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Report

Flowering as a Condition for Xylem Expansion in *Arabidopsis* Hypocotyl and Root

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Summary

In dicotyledons, biomass predominantly represents cell-wall material of xylem, which is formed during the genetically poorly characterized secondary growth of the vasculature. In *Arabidopsis* hypocotyls, initially proportional secondary growth of all tissues is followed by a phase of xylem expansion and fiber differentiation. The factors that control this transition are unknown. We observed natural variation in *Arabidopsis* hypocotyl secondary growth and its coordination with root secondary growth. Quantitative trait loci (QTL) analyses of a recombinant inbred line (RIL) population demonstrated separate genetic control of developmentally synchronized secondary-growth parameters. However, major QTL for xylem expansion and fiber differentiation correlated tightly and coincided with major flowering time QTL. Correlation between xylem expansion and flowering was confirmed in another RIL population and also found across *Arabidopsis* accessions. Gene-expression analyses suggest that xylem expansion is initiated after flowering induction but before inflorescence emergence. Consistent with this idea, transient activation of an inducer of flowering at the rosette stage promoted xylem expansion. Although the shoot was needed to trigger xylem expansion and can control it in a graft-transmissible fashion, the inflorescence stem was not required to sustain it. Collectively, our results suggest that flowering induction is the condition for xylem expansion in hypocotyl and root secondary growth.

Results and Discussion

Vascular Development and Secondary Growth in *Arabidopsis*

Carbon skeletons in nearly all biomass are directly or indirectly provided by photosynthesis, which uses solar energy to transform water and carbon dioxide into sugars. In higher land plants, excess sugar is invested into growth and transported from photosynthetic source organs toward sink organs through the phloem tissue of the vasculature [1]. The vasculature also comprises xylem, which transports soil water and minerals to the leaves. In most physiological conditions, xylem is a sink tissue. In perennial dicotyledons, it represents the principal site of biomass accumulation [2].

Mutagenesis in *Arabidopsis thaliana* has identified numerous regulators of vascular development [1], including genes required for vascular patterning (e.g., [3, 4]) or proper vascular-tissue differentiation (e.g., [5]). Several of these genes implicate plant-hormone pathways in vascular development. For instance, polar auxin transport is needed for de novo vascular specification and patterning [6], whereas cytokinin signaling has a crucial role in xylem versus phloem differentiation [7, 8]. Finally, the brassinosteroid pathway has been implicated in vascular patterning as well as differentiation [9, 10].

Radial expansion growth of the vasculature is of pivotal importance because water and solute transport through the xylem eventually limits plant growth. Thus, vascular expansion or secondary growth continues throughout the life cycle in a more or less complex pattern and often displays developmentally regulated organ- or species-specific transitions [1]. In *Arabidopsis* hypocotyls, the seedling stems, secondary growth is characterized by an early phase of proportional growth of all vascular tissues ("proportional phase" in the following) and then a later phase of overproportional expansion of the xylem as compared to phloem ("xylem-expansion phase" in the following) [11]. The factors that are responsible for this transition are not known [11–13].

The Hypocotyl as a Model Organ for Secondary Growth

It has been previously proposed that the hypocotyl is a credible model for secondary growth [2, 11]. Importantly, unlike inflorescence stems, hypocotyls offer the distinct advantage of uncoupled elongation and secondary growth and the interesting feature of a xylem development that is highly reminiscent of trees. In the constant-light conditions used throughout our study, hypocotyl elongation ceased 4–5 days after germination (dag) and secondary growth started 5–6 dag, after formation of the cambium, the vascular stem cells. Our conditions reproduced the developmental pattern described previously [11]. Initially, radial growth of the concentric tissue layers, epidermis, cortex, endodermis, phloem, and xylem, was proportional (Figures 1A–1C and 1F). Later, while overall growth continued, the xylem area started to expand and steadily increase as compared to phloem (Figures 1C–1F). Xylem consists of water-conducting xylem vessels and nonconducting parenchyma cells. Coincident with xylem expansion, parenchyma cells started to differentiate into fibers, cells with lignified secondary cell walls that provide structural support (Figures 1E and 1G). Thus, xylem-to-phloem ratio and fiber differentiation are good indicators of the observed developmental transition.

Natural Variation of Hypocotyl Secondary Growth in *Arabidopsis*

We quantified secondary-growth parameters (transverse total hypocotyl, cortex, phloem, xylem, and fiber area) at 28 dag (Figures 1H and 1I) across a sample of 32 natural accessions. These traits displayed natural variation (Figure 1J), including xylem-to-phloem ratio (Figure 1K) (broad sense heritability, $h^2 = 0.89$). Among the accessions with low xylem-to-phloem ratio, we had previously analyzed Uk-1 for its short-root phenotype [14]. This phenotype results from loss of function of the *BREVIS RADIX* (*BRX*) gene, which has been implicated in connecting the auxin and brassinosteroid pathways [15]. Because certain brassinosteroid mutants display decreased xylem and increased phloem formation [16], low xylem-to-phloem ratio in Uk-1

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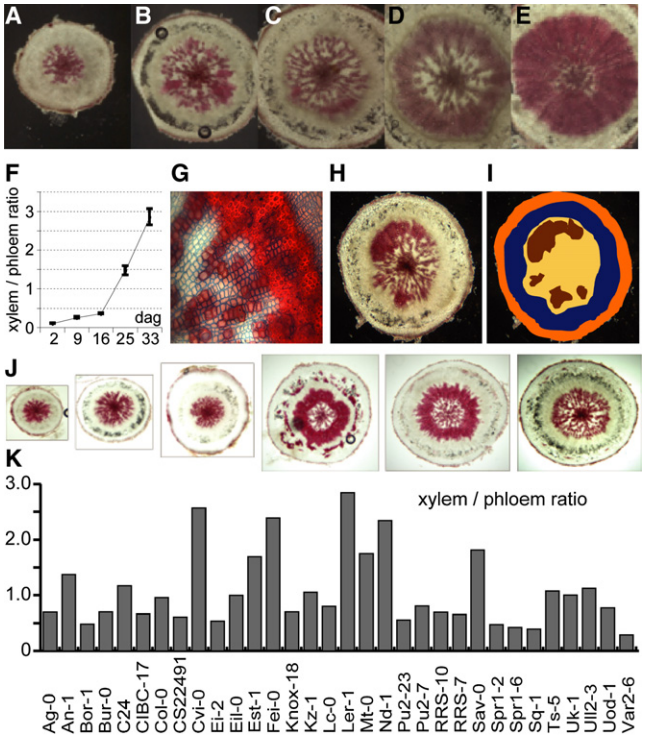

Figure 1. Secondary Growth of *Arabidopsis* Hypocotyls

Images show transverse sections after Wiesner staining of cell walls. The thick cell walls of water-conducting xylem vessels appear in lighter red, and those of nonconducting fibers appear in darker red, because of different lignin composition [20]. As shown in (A)–(E), hypocotyl secondary growth can be divided into a phase of balanced growth of all tissues (proportional phase [A–C]) followed by xylem expansion and fiber differentiation (xylem-expansion phase [D and E]). In (F), quantification of xylem-to-phloem ratio in hypocotyls of the accession Sav-0 illustrates the acceleration of xylem expansion, observed between 16 and 25 days after germination (dag). As shown in (G), fiber differentiation (dark red) occurs from nonconducting parenchyma cells within the xylem area. (H) and (I) shows methodology: Images were loaded into analysis software to mark and measure different tissue areas. The schematic (I) on the right represents the section (H) on the left. Orange represents cortex area plus the single cell epidermal layer. Blue represents the phloem area. Yellow represents the xylem area. Within the xylem, brown represents differentiated fibers, which appear in clusters. (J) shows examples of natural variation at 28 dag. (K) shows the quantification of xylem-to-phloem ratio in 32 wild accessions. Images were taken at 10× magnification.

was not unexpected. Analysis of Uk-1 hypocotyl and root vasculature revealed generally decreased vascular proliferation as compared to Uk-1 plants complemented by a *BRX* transgene (Figures 2A, 2C, and 2D). Moreover, we also noticed slightly decreased hypocotyl elongation in Uk-1 (Figure 2B). Thus, *brx* loss of function results in decreased growth in the longitudinal as well as radial dimension in both hypocotyl and root. Importantly, however, although vascular cell proliferation was restored by functional *BRX* (Figures 2E–2H), xylem-to-phloem ratio was not altered (Figures 2F–2H).

Separate Genetic Control of Secondary-Growth Parameters in an *Arabidopsis* RIL Population

To determine which factors, if not *BRX*, determine Uk-1 xylem-to-phloem ratio, we turned to quantitative trait loci (QTL) analysis of recombinant inbred lines (RILs) derived from a cross to the Sav-0 accession [14]. Sav-0 has a functional *BRX* allele and displays stronger vascular proliferation and earlier transition to the xylem-expansion phase than Uk-1 (Figure S1 available online). Interestingly, these trait differences were also evident in the root (Figures S1C–S1F), suggesting that hypocotyl and root secondary growth are coordinated. Secondary-growth phenotypes were determined for the RILs and combined with single-nucleotide polymorphism (SNP) marker genotyping data (Table S1) for respective QTL mapping. As a control, root elongation, as expected, mapped *BRX* as the single highly significant QTL (Figure S2A). Total transverse hypocotyl area did not reveal any significant QTL (Figure S2B), which could result from limited SNP marker resolution or little segregating variation in the population. By contrast, a significant QTL for cortex area was found on chromosome 1 (Figure S2C). Mapping of xylem-to-phloem ratio revealed a major, highly significant QTL on chromosome 5 (Figure S2D), which coincided with the major QTL for fiber content (Figure S2E). Thus, of the four secondary growth traits investigated, only xylem expansion and fiber differentiation were genetically linked. This is remarkable because shared genetic control could facilitate coordinated growth of neighboring tissues [17].

Coincidence of Xylem-to-Phloem Ratio and Flowering Time QTL

The major QTL controlling xylem-to-phloem ratio and fiber content in the Uk-1 × Sav-0 RILs coincided with the major flowering time QTL determined in an independent experiment (Figure S2F). A tightly linked candidate gene for this QTL is *FLOWERING LOCUS C* (*FLC*), a central negative regulator of flowering [18]. *FLC* expression level is a quantitative determinant of flowering, and its variation is predominantly responsible for natural flowering time variation [19]. Increased *FLC* expression at the vegetative stage delays flowering. Indeed, consistent with early flowering of Sav-0 and late flowering of Uk-1, *FLC* expression was approximately nine times higher in Uk-1 than Sav-0 rosettes (Figure 3A). Given the well-documented role of *FLC* in natural variation of flowering, this suggests that *FLC* and the major flowering QTL are identical. Moreover, coincidence of this QTL with the secondary-growth QTL suggests that *FLC* expression levels also explain RIL variation in xylem-to-phloem ratio and fiber content. Indeed, in the RIL population, all three traits correlated tightly ($R^2 > 0.60$) (Figures 3B–3D). To corroborate the observed correlation, we determined xylem-to-phloem ratio, *FLC* expression level, and flowering time in eight individual RILs. Consistent with the population analysis, RILs with high xylem-to-phloem ratio at 28 dag displayed much lower levels of *FLC* expression at 10 dag and had flowered much earlier than plants with low xylem-to-phloem ratio (Figures 3E and 3F). Therefore, low *FLC* expression correlated with earlier flowering and transition to the xylem-expansion phase.

The Correlation between Flowering and Hypocotyl Xylem Expansion Is a General Rule

To elucidate whether *FLC* in particular or flowering as a physiological condition per se determines hypocotyl xylem-to-phloem ratio, we analyzed secondary-growth traits and flowering time in another RIL population. The parental accessions, Eil-0 and Lc-0, displayed similar flowering time, similar *FLC* expression levels, and moderately different xylem-to-phloem ratio. The RIL population displayed transgressive phenotypes for both flowering and xylem-to-phloem ratio (Figure S3A). Consistent with weak (xylem-to-phloem ratio) or absent (flowering) parental trait differences, we could not detect

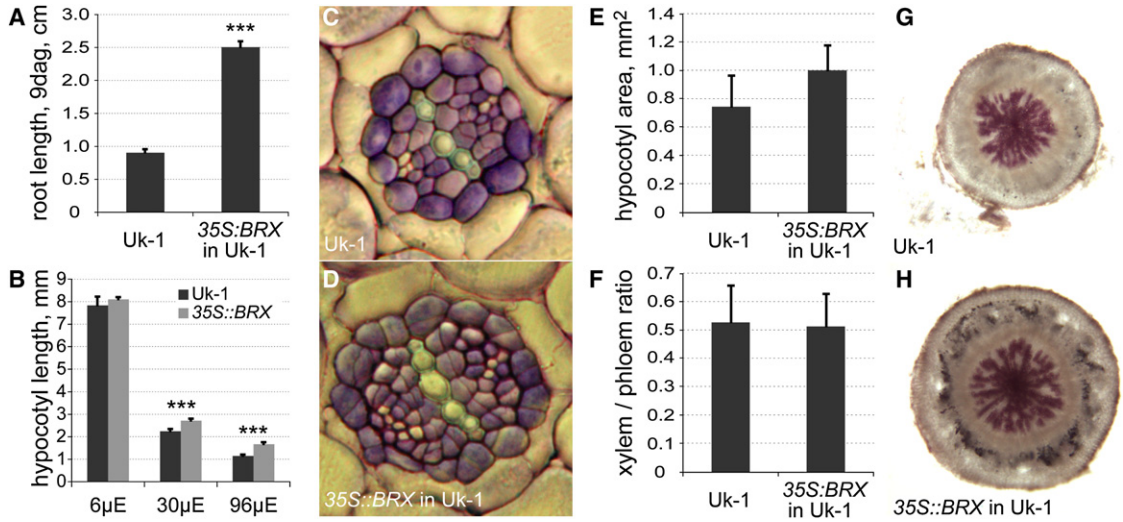

Figure 2. Vascular and Hypocotyl Phenotypes of the Uk-1 Accession

(A) Introduction of a transgenic functional *BRX* open-reading-frame unit under control of the constitutive *35S* promoter complements the Uk-1 root growth defect.

(B) Hypocotyl elongation in complemented lines relative to Uk-1 background at 6 dag under increasing light intensity.

(C) Vascular cylinder of a mature root section of Uk-1 at 7 dag after toluidine blue staining. The outermost stained cell layer, the pericycle, surrounds the stained phloem, parenchyma, and cambial cells as well as the xylem vessels.

(D) Same as (C) for a complemented line.

(E) Total transverse hypocotyl area in Uk-1 and complemented lines at 21 dag.

(F) Xylem-to-phloem ratio of hypocotyls measured in (E).

(G and H) Images of hypocotyl sections (Wiesner staining) from Uk-1 and a complemented line at 28 dag. Magnification is shown at 63× in (C) and (D) and 10× in (G) and (H). Error bars represent standard error of the mean. Significant differences as judged by t test are indicated as * for $p < 0.05$, ** for $p < 0.01$, and *** for $p < 0.001$.

significant QTL for flowering time nor xylem-to-phloem ratio. Nevertheless, correlation between xylem-to-phloem ratio and flowering was again evident ($R^2 = 0.2889$) (Figures S3A and S3B), suggesting that flowering per se determines the transition of hypocotyl secondary growth to the xylem-expansion phase in this RIL population as well. Moreover, a survey of flowering time and secondary growth across a sample of accessions revealed again correlation between flowering and xylem-to-phloem ratio ($R^2 = 0.5523$) (Figure S3C), suggesting once more that these traits are under shared genetic control.

Global Gene-Expression Analysis of the Transition to the Xylem-Expansion Phase

To detect a molecular signature of xylem expansion, we compared global gene-expression profiles of hypocotyls during their incipient transition to the xylem-expansion phase. To this end, we planted three cohorts of seedlings at 4 day intervals and grew them in identical conditions. As soon as an inflorescence was visible in the oldest plants, the hypocotyls of the 4 and 8 day younger plants were harvested and compared by microarrays. A total of 207 genes were differentially expressed (false-discovery rate <1%, fold change >2-fold) (Table S2), with most (177) displaying increased expression in the older sample, including genes described in the context of secondary-cell-wall thickening, a characteristic of xylem formation (Figure 4A). These are for instance cellulose or lignin biosynthesis enzymes [20] as well as transcription factors that have been implicated in vascular differentiation (e.g., [21–23]). Overall, the pattern was reminiscent of previous analyses of inflorescence stem secondary growth (e.g., [17, 24]). Direct comparison with other studies revealed significant overlap with published marker genes for secondary-cell-wall formation [25] and genes preferentially expressed in the xylem of hypocotyls [26] (Figures 4B and 4C). Thus, the data suggest that a developmental shift toward increased xylem proliferation, i.e., xylem expansion, had occurred between the two time points assayed.

Flowering Triggers the Transition to the Xylem-Expansion Phase

Notably, *FLC* as well as other pivotal regulators of flowering time, such as *CONSTANS* (*CO*) or the florigen, *FLOWERING LOCUS T* (*FT*), are expressed in the vasculature. There, FLC counteracts *FT* activation by other factors, such as CO [18]. Given that transcriptional initiation of flowering precedes inflorescence bud formation by more than a week [18], our microarray analysis suggests that upregulation of genes involved in xylem formation occurred after initiation of flowering. The idea that flowering is a condition for hypocotyl xylem expansion was further supported by our analysis of accessions that flowered very late. None of them displayed xylem expansion before flowering (e.g., in Figure 4D), again suggesting that its occurrence is not simply a matter of age.

Finally, we directly tested whether flowering promotes xylem expansion by taking advantage of transgenic lines that allow dexamethasone-inducible activation of CO in the phloem of *co* null mutants [27]. *CO* acts in the phloem to induce flowering non-cell autonomously in response to photoperiod, but constitutive expression of *CO* induces flowering irrespective of day length [28]. In our experiment, we transiently induced CO activity at the rosette stage by watering plants with dexamethasone between 10 and 13 dag. Compared to noninduced controls, these plants flowered only slightly earlier (1–2 days) in our constant-light conditions. Examination of the hypocotyls

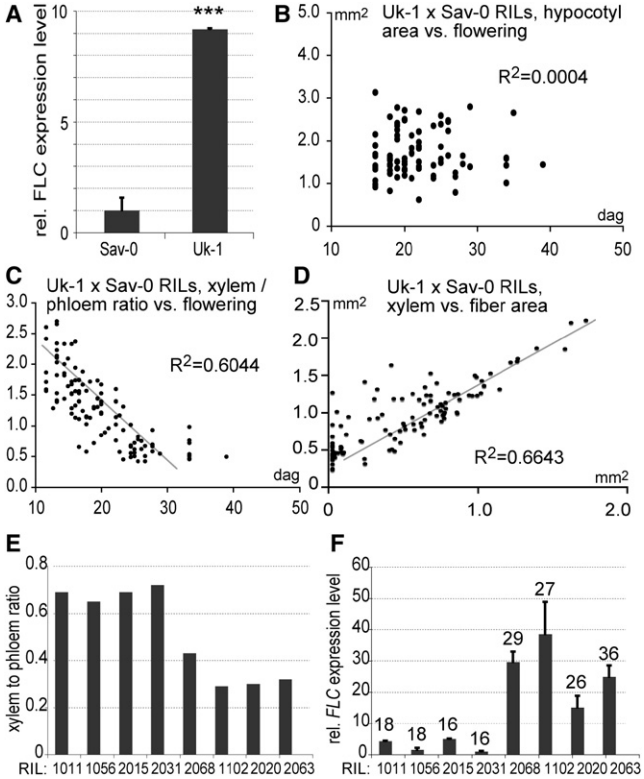

Figure 3. Correlation of Flowering Time and *FLC* Expression Levels with the Xylem-Expansion Phase of Hypocotyl Secondary Growth in the Uk-1 × Sav-0 RIL Population

(A) Relative levels of *FLC* expression in Sav-0 and Uk-1, measured by quantitative real-time PCR and calibrated against the *EF1* house-keeping gene.
(B) Correlation plot of the Uk-1 × Sav-0 RILs for hypocotyl area versus flowering time.
(C) Correlation plot of the Uk-1 x Sav-0 RILs for xylem-to-phloem ratio versus flowering time.
(D) Correlation plot of the Uk-1 × Sav-0 RILs for xylem area versus fiber area.
(E) Xylem-to-phloem ratio determined at 28 dag for a sample of individual Uk-1 × Sav-0 RILs.
(F) Columns represent relative *FLC* expression levels (calibrated against the house-keeping gene *EF1*) in the RILs of (E), measured at the seedling stage at 10 dag. Numbers above columns indicate actual time of flowering of the corresponding RIL (dag). The Pearson correlation values are $R^2 = 0.6533$ for *FLC* levels versus flowering time, $R^2 = 0.7354$ for *FLC* levels versus xylem-to-phloem ratio, and $R^2 = 0.7895$ for flowering time versus xylem-to-phloem ratio. Error bars represent standard error of the mean. Significant differences as judged by t test are indicated as * for $p < 0.05$, ** for $p < 0.01$, and *** for $p < 0.001$.

at 20 dag, after floral buds were detectable but before emergence of the inflorescence stem, revealed a striking difference in secondary growth (Figures 4E and 4F). Overall secondary thickening was enhanced in the induced plants, and xylem expansion had strongly progressed (Figure 4G). Thus, transient upregulation of the *CO* flowering initiation pathway triggered secondary growth in general and transition to the xylem-expansion phase in particular.

Induction of the Xylem-Expansion Phase Depends on the Presence of the Shoot

To determine whether this reflects an autonomous vascular function of *CO* or requires feedback from the shoot, we monitored the expression levels of the CO target *FT* and *NAC SECONDARY WALL THICKENING PROMOTING FACTOR 1* (*NST1*), a key regulator of secondary xylem formation [22] (Figures 4H and 4I). To this end, we transferred one set of dexamethasone-inducible CO seedlings onto dexamethasone media at 6 dag, whereas a second set was transferred onto mock media. In both sets, the shoot was removed from one-half of the seedlings. At 9 dag, RNA was isolated from excised hypocotyls, and *FT* and *NST1* levels were determined by qPCR, relative to the housekeeping gene *EF1* [29]. In a parallel setup, seedlings were transferred back to standard media at 9 dag and kept for another 4 days before a similar analysis. As expected, *FT* expression was strongly induced in all dexamethasone-treated samples (Figures 4H and 4I). By contrast *NST1* was not expressed at appreciable levels at 9 dag (Figure 4H), suggesting that unlike *FT*, it is not a direct CO target. However, *NST1* was strongly upregulated at 13 dag, but only so if the shoot had been present (Figure 4I). Flowering induction was confirmed in the dissected shoots by monitoring *APETALA1* expression as well as visual observation. Notably, again inflorescence emergence had not yet occurred (Figure S4). These data suggest that CO-induced xylem expansion was not a direct result of CO action in the vasculature. Rather, it appears to reflect feedback from the flowering program induced in the shoot.

To independently corroborate this idea, we performed grafting between hypocotyls at 6 dag, by using Sav-0 root stocks and Sav-0 (expressing low *FLC* levels) or Uk-1 (expressing high *FLC* levels) shoot stocks. At 20 dag, we determined xylem-to-phloem ratio in the root stock, just below the hypocotyl-root junction. Plants that had received a Sav-0 shoot stock displayed a significantly higher ratio than plants that had received a Uk-1 shoot stock (Figure 4J). Thus, a late flowering shoot suppressed the transition to xylem expansion in the root of a genotype in which this transition would normally already have occurred. This finding is consistent with the idea that flowering-related signals from the shoot control xylem expansion in hypocotyl and root.

Conclusions

In summary, our findings demonstrate separate genetic control of secondary-growth traits, coordination of secondary growth between hypocotyl and root, and flowering as the elusive condition for the switch to the xylem-expansion phase [11–13]. This coordination of flowering and xylem expansion could serve to structurally reinforce the plant in preparation for emergence ("bolting") of the inflorescence. Notably, bolting itself does not appear to be required for hypocotyl xylem expansion because our gene-expression analyses and flowering-induction experiments suggest that xylem expansion is initiated earlier. Consistent with this idea, artificial excess weight placed on rosettes before flowering did not trigger xylem expansion, and xylem expansion continued even if the inflorescence was cut immediately after formation (data not shown) [13].

The causal relationship between flowering, xylem expansion, and fiber differentiation should be taken into account to isolate genes that genuinely affect secondary growth in a quantitative manner. For instance, xylem-to-phloem-ratio variation between accessions is strongly diminished if lines are assayed at their respective time of flowering rather than a given age. Thus, it would be interesting to revisit mutants with a xylem-to-phloem-ratio phenotype and determine whether this is accompanied by a flowering phenotype. For example, altered xylem-to-phloem ratio has been reported for the stems of certain brassinosteroid pathway mutants [16]. We observed the

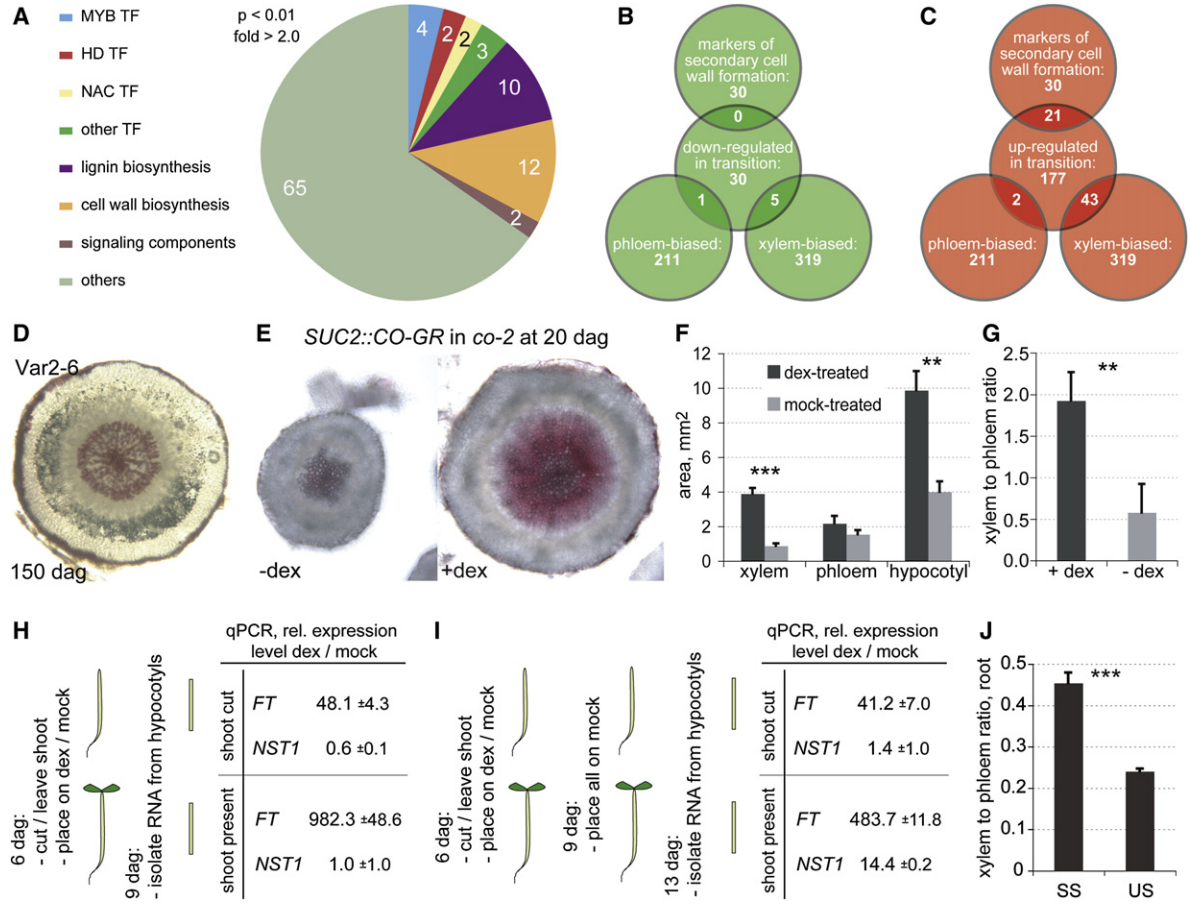

Figure 4. Gene-Expression Analysis of the Transition to Xylem Expansion

(A) Functional categorization of 177 genes that were upregulated in hypocotyls during their transition to the xylem-expansion phase. The numbers indicate percentage of the total.

(B) Comparison of 30 genes downregulated during transition to hypocotyl xylem expansion with secondary-cell-wall biosynthesis marker genes [25] and genes expressed specifically in phloem or xylem of hypocotyls [26]. The number of genes shared by different sets is indicated as overlaps.

(C) As in (B), for the 177 genes upregulated during transition to hypocotyl xylem expansion.

(D) Hypocotyl of a late-flowering accession at 150 dag. No signs of xylem expansion are visible.

(E) Comparison of hypocotyls of transgenic *co* mutant plants carrying a *SUC2::CO-GR* transgene that allows dexamethasone (dex)-inducible activation of CO in the phloem. Transiently induced (+dex) and noninduced (−dex) plants at 20 dag. Induction was performed by watering with 50 μM dex between 10 and 13 dag.

(F) Quantification of secondary-growth traits in the hypocotyls of dex- and mock-treated plants.

(G) Quantification of the xylem-to-phloem ratio of the plants measured in (F).

(H) Schematic representation of the tissue-culture treatment of the plants used in (E) before quantification of *FT* and *NST1* expression levels in excised hypocotyls at 9 dag by qPCR. *CO* was induced with 50 μM dexamethasone (dex).

(I) As in (H), except that expression levels were assayed at 13 dag, after an additional 4 days on standard media.

(J) Quantification of xylem-to-phloem ratio at 20 dag in the root vasculature just below the hypocotyl-root junction in plants grafted at 6 dag. Root genotype was Sav-0; shoot genotype was Sav-0 (low *FLC* levels) or Uk-1 (high *FLC* levels). "SS" represents Sav-0 shoot stock and Sav-0 root stock. "US" represents Uk-1 shoot stock and Sav-0 root stock. Error bars represent standard error of the mean. Significant differences as judged by t test are indicated as * for $p < 0.05$, ** for $p < 0.01$, and *** for $p < 0.001$.

equivalent phenotype in the hypocotyl of *brassinosteroid insensitive 1* and *constitutive photomorphogenic and dwarf* mutants if both mutants and wild-type controls were assayed at the same age. However, we did not observe a significant difference if plants were assayed at their respective time of flowering (R.S., unpublished data). This finding is consistent with a recently reported delay of flowering in these mutants [30]. Finally, it is worth mentioning that we noticed two atypical accessions in which xylem expansion was strongly delayed or accelerated with respect to flowering. Molecular genetic analyses of these lines might reveal the identity of novel rate-limiting factors of secondary growth.

Supplemental Data

Experimental Procedures, four figures, and two tables are available at http://www.current-biology.com/cgi/content/full/18/6/458/DC1/.

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