specific primers for polymerase chain reaction. *BMC Bioinformatics* **13**.

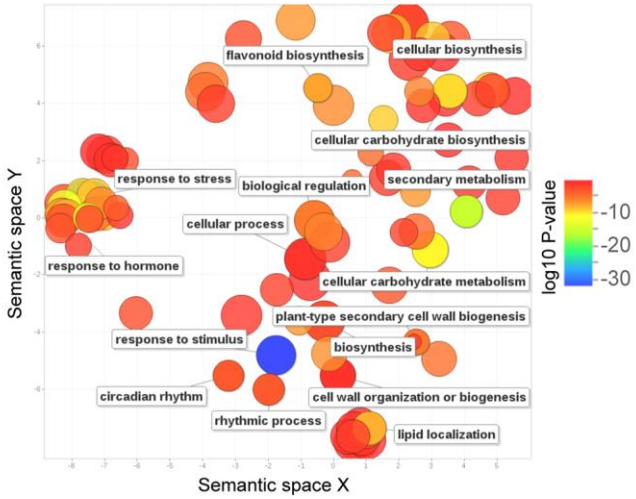
Zhong, R., Richardson, E. A. and Ye, Z. H. (2007). The MYB46 transcription factor is a direct target of SND1 and regulates secondary wall biosynthesis in Arabidopsis. *Plant Cell* **19**, 2776-2792.

Figures

A



B



C

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

Figure 1 - An expression analysis of xylem expansion. (A) Representative cross-sections of 5 time-points used for the 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.

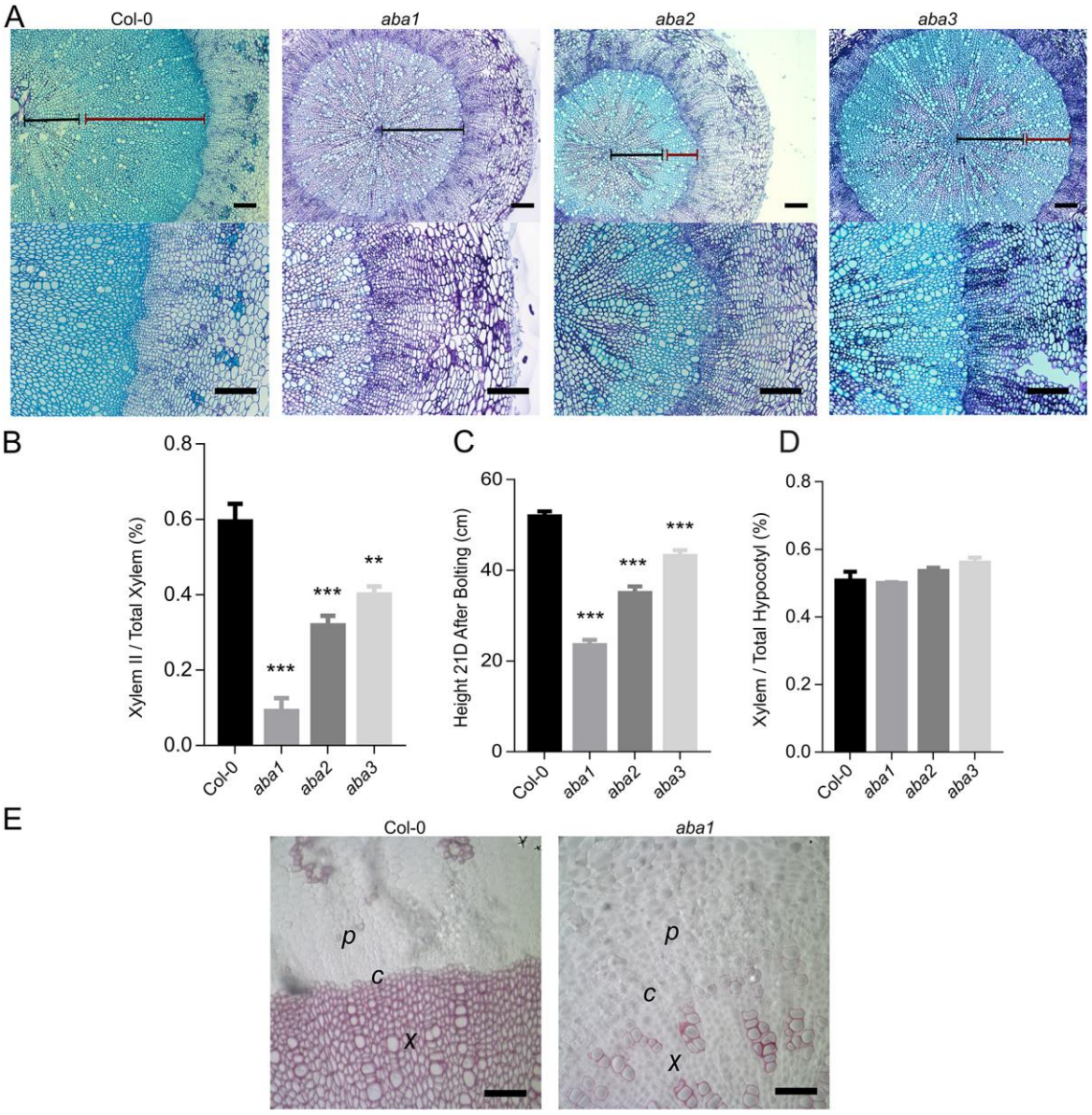


Figure 2 – ABA biosynthesis mutants have a disrupted phase transition. (A) Representative cross-sections of Col-0, *aba1, aba2* and *aba3* plants 21 days after the first signs of bolting. Xylem I (black line) and Xylem II (maroon line) are indicated. (B) Ratio of size of xylem II as a proportion of the 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), (C) and (D) show the S.E.M. Asterisks indicate significance versus the Col-0 control. At least five plants of each genotype were analysed. The means in B-D were compared to wild type using a two-tailed t-test. (* = $p<0.05$, ** = $p<0.01$, *** = $p<0.001$). (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 bar represents 100 µm in A and 50 µm in E.

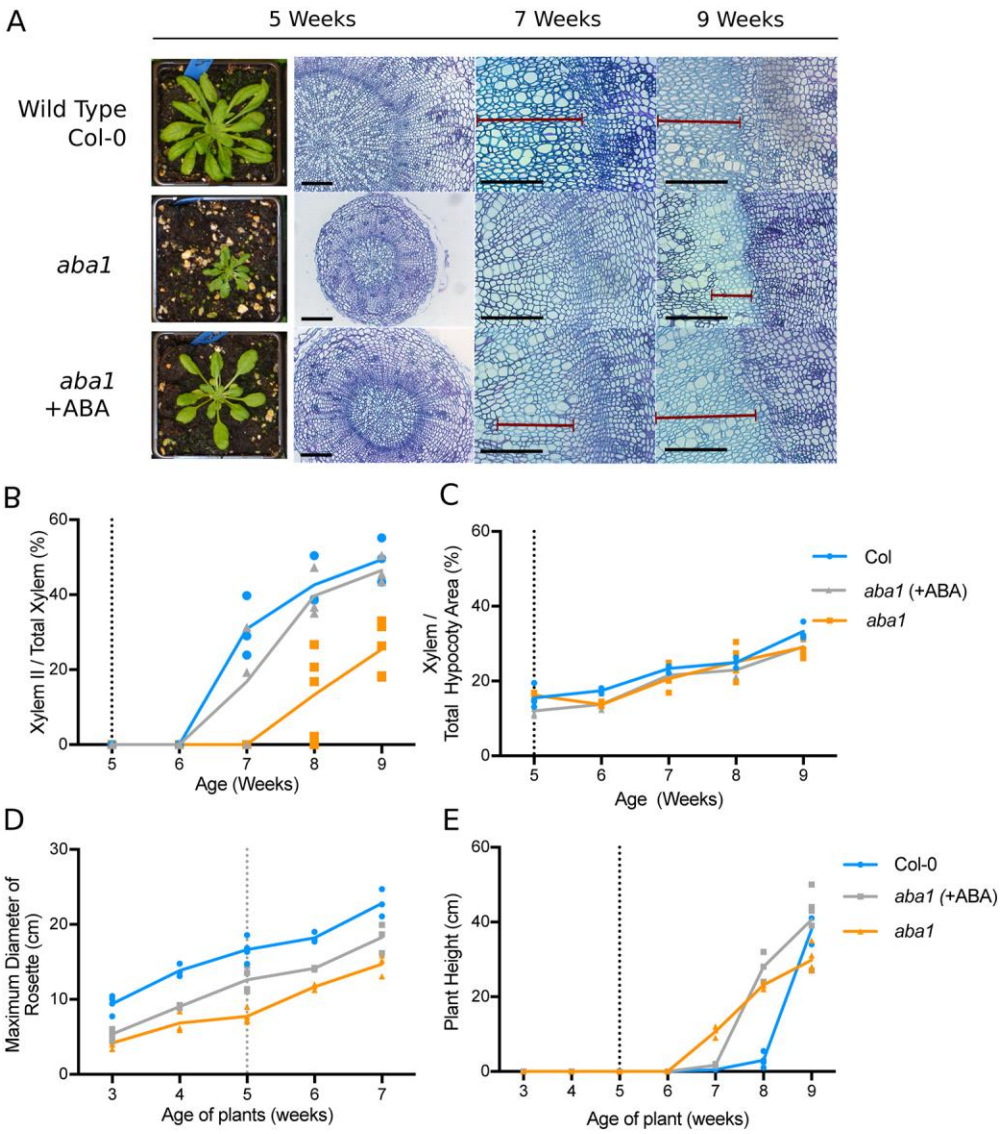


Figure 3 – Phenotype of *aba1* mutant plants following a switch to long days. (A) Representative plant photos at 5 weeks and hypocotyl cross-sections at 5, 7 and 9 weeks. Scale bars represent 100µm, Phase II xylem (maroon line) is indicated. (B) Ratio of xylem II as a percentage of the total xylem versus plant age. (C) Ratio of xylem as a percentage of the total hypocotyl area versus plant age. (D) Rosette Diameter. (E) Plant height. Individual data points are shown, the line connects the means at each time point. Results were analysed using 2 way ANOVA, differences were considered significant if $P < 0.05$. Xylem II/total xylem, *aba1* mutant was significantly different from Col-0 and *aba1* (+ABA) at weeks 7, 8, and 9. Xylem/total: there were no significant differences at any stage. Rosette diameter, *aba1* (+ABA) was significantly different from Col-0 at all stages and from aba1 at weeks 5-7. Plant height, *aba1* was significantly different from Col-0 at weeks 7-9 and from *aba1* (+ABA) at weeks 7 and 9.

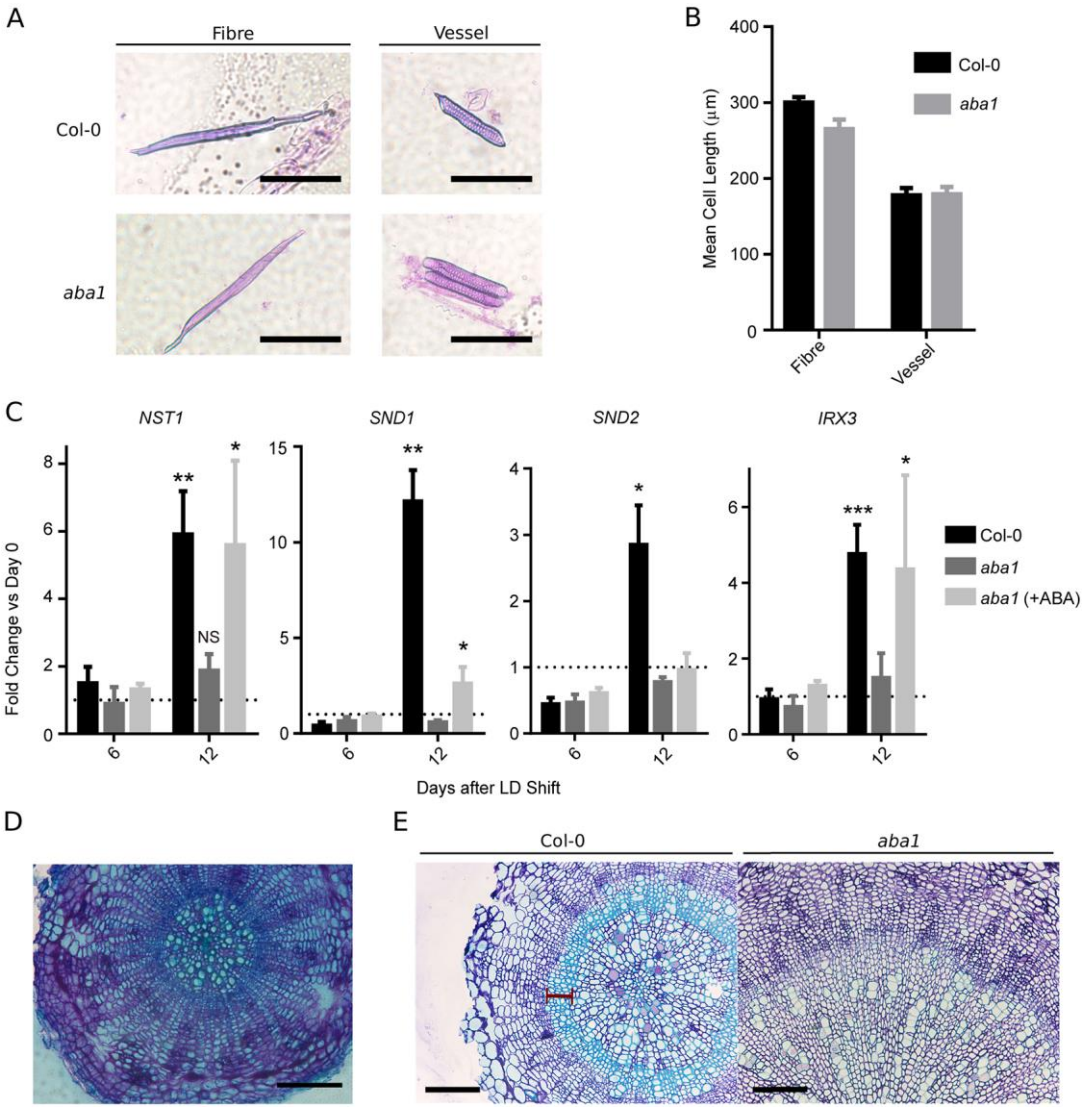
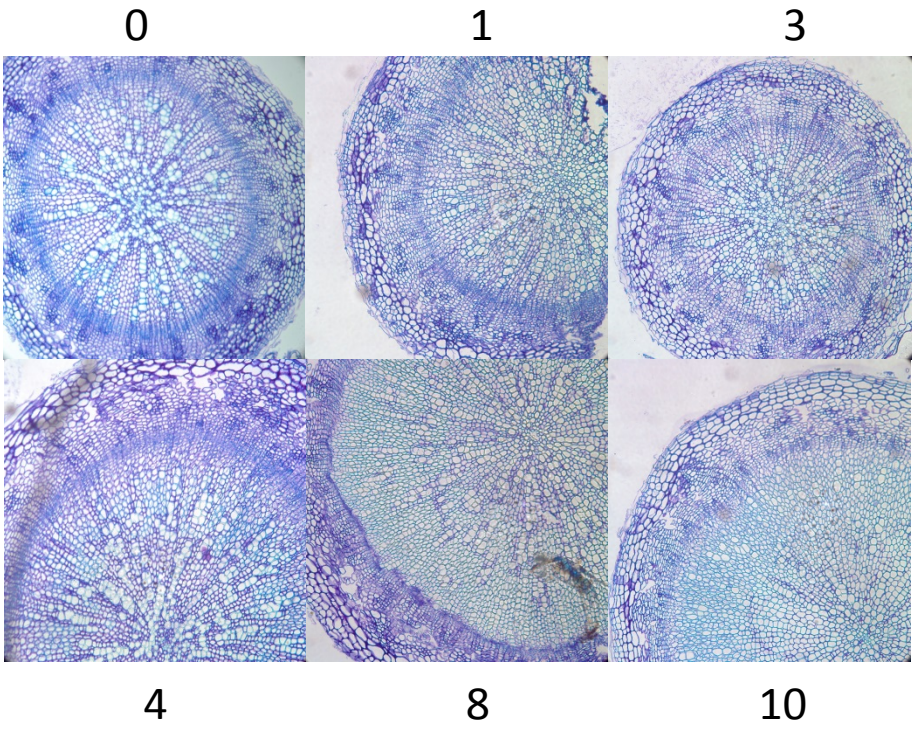


Figure 4 – Characterisation of fibre differentiation in *aba1* mutants. (A) Representative images of xylem vessel and fibre cells from macerated Col-0 and *aba1* hypocotyl tissue. (B) The mean cell lengths ±SD of xylem vessel and fibre cells taken from three biological replicates. More than 20 cells of each type were measured from each plant. Means were compared using a two-tailed t-test and no significant difference were 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. Asterisks indicate a significant difference from the day 0 expression level. 3 samples were collected for each point and the qPCR was carried using technical duplicates using elongation Factor-1α (At5g60390) as an endogenous control. Error bars indicate the S.E.M. Means were compared using a two-tailed t-test. * = $p<0.05$, ** = $p<0.01$, *** = $p<0.001$. (D) A representative cross-section of 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(16 hour day, 8 hour night). Phase II xylem is indicated (maroon line). Scale bars represent 100 µm.

A



B

Figure S1. Plant used for floral induction with Dexamethasone. (A) Hypocotyl sections of the 35S::CO:GR *co-2 tt4* plant in the days following induction (day 0) up to day 10. (B) Representative images of a *co-2 tt4* mutant and an induced 35S::CO:GR *co-2 tt4* plant 14 days after dexamethasone application.

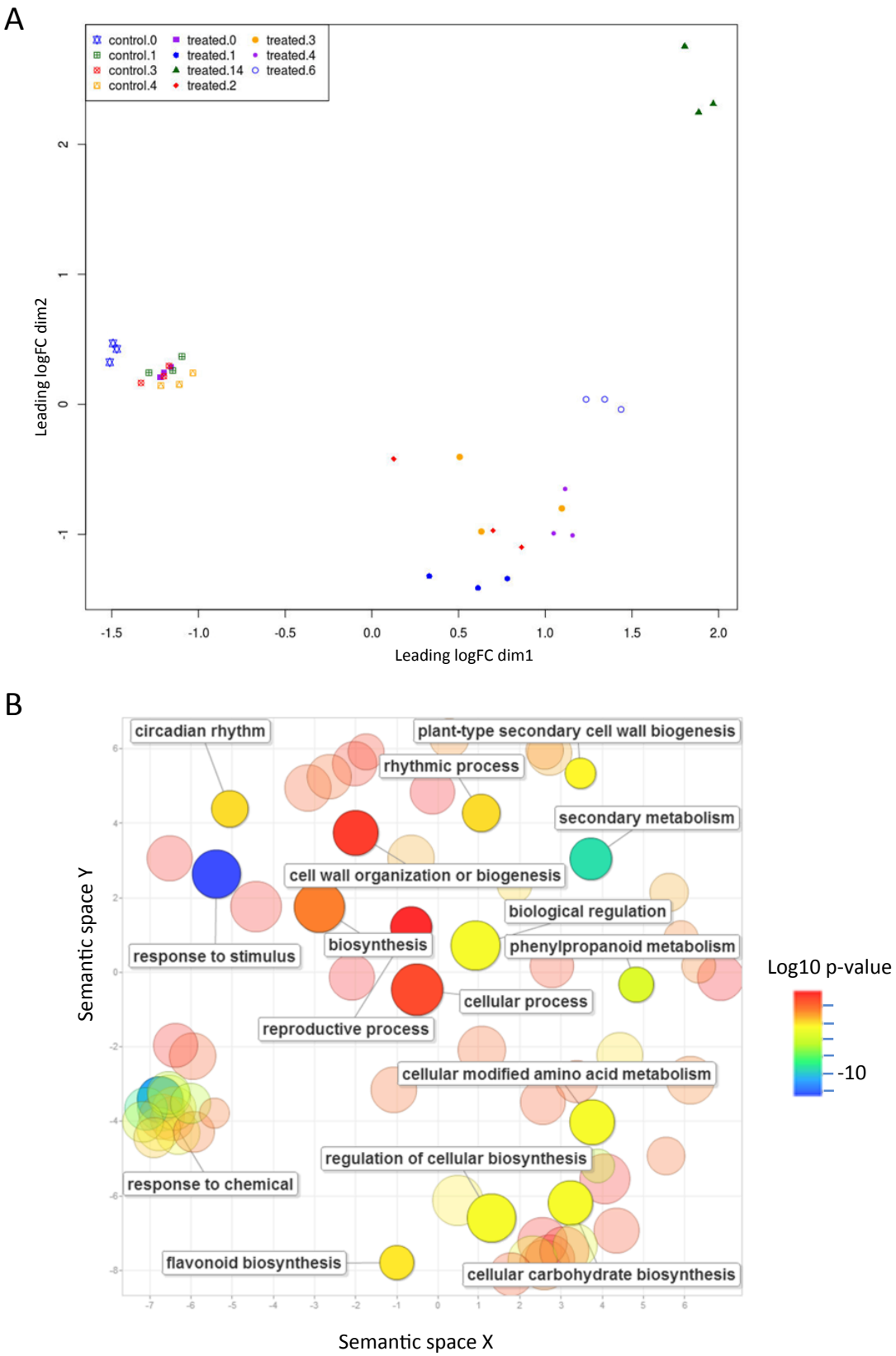


Figure S2 Analysis of RNAseq dataset. (A) An MDS plot of the 33 samples used to create the RNAseq dataset. (B) A visual representation of the most significantly altered GO terms in the RNAseq dataset . Each GO term is represented by an circle and selected GO terms of particular relevance to the current 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).

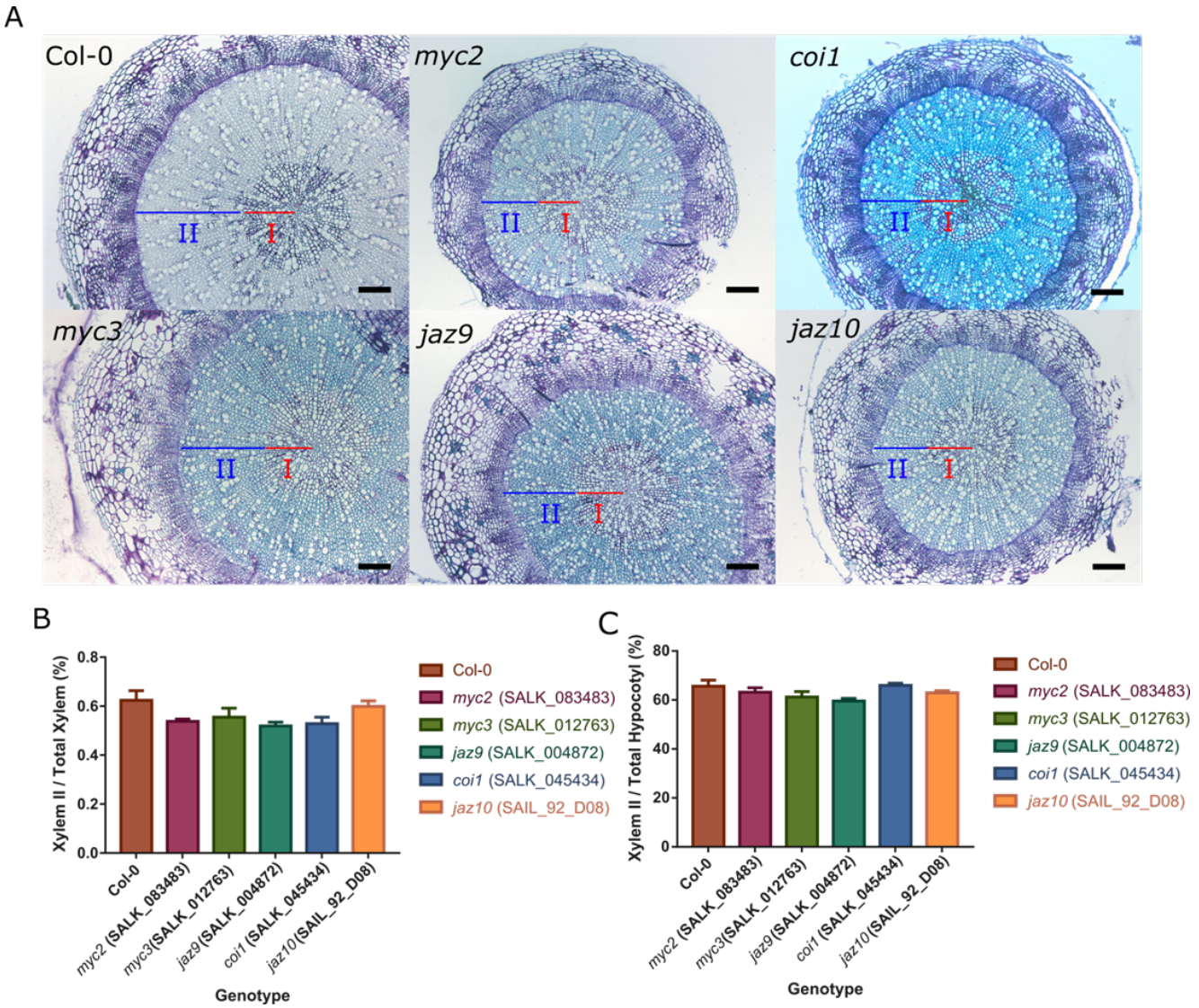


Figure S4 − Analysis of hypocotyl phase transition in known JA signalling mutants. (A) Cross-sections of five JA-signalling mutants taken 3 weeks after flowering, with a Col-0 control. Phase I and II are indicated by blue and red lines respectively. (B) The amount of phase II xylem as a proportion of the total xylem. Scale bars represent 100 µm. (C) The amount of the hypocotyl occupied by xylem. JAZ proteins repress JA signalling, while COI1 and MYC2 are required for JA signalling. Error bars in B and C indicate the S.E.M.Mutant were compared to the wild type in a T-test, no significant differences were identified. At least three plants of each genotype were analysed.

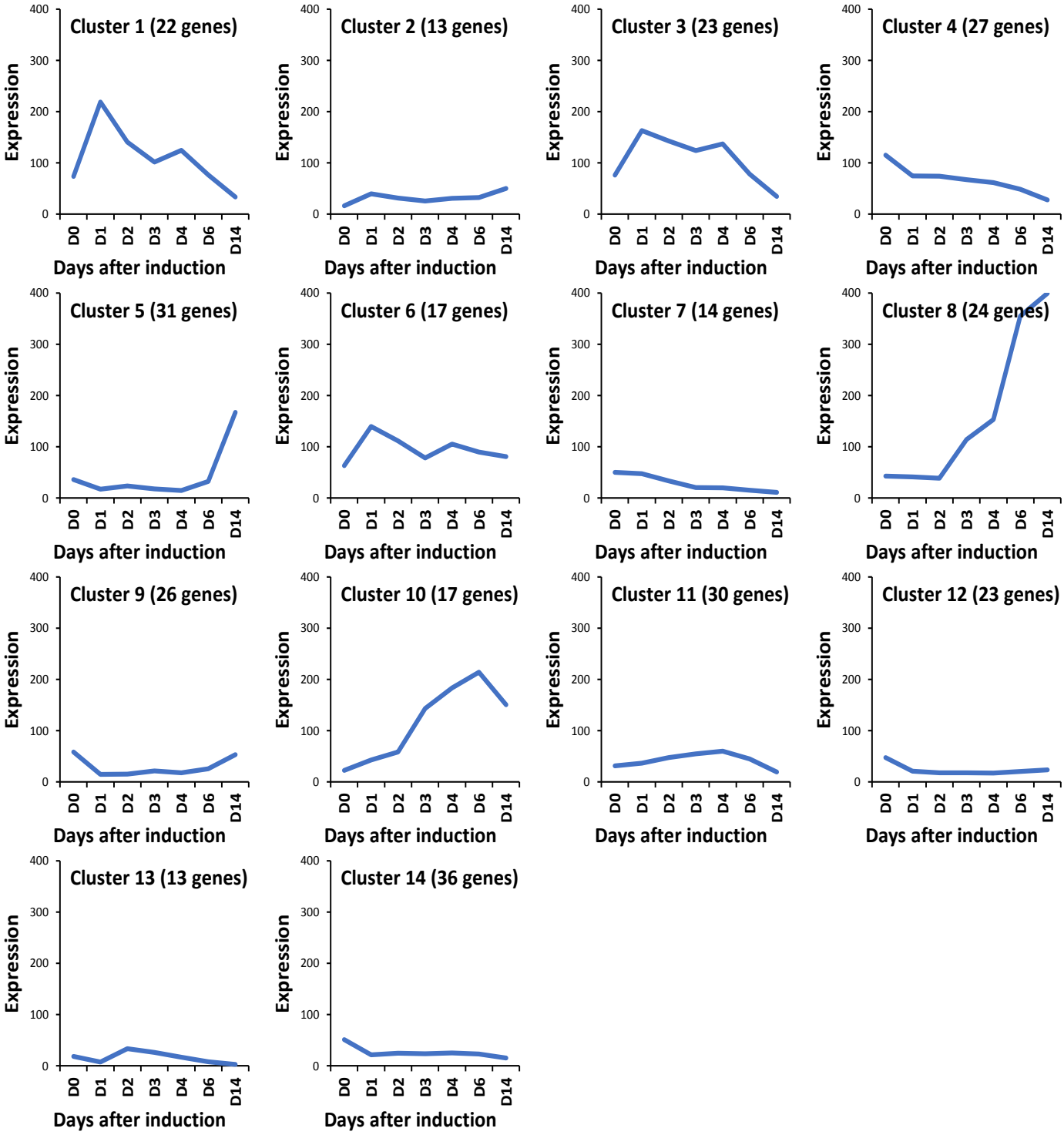


Figure S4. **Expresssion patterns of core genes during hypocotyl phase change.** Clustering of 313 genes whose expression is significantly altered in hypocotyl that are common to both daylength and CO-based induction of phase change. The full list of genes that are found in each cluster are shown in supplementary table SX.

Gene	Name	Induced (day)							Control (day)				Function
		0	1	2	3	4	6	14	0	1	3	4	
AT5G67030	ABA1	0.82	0.99	0.89	0.73	0.87	0.86	1.00	0.58	0.58	0.57	0.56	Core enzyme of ABA biosynthesis
AT2G27150	AAO3	0.96	0.81	0.88	0.89	0.86	0.81	1.00	0.84	0.89	0.84	0.84	Aldehyde oxidase, catalyses final step of aba biosynthesis
AT1G30100	NCED5	0.12	0.25	0.87	0.51	0.93	0.84	1.00	0.02	0.13	0.14	0.05	Required for the production of ABA
AT1G67080	abi4	0.89	0.38	0.38	0.47	0.51	0.72	1.00	0.65	0.66	0.62	0.70	Required for neoxanthin biosynthesis, an intermediary step in abscisic acid biosynthesis
AT1G50030	TOR	0.65	0.66	0.66	0.62	0.63	0.69	1.00	0.71	0.72	0.74	0.77	Prevents aba signalling
AT1G04580	AAO4	0.01	0.01	0.02	0.12	0.16	0.28	1.00	0.01	0.05	0.07	0.11	Aldehyde oxidase, abscisic acid biosynthesis
AT2G04240	XERICO	0.47	0.46	0.35	0.41	0.48	0.57	1.00	0.54	0.46	0.50	0.55	Upregulation of XERICO results in increased cellular ABA content
AT2G38470	WRKY33	0.68	0.19	0.66	0.29	0.24	0.23	1.00	0.85	0.39	0.33	0.42	Negative regulator of ABA biosynthesis, represses NCED3 and NCED5 expression
AT2G47130	SDR3	0.17	0.20	1.00	0.45	0.47	0.26	0.98	0.26	0.26	0.27	0.23	Short-chain dehydrogenase/reductase
AT1G16540	ABA3	0.77	1.00	0.80	0.86	0.81	0.82	0.84	0.68	0.66	0.71	0.65	Core enzyme of ABA biosynthesis
AT5G20960	AAO1	0.96	0.30	0.39	0.33	0.23	0.21	0.58	1.00	0.89	0.89	0.75	Adehyde oxidase
AT1G52340	ABA2	0.63	1.00	0.87	0.77	0.84	0.77	0.50	0.57	0.67	0.64	0.71	Core enzyme of ABA biosynthesis
AT3G14440	NCED3	0.33	0.86	0.82	0.51	1.00	0.83	0.47	0.36	0.51	0.62	0.57	Required for the production of ABA
AT3G43600	AAO2	0.95	0.57	0.61	0.46	0.47	0.42	0.42	1.00	0.79	0.73	0.73	Aldehyde oxidase

Figure S5. Expression pattern of genes involved in ABA biosynthesis in the hypocotyl during floral induction. Genes were taken from TAIR and relative expression is from RNAseq data following Dexamethasome application. Red is maximum expression, green is minimal expression.

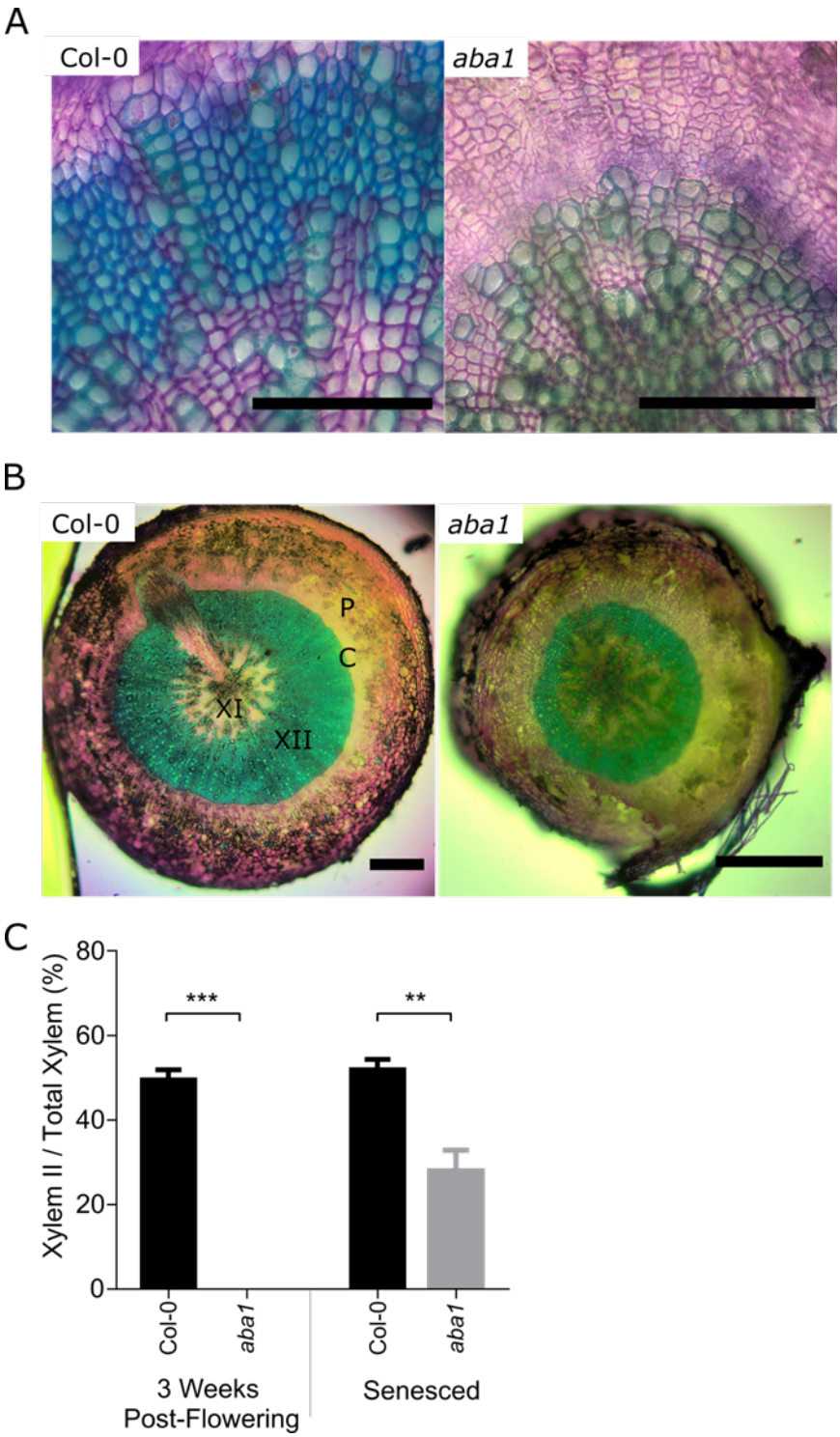


Figure S6 – ABA biosynthesis mutants have reduced fibre formation under constant long day conditions. (A) Representative Col-0 and *aba1* sections 21 days after flowering under continual long day (16hr day/8hr night) conditions. Scale bars represent 100 µm. (B) Representative Col-0 and *aba1* sections from plants grown until senescence under continual long day conditions. Scale bars represent 200 µm. XI = xylem I, XII = xylem II, C = cambium, P = phloem. (C) Quantitative xylem measurements from the experiments shown in (A) and (B). * = $p<0.05$, ** = $p<0.01$, *** = $p<0.001$. Error bars indicate the S.E.M. Measurements were taken from at least 7 biological replicates of each genotype.

Tables

Table S1. A list of primer sequences used in this study.

Primer Name	Sequence
eEF1a1_F	TCCAGCTAAGGGTGCC
eEF1a1_R	GGTGGGTACTCGGAGA
IRX3_F	cagcacagtgttggatgtgacga
IRX3_R	ccgctccatctcaattccaagattc
KNAT1_F	TCCCATTCACATCCTCAACA
KNAT1_R	CCCCTCCGCTGTTATTCTCT
NST1_F	GATGTCACCGTTCATGAGGTC
NST1_R	GGACTGTTTAGGGTTTTGTGAAG
SALK_059469_F	GATGTTGGTGGTGGAAAAATG
SALK_059469_R	ACGTTCAAGAGCATCGTCATC
SALK_LBP1.3	ATTTTGCCGATTTCGGAAC
SND1_F	CAAGCTTGAGCCTTGGGATA
SND1_R	TGGTCCCGGTTGGATACTT
SND2_F	TGCAATGACCGTAGCGATGT
SND2_R	TCGGTGGAAGAAATGACGCA

Table S2. Expression of core 331 genes significantly altered in both daylength shift and CO induction. Those genes idenfitied as related to ABA signalling in the GO analysis are hightlighted in bold.

Click here to Download Table S2