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Mobile Gibberellin Directly Stimulates *Arabidopsis* Hypocotyl Xylem Expansion

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Secondary growth of the vasculature results in the thickening of plant structures and continuously produces xylem tissue, the major biological carbon sink. Little is known about the developmental control of this quantitative trait, which displays two distinct phases in *Arabidopsis thaliana* hypocotyls. The later phase of accelerated xylem expansion resembles the secondary growth of trees and is triggered upon flowering by an unknown, shoot-derived signal. We found that flowering-dependent hypocotyl xylem expansion is a general feature of herbaceous plants with a rosette growth habit. Flowering induction is sufficient to trigger xylem expansion in *Arabidopsis*. By contrast, neither flower formation nor elongation of the main inflorescence is required. Xylem expansion also does not depend on any particular flowering time pathway or absolute age. Through analyses of natural genetic variation, we found that *ERECTA* acts locally to restrict xylem expansion downstream of the gibberellin (GA) pathway. Investigations of mutant and transgenic plants indicate that GA and its signaling pathway are both necessary and sufficient to directly trigger enhanced xylogenesis. Impaired GA signaling did not affect xylem expansion systemically, suggesting that it acts downstream of the mobile cue. By contrast, the GA effect was graft transmissible, suggesting that GA itself is the mobile shoot-derived signal.

INTRODUCTION

In higher land plants, any excess sugar provided by photosynthesis is not needed for maintenance of the general metabolism; it is invested into growth and transported from photosynthetic source organs toward sink organs (Ye, 2002; Thompson, 2006). This transport occurs in the vascular phloem, a highly specialized tissue comprised of various cell types, such as the sieve elements, which perform the actual transport of the phloem sap; companion cells, which are responsible for loading and unloading of phloem sap cargo; phloem parenchyma cells, which transfer metabolites to and from companion cells; and phloem fibers, cells with thick secondary cell walls that provide structural support. The vasculature also comprises xylem tissue, which transports soil water and minerals to the leaves. Xylem is a sink tissue, which incorporates sugar into novel cell wall material and represents the principal site of biomass accumulation in perennial dicotyledons (Demura and Ye, 2010). Three general cell types are found in the xylem of nearly all vascular plants: xylem vessels, which are the actual conduits for water and solutes; xylem fibers, which possess thick secondary cell walls to provide

structural support; and xylem parenchyma cells, which can differentiate into fibers.


The extent of xylem tissue is an important factor for plant growth because water and solute transport capacity limits shoot growth as plants become bigger. The major group of extant plants, the dicotyledons, have solved this developmental problem by continuously expanding their vascular tissues throughout their life cycle, resulting in the radial expansion of stems and roots. This so-called secondary growth also offers the advantage that nonfunctional xylem vessels can be replaced, as they do not forever resist the strain of the negative pressure created by the transpiration stream. In perennial plants, for instance, this permits the replacement of water transport capacity lost through cavitation of xylem vessels. Moreover, because negative pressure and thus mechanical strain increase with height, secondary growth permits plants to grow taller and thus represents the key invention that permitted the evolution of trees (Spicer and Groover, 2010).


Secondary growth is driven by the vascular cambium, a secondary meristem that is located between phloem and xylem within a vascular bundle (Ye, 2002; Elo et al., 2009). The cambial stem cells produce daughter cells that will acquire phloem or xylem fate, depending on the given polarity. Studies in *Arabidopsis thaliana* have identified various genes that are critical for the proper differentiation of vascular tissue types (e.g., Bonke et al., 2003; Kubo et al., 2005; Mitsuda et al., 2007), and several among them implicate plant hormone pathways in vascular development. For instance, polar auxin transport and auxin signaling are required for de novo vascular specification and

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patterning (Scarpella et al., 2006; Nilsson et al., 2008), while cytokinin signaling has a crucial role in the determination of xylem versus phloem identity (Mähönen et al., 2000; Carlsbecker and Helariutta, 2005; Mähönen et al., 2006).

In various species, the gibberellin (GA) pathway has been implicated in promoting xylogenesis in combination with the auxin pathway, as well as in the induction of fiber differentiation (Digby and Wareing, 1966; Eriksson et al., 2000; Biemelt et al., 2004; Mauriat and Moritz, 2009; Dayan et al., 2010). However, in these studies, GA also exerted a general growth-promoting effect, suggesting that elevated GA signaling might merely enhance xylem production along with the overall growth increase in height. For instance, while *Populus* trees overexpressing a GA biosynthetic gene produced more total fibers than the control trees, relative abundance of fibers remained approximately constant (Eriksson et al., 2000). In summary, it remains unclear to what degree the effects of GA on xylem production and fiber development are specific.

Other hormone pathways that impinge on vascular development in *Arabidopsis* have been shown to play an equally important role in *Populus* (Schrader et al., 2003; Dettmer et al., 2009; Nieminen et al., 2008; Nilsson et al., 2008). Moreover, despite being an annual, herbaceous plant, *Arabidopsis* displays significant secondary growth, including lignification of cells walls, suggesting that *Arabidopsis* can serve as a model to investigate this trait (Chaffey et al., 2002; Nieminen et al., 2004). This potential is underpinned by a recent study that found that manipulation of flowering time regulators can turn *Arabidopsis* into a quasiperennial plant that displays strong secondary growth and bush-like morphology (Melzer et al., 2008). Already in wild-type *Arabidopsis* lines, secondary growth is observed in the hypocotyl, the seedling stem (Chaffey et al., 2002; Sibout et al., 2008). Moreover, in the hypocotyl, elongation growth and thickening are uncoupled; therefore, unlike in stems, secondary growth at any position along the hypocotyl is not obscured by parallel ongoing elongation growth and formation of novel vasculature at the apex (Sibout et al., 2008). Finally, secondary growth in *Arabidopsis* hypocotyls proceeds in two distinct phases, an early phase of proportional secondary growth of all tissues followed by a later phase of xylem expansion and fiber differentiation, which is highly reminiscent of the mode of secondary growth in trees (Chaffey et al., 2002; Nieminen et al., 2004). Collectively, these characteristics render the *Arabidopsis* hypocotyl a particularly suitable model for the investigation of basic mechanisms of secondary growth.

The transition between the early and late stages of hypocotyl secondary growth is triggered by flowering (Sibout et al., 2008). Transient transgenic expression of flowering inducers, such as *CONSTANS* (CO), can therefore be used to accelerate xylem production at will (Sibout et al., 2008). A combination of micrografting and gene expression analyses has suggested that this requires signaling from the shoot via a mobile shoot-derived signal (Sibout et al., 2008). In this study, we set out to identify the factors that limit and induce hypocotyl xylem expansion in response to flowering. Guided by the analysis of natural genetic variation in this trait, we identified local determinants as well as a systemic determinant, which appears to be identical with the mobile signal.

RESULTS

Flowering as a General Condition for Hypocotyl Xylem Expansion in Rosette Plants

The aerial part of plants consists of repeating units called phytomeres, which each comprise a stem internode, one or several organs, and an axillary meristem. The internodes of vegetative *Arabidopsis* phytomeres do not elongate significantly before bolting, and *Arabidopsis* is thus classified as a plant with rosette habit. The switch from vegetative to reproductive growth (flowering) reprograms the shoot apical meristem to produce inflorescence rather than leaf primordia and coincides with the appearance of elongated internodes bearing inflorescences (bolting). Since flowering also triggers xylem expansion along *Arabidopsis* hypocotyls (Figures 1A to 1C) (Sibout et al., 2008), we investigated whether this phenomenon is conserved in other species and whether it correlates with the rosette growth habit. To this end, we selected species within the *Brassicaceae* and *Astereaceae* (rosette plants mostly belong to these two families) that do or do not display a rosette growth habit. In addition, we analyzed two species from the *Solanaceae*, which do not encompass rosette plants. For all species, hypocotyl cross sections were obtained at different developmental stages and stained with phloroglucinol to highlight xylem tissue. In all non-rosette plants analyzed (*Arabis alpina*, *Aster alpinus*, *Nicotiana benthamiana*, and *Solanum lycopersicum*), xylem started to expand during the vegetative phase (Figures 1D to 1G), as indicated by the appearance of fibers, whereas in rosette plants, (*Arabidopsis*, *Cardamine hirsuta*, *Barbarea verna*, and *Taraxacum officinalis*), no xylem expansion was observed before flowering (Figures 1H to 1K). Thus, flowering appears to be a general condition for xylem expansion in the hypocotyl of rosette plants.

Flower Specification and Bolting Are Not Required for Hypocotyl Xylem Expansion

An open question that we also aimed to answer was whether hypocotyl xylem expansion depends on the actual formation of flowers? To this end, we analyzed mutants in the *FLOWERING LOCUS T* (FT) gene, which produces a mobile protein that moves from the leaf vasculature to induce flowering in the shoot apical meristem in long-day conditions (Corbesier et al., 2007). In addition, FT acts as a floral pathway integrator (a central regulator of flowering) in the inflorescence meristem. *ft-1* mutants flower late compared with the wild type; thus, xylem expansion was determined at the same developmental stage (i.e., at or shortly after the appearance of the inflorescence, as throughout this article unless indicated otherwise) rather than the same age to allow meaningful comparison of secondary growth vigor (Sibout et al., 2008). In these assays, *ft-1* mutants displayed wild-type levels of xylem expansion (see Supplemental Figures 1A and 1B online), also suggesting that FT is not the elusive mobile signal for hypocotyl expansion. *LEAFY* (LFY) is another flowering pathway integrator and also controls flower and inflorescence identity (Weigel et al., 1992). *lfy ft* double mutants bolt very late and produce leaf-like structures instead of flowers (Kardailsky

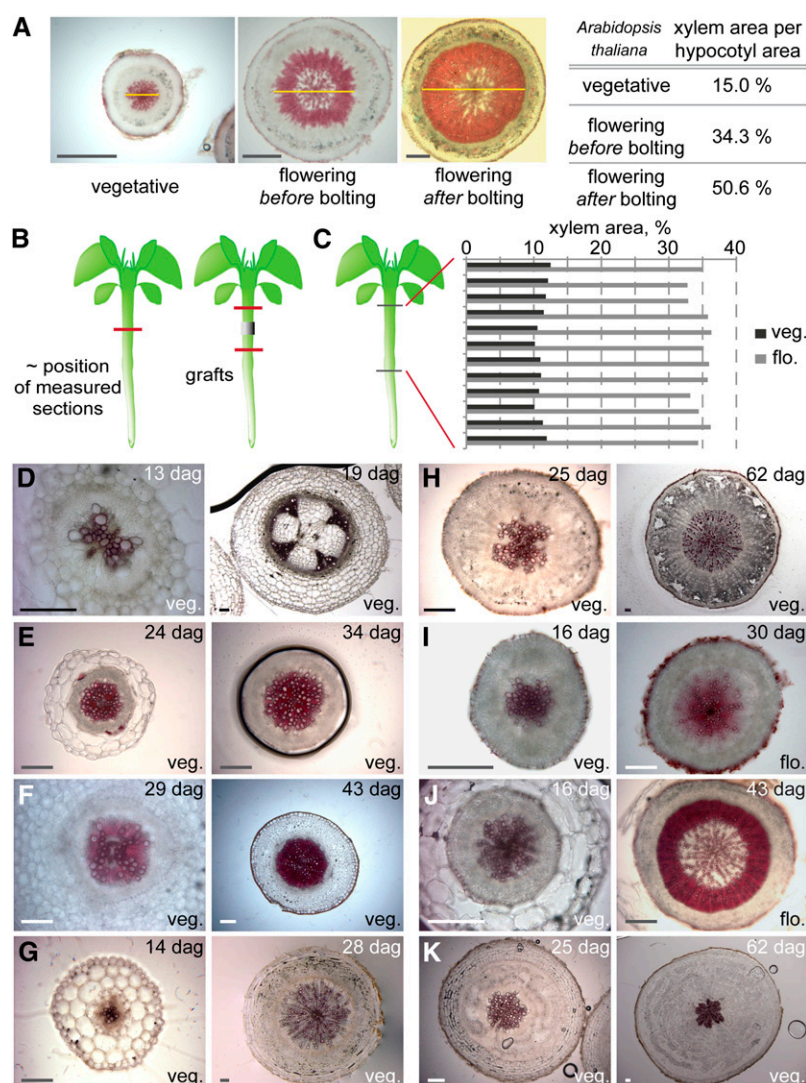


Figure 1. Flowering as a Condition for Hypocotyl Xylem Expansion in Plants with Rosette Growth Habit.

(A) Progression of xylem expansion in *Arabidopsis* illustrated by transverse sections, stained for lignin with phloroglucinol to highlight xylem vessels and fibers (yellow line indicates xylem diameter). Xylem area compared with total hypocotyl area increases slowly during the vegetative phase but accelerates considerably once flowering occurs.

(B) Schematic illustration of the approximate position of sections (red lines) sampled to measure the relative xylem area in the genotypes analyzed in this study. Sections were taken from the hypocotyl center, except in the case of grafting experiments. For the latter, scion and stock sections were taken about halfway between the apical and basal ends of the hypocotyls, respectively, and the silicon tubing collar of the grafts.

(C) Relative xylem area as determined from serial sections along individual *Arabidopsis* hypocotyls before and after flowering.

(D) to (K) Hypocotyl cross sections of species with nonrosette growth habit (i.e., the stem internodes elongate already during vegetative growth) (**[D]** to **[G]**) and with rosette growth habit (i.e., the stem internodes elongate only once flowering has been induced) (**[H]** to **[K]**), demonstrating that in nonrosette plants, xylem expansion already starts during vegetative growth. *S. lycopersicum* (**D**), *A. alpina* (**E**), *Aster alpinus* (**F**), *N. benthamiana* (**G**), *B. verna* (**H**), *Arabidopsis* (**I**), *C. hirsute* (**J**), *T. officinalis* (**K**). dag, days after germination; veg., vegetative growth phase; flo., flowering, at appearance of inflorescence meristem. Bars = 200 μ m.

et al., 1999). Still, the double mutants displayed wild-type xylem expansion (see Supplemental Figure 1C online), suggesting that floral identity is not a prerequisite for this process.

Somewhat opposite to *lfy ft* double mutants, *terminal flower1* (*tfl1*) mutants are tiny plants, which flower early after producing only few leaves and terminate their inflorescence prematurely

(Kobayashi et al., 1999). Again, as in the wild type, xylem expansion in *tfl1* occurred at flowering (see Supplemental Figure 1D online), corroborating that neither inflorescence weight nor bolting are the triggering factor (Sibout et al., 2008). Additional evidence that bolting is not required for xylem expansion was obtained from investigation of *pennywise pound-foolish* (*pnf pnf*)

double mutants. PNY and PNF are two homeodomain transcription factors that promote flowering. Their combined loss of function results in plants that never flower and continue to produce leaves instead. *pnf pnf* mutants respond to flowering stimuli but do not complete floral induction and fail to express the majority of floral meristem identity genes (Smith et al., 2004; Kanrar et al., 2008). Surprisingly, we observed some xylem expansion in *pnf pnf* plants, although with a long delay compared with the wild type (see Supplemental Figures 1E to 1G online). In summary, these analyses of key flowering regulators suggest that neither floral specification nor bolting is required for hypocotyl xylem expansion.

Hypocotyl Xylem Expansion Is Not an Age-Related Trait

The result from the analysis of the *pnf pnf* double mutants suggested that age might be a factor in triggering hypocotyl xylem expansion. Indeed, a novel, age-regulated flowering induction pathway that acts in parallel to FT and might still be functional in *pnf pnf* has been identified; this pathway is based on the interplay of two microRNAs, *miR156* and *miR172*, which control the general transition from the juvenile to the adult phase (Wu et al., 2009). The direct targets of *miR156* are transcription factors of the SQUAMOSA PROMOTER BINDING PROTEIN-LIKE family (Cardon et al., 1999), whereas the targets of *miR172* are APETALA2-like transcription factors (Aukerman and Sakai, 2003). To determine whether these miRNAs could trigger xylem expansion, we analyzed both overexpressor lines of *miR156* and *miR172* as well as so-called mimic lines, in which the activity of the respective miRNA is reduced by expression of a complementary, artificial miRNA (Franco-Zorrilla et al., 2007; Todesco et al., 2010). Both overexpressor and mimic lines did not show any xylem phenotype during development, whether assayed before or at flowering (see Supplemental Figures 1H to 1M online). Moreover, loss of function in *miR156* targets also displayed wild-type xylem expansion (see Supplemental Figure 2 online). Collectively, these data suggest that the *miR156/miR172* pathway does not control xylem expansion and that xylem expansion is in sync with the transition to reproductive development irrespective of absolute age.

Exploring Natural Variation to Identify Novel Rate-Limiting Factors for Xylem Expansion

In summary, the investigation of mutant and transgenic lines affected in various flowering pathways, as well as variation in relevant environmental conditions, such as photoperiod, did not yield any candidate regulators of xylem expansion. Since the same was true for genes involved in bolting and flower specification, it appears that xylem expansion is a consequence of the incipient reprogramming of the shoot apical meristem upon flowering. To find relevant regulators, we turned to the analysis of natural genetic variation, which enabled us previously to isolate a major QTL for xylem expansion, the central flowering time regulator *FLOWERING LOCUS C* (Sibout et al., 2008). As suggested by our earlier study, we revisited this trait to assay *Arabidopsis* accessions at their individual time of flowering rather than the same age to select against identification of flowering

time genes and reveal natural variation in genuine regulators of hypocotyl secondary growth (Sibout et al., 2008). Indeed, natural variation in our indicator trait for xylem expansion, the ratio of xylem area to total hypocotyl area in transverse sections, was observed. Among the few dozen investigated, two accessions stood out because of their atypical secondary growth phenotypes: whereas xylem expansion was strongly enhanced in the commonly used Landsberg *erecta* (*Ler*) accession, xylem expansion was significantly delayed in Kazakhstan-1 (Kz-1) (Figure 2A).

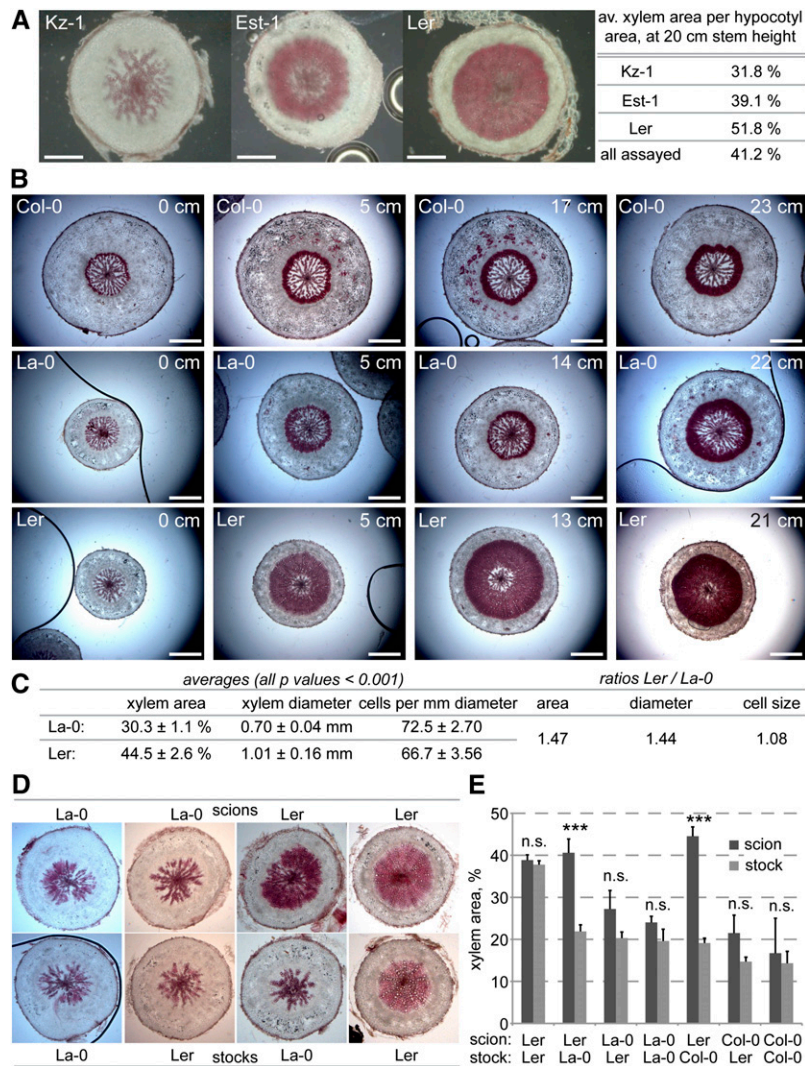
ERECTA Is a Locally Acting Negative Regulator of Xylem Expansion

Detailed analysis of the *Ler* accession revealed that the onset of xylem expansion is normal at the time of bolting but proceeds at an enhanced rate afterwards compared with other accessions, such as the *Arabidopsis* reference accession Columbia (Col-0) (Figure 2B). *Ler* is a mutant derived from the original Landsberg-0 (La-0) accession and carries a loss-of-function mutation in the *ERECTA* (*ER*) gene (Redei, 1962), which encodes a Leu-rich receptor-like Ser/Thr kinase (Torii et al., 1996). The La-0 accession displayed normal, less prominent xylem expansion, comparable to Col-0 (Figures 2B and 2C), suggesting that the *Ler* phenotype is due to the *er* loss of function. This idea fits with the fact that *ER* acts as a major general regulator of growth (van Zanten et al., 2009). However, while *ER* is a positive regulator of elongation growth, it appears to be a negative regulator of secondary growth. Notably, although xylem cells in *Ler* are somewhat bigger, the increased xylem area in *Ler* was only to a minor part due to cell size (Figure 2C), corroborating that the effect of the *er* mutation manifests primarily at the level of enhanced xylogenesis rather than cellular anisotropy.

To determine whether *ER* could have a role in the mobile signal, we took advantage of the micrografting technique (Turnbull et al., 2002). To facilitate this approach, we germinated seedlings at 25°C to enhance hypocotyl elongation before performing reciprocal grafts between hypocotyls of the different genotypes. Xylem expansion was then measured in the upper hypocotyl, derived from the scion, and the lower hypocotyl, derived from the stock, at 8 d after appearance of the inflorescence meristem. These experiments revealed that the stimulation of xylem expansion observed in *Ler* scions was not graft transmissible and that this stimulation was suppressed in *Ler* stocks if the scion was derived from La-0 or Col-0 (Figures 2D and 2E). This might mean that the extent of secondary growth in the scion and, thus, *ER* limits the secondary growth of the stock, possibly by limiting transmission of the mobile signal. In summary, it appears that *ER* is not involved in generating or suppressing the mobile signal and that *ER* acts locally, downstream of the mobile signal.

Analysis of the Kz-1 Accession Suggests a Role of the GA Pathway in Hypocotyl Xylem Expansion

Opposite to *Ler*, xylem expansion in the Kz-1 accession was strongly delayed and did not occur even well after flowering and bolting (Figure 2A). Interestingly, in an independent survey of



(A) Natural quantitative variation in hypocotyl xylem expansion between *Arabidopsis* accessions, exemplified as xylem area per total transverse hypocotyl area in plants with 20-cm-tall main inflorescence stems. Est-1, Estland-1.

(B) Progression of hypocotyl xylem expansion in the Col-0 reference strain and the *er* mutant (*Ler*) in La-0 background. Plants with roughly equal stem height are shown for comparison; 0-cm stem height represents flowering (i.e., appearance of inflorescence meristem).

(D) Examples of scion and stock hypocotyl sections from micrograftings of indicated genotypes, sampled at 8 d after flowering.

(E) Xylem area quantification of scion and stock hypocotyls obtained from indicated micrograftings, sampled at 8 d after flowering. Error bars are

standard error. n.s., not significant; * $P < 0.05$; ** $P < 0.01$; *** $P < 0.001$. Bars = 200 μm .

To further investigate a role of the GA pathway in xylem expansion, we took advantage of transgenic lines that allow dexamethasone-induced nuclear import of a CO-glucocorticoid receptor fusion protein (CO-GR) expressed under control of the *SUCROSE TRANSPORTER2* promoter in a *co-2* null mutant background (*SUC2:CO-GR*). Induction enables the CO-GR fusion protein to trigger the expression of *FT* and, thus, flowering and consequently hypocotyl xylem expansion (Sibout et al., 2008). Combination of this induction with paclobutrazol

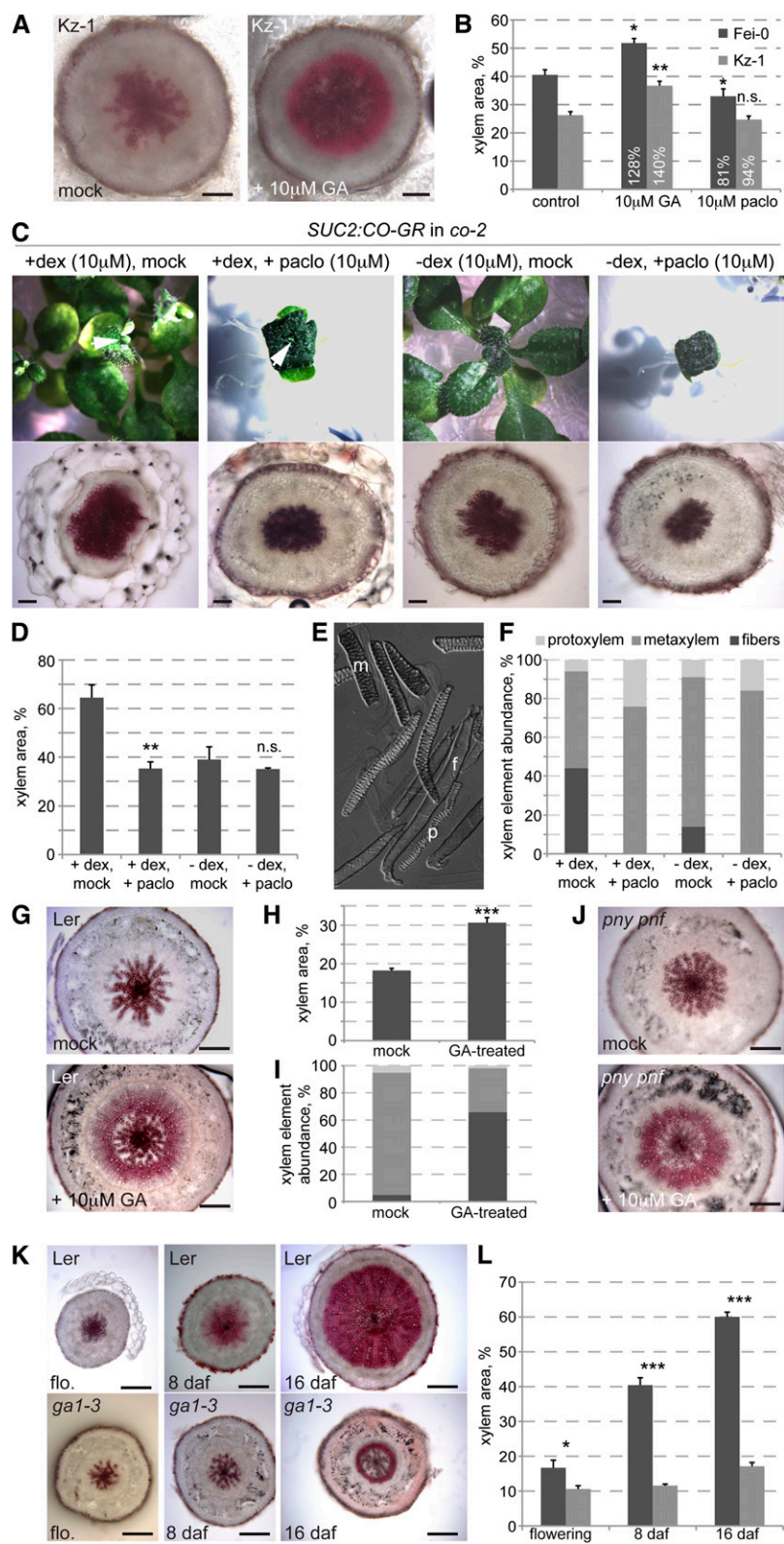


Figure 3. GA Is Necessary for Hypocotyl Xylem Expansion.

(A) Rescue of the reduced xylem expansion phenotype of the Kz-1 accession (assayed at 26 d after germination) by treatment with 10 μ M GA.

treatment prevented xylem expansion as well as fiber formation (Figures 3C and 3D). Maceration of the hypocotyls allowed us to score the abundance of the different xylem elements (Muñiz et al., 2008), confirming the absence of fibers and an increased amount of vessels in response to paclobutrazol treatment (Figures 3E and 3F). We also conducted the inverse experiment and investigated whether GA application could promote xylem expansion before flowering. To this end, plants were grown in short-day conditions to prevent flowering and sprayed with GA at 28 d. Hypocotyls were then sectioned and macerated once the inflorescence meristem became visible (at 35 d for GA-treated plants and at 41 d for the control plants). The GA-treated plants displayed a significant increase in the xylem area to total area ratio and in the proportion of fibers (Figures 3G to 3I). This finding was corroborated by GA treatment of the *pnf pnf* double mutant, which did not restore flowering, but again induced xylem expansion compared with mock-treated plants (Figure 3J).

We confirmed our results by analysis of the prototypical GA-deficient mutant *ga1-3* (Sun and Kamiya, 1994). *ga1-3* plants are dwarfs with small, dark-green leaves and severely stunted inflorescences. They also flower very late, as is typical for GA-deficient plants (Willige et al., 2007) (i.e., in our conditions, *ga1-3* flowered 50 d later than the *Ler* control). Hypocotyl sections of both genotypes were again taken at the same stage after flowering. Consistent with our earlier observations, *ga1-3* mutants displayed severely reduced xylem expansion, although fibers were still present (Figures 3K and 3L).

GA Signaling Is Necessary to Permit Hypocotyl Xylem Expansion

To investigate whether the GA effect on xylem expansion is mediated by the described GA signaling pathway, we also assayed GA signaling mutants. First, we sought to analyze mutants in the GA receptor, which is encoded by three redundantly acting genes, *GA INSENSITIVE DWARF (GID) 1a*, *GID1b*,

and *GID1c* (Griffiths et al., 2006; Nakajima et al., 2006; Willige et al., 2007). *gid1a-b-c* triple null mutants (as described in Willige et al., 2007) largely resemble *ga1-3* both in their phenotypic and molecular characteristics; however, their phenotype is even more severe in that they never flower (Willige et al., 2007). This is unlike another *gid1a-b-c* triple mutant (Griffiths et al., 2006), which was constructed with a *GID1C* mutant allele (*gid1c-1*) that carries an intronic T-DNA insertion. Although it has been reported that wild-type transcript was not found in this allele and it should be considered null, the flowering phenotype suggests that it might be leaky. By contrast, the *gid1a-b-c* triple used in our study has been constructed using the *gid1c-2* allele, an exon insertion (Willige et al., 2007). This line never flowered. Therefore, we introduced either a *SUC2:FT* or a *35S:FT* transgene into segregating lines to obtain *gid1a-b-c* triple mutants that constitutively express the *FT* florigen. In these lines, we eventually observed flower-like structures, indicating that reprogramming of the shoot apical meristem by the transgene was successful (Figure 4A). However, no hypocotyl xylem expansion was observed in these plants, suggesting that the GA receptors are required for this process.

The targets of the GA receptors are transcriptional regulators of the so-called DELLA class (Silverstone et al., 2001), which repress growth and are targeted for degradation upon GA binding to the GID1 receptors. In *Arabidopsis*, the DELLA family comprises five members, *GA-INSENSITIVE (GAI)*, *REPRESSOR OF *ga1-3* (RGA)*, and *RGA-LIKE (RGL) 1-3* (Davière et al., 2008), which were identified by mutant analysis (Peng et al., 1997; Dill et al., 2001). Dominant *gai* and *rga* mutants encode stabilized variants of the proteins that are insensitive to GA-triggered degradation. Thus, the mutant proteins continue to suppress elongation growth even in the presence of GA, leading to a stunted dwarf phenotype. We obtained transgenic plants that express functional fusions of these dominant versions with the GR domain under control of the respective endogenous promoters (*GAI:gai^D-GR* and *RGA:rga^D-GR*), enabling their dexamethasone-inducible activation as described above.

Figure 3. (continued).

(B) Quantification of xylem expansion in Kz-1 (assayed at 29 d after germination) and an average accession, Fei-0 (St. Maria d. Feiria) (assayed at 34 d after germination) with or without GA treatment.

(C) Suppression of hypocotyl xylem expansion in *SUC2:CO-GR* transgenic plants (*co-2* mutant background) upon interference with GA biosynthesis by treatment with the inhibitor paclobutrazol (paclo). Plants were first treated with dexamethasone (dex) from 0 to 5 d after germination to induce flowering and thereby xylem expansion. This was followed by a 14-d treatment with paclobutrazol, inducing a GA-deficient phenotype (top). Induced plants flowered at 19 d after germination, and hypocotyls were sampled and sectioned at that point (bottom). Arrowheads point out the inflorescence meristem in dexamethasone-induced plants.

(D) Quantification of xylem area in the experiment illustrated by examples in **(C)**. Error bars are SE.

(E) Example of xylem elements released from a hypocotyl sample by maceration. Elements can be distinguished by their morphology and counted to determine relative abundance. f, fiber; m, metaxylem; p, protoxylem.

(F) Quantification of xylem element abundance in the experiment illustrated by examples in **(C)**.

(G) Stimulation of xylem expansion in the *Ler* accession by GA treatment, sampled at vegetative state.

(H) Quantification of xylem area in the experiment illustrated by examples in **(G)**. Error bars are SE.

(I) Quantification of xylem element abundance in the experiment illustrated by examples in **(G)**. See **(F)** for xylem element coding.

(J) Stimulation of xylem expansion in the *pnf pnf* double mutant by GA treatment, sampled at vegetative state.

(K) Reduction of xylem expansion in the GA-biosynthetic mutant *ga1-3* compared with its background, *Ler*.

(L) Quantification of xylem area in the experiment illustrated by examples in **(K)**. Error bars are SE. daf, days after flowering; n.s., not significant; **P* < 0.05; ***P* < 0.01; ****P* < 0.001. Bars = 200 μ m.

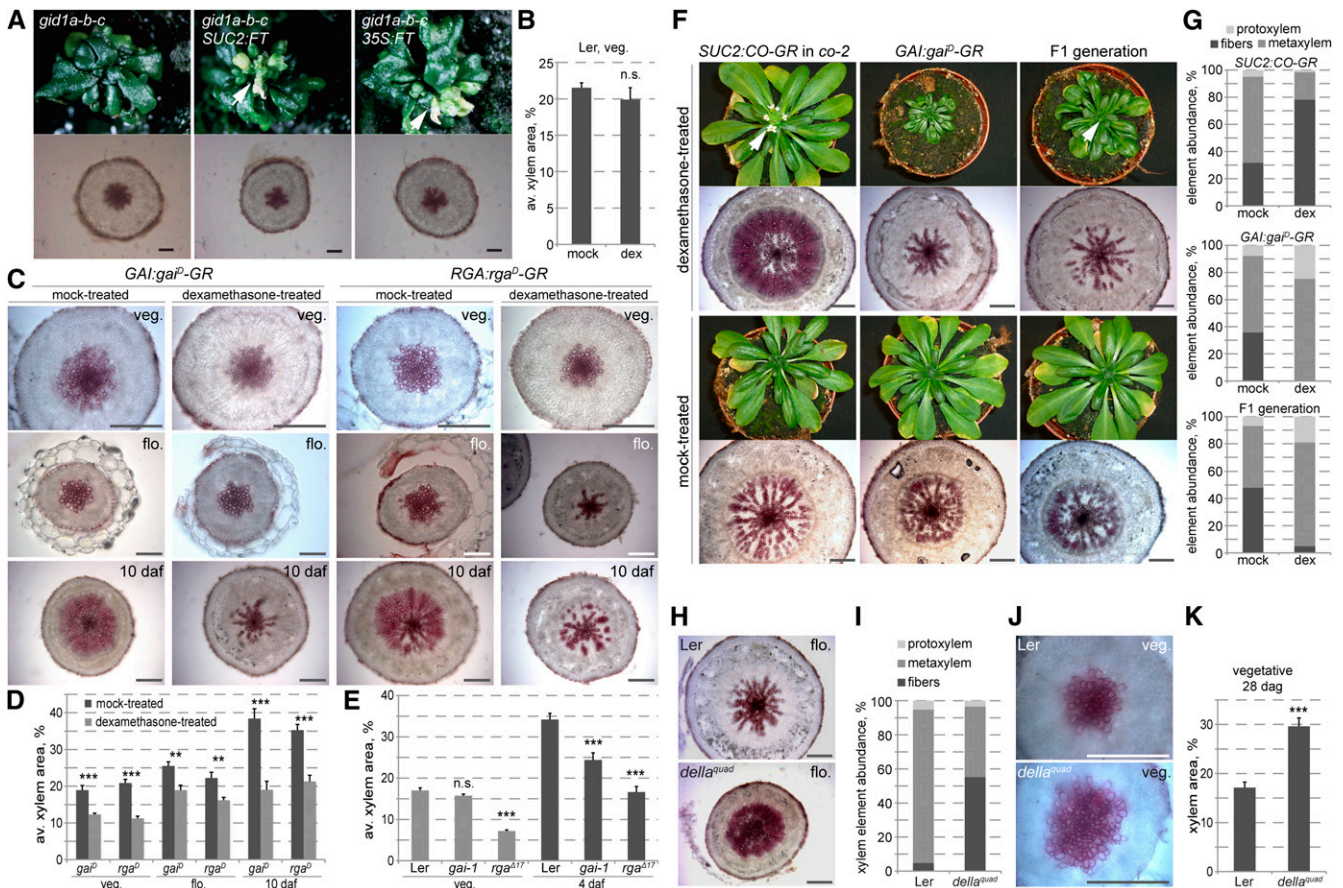


Figure 4. Control of Hypocotyl Xylem Expansion by GA Signaling.

(A) *gid1a-b-c* triple mutants that fail to flower (top) unless transgenes driving constitutive expression of the florigen, *FT*, are introduced. Transverse hypocotyl sections (bottom) were taken at 14 days after flowering of the transgenic lines for all lines. Arrowheads point out the inflorescence meristem and flower-like structures in transgenic plants.

(B) Control experiment demonstrating the absence of dexamethasone (dex) effects on xylem expansion in the *Ler* control background.

(C) Suppression of xylem expansion by dex activation of dominant-negative versions of transgenic *GAI* and *RGA* genes (*gai^D* and *rga^D*) fused with the glucocorticoid receptor domain (GR).

(D) Quantification of xylem area in the experiments illustrated in (C). Error bars are SE.

(E) Reduced xylem expansion in the *rga^{Δ17}* and the *gai-1* mutants compared with their *Ler* background at vegetative state and after flowering. Error bars are SE.

(F) Suppression of xylem expansion by dex activation of dominant-negative transgenic *GAI* and simultaneous dex induction of flowering and, thus, xylem expansion through the *SUC2:CO-GR* transgene. Arrowheads point out the inflorescence meristems.

(G) Quantification of xylem element abundance in the hypocotyls sampled in the experiment illustrated in (F).

(H) Enhanced xylem expansion at flowering in the *rga-24 gai-t6 rgl1-1 rgl2-1* quadruple loss-of-function mutant (*della^{quid}*) compared with its *Ler* background (*della^{quid}* at 36 d after germination; *Ler* at 42 d after germination).

(I) Quantification of xylem element abundance in the hypocotyls sampled in the experiment illustrated in (H).

(J) Xylem expansion in hypocotyls of *Ler* and *della^{quid}* plants at same age vegetative state (28 d after germination).

(K) Quantification of xylem area in the experiment illustrated by examples in (J). Error bars are SE. daf, days after flowering; n.s., not significant; **P* < 0.05; ***P* < 0.01; ****P* < 0.001. Bars = 200 μm.

Dexamethasone treatment as such had no effect on the xylem expansion phenotype of the wild-type background (Figure 4B). However, dexamethasone activation of the transgenic proteins not only resulted in a dwarf phenotype similar to the *gai-1* and *rga^{Δ17}* mutants but also in a large reduction in xylem expansion from the vegetative stage onwards compared with controls (Figures 4C and 4D). This effect was confirmed by analysis of

the original dominant *gai-1* and *rga^{Δ17}* mutants; however, at the vegetative stage, only the *rga^{Δ17}* mutants were significantly affected at earlier stages. Reduced xylem expansion also became obvious in *gai-1* later, but the phenotype remained quantitatively milder than the one observed in *rga^{Δ17}*, suggesting that *RGA* has a genuinely more prominent role in xylem expansion than *GAI* (Figure 4E).

One shortcoming of the experiments described above is that GA itself has an effect on flowering time (Willige et al., 2007), for instance, flowering was suppressed in paclobutrazol-treated plants. We thus sought to corroborate our results by uncoupling flowering and GA signaling. To this end, we crossed the *SUC2:CO-GR* and the *GAI:gai^D-GR* lines and investigated the resulting F1 generation that carried both transgenes. In these plants, dexamethasone-treatment thus triggered flowering (which occurred simultaneously in the *SUC2:CO-GR* parents and the F1 plants) and at the same time interfered with GA signaling. Again, this resulted in the suppression of xylem expansion (Figure 4F). Quantification of xylem elements by the maceration assay confirmed that this coincided with a shift toward preferentially early cell types (Figure 4G). In summary, our results thus suggest that both GA biosynthesis and signaling are necessary for xylem expansion to occur.

GA Signaling Is Sufficient to Induce Xylem Expansion in the Absence of Flowering

Opposite to the dominant *della* mutants is a *della* quadruple mutant that combines loss-of-function alleles (*rga-24 gai-t6 rgl1-1 rgl2-1*) (Achard et al., 2006), largely abolishing the redundancy between the DELLA proteins and resulting in constitutively elevated GA signaling even in the absence of the hormone (Cao et al., 2005). In this *della* quadruple mutant, xylem expansion was increased as compared with the wild type at flowering (Figure 4H), concomitant with a higher abundance of fibers (Figure 4I). This quadruple mutant also enabled us to determine whether GA signaling is sufficient to trigger xylem expansion, as implied by the stimulatory effect of GA treatment described above. To this end, we analyzed the mutants during the early, proportional phase of hypocotyl secondary growth, prior to flowering. To increase the trait resolution, we conducted these experiments in short-day conditions, which promoted overall secondary growth but not xylem expansion, and collected samples at 26 to 28 d after germination, well before any signs of flowering as determined by parallel grown control groups. In the hypocotyls of *della* quadruple mutants, the area occupied by xylem vessels and parenchyma cells was increased relative to the total area compared with the *Ler* control plants (Figures 4J and 4K). Thus, GA signaling appears to be sufficient to stimulate xylem production, even in the absence of flowering. Notably, in this experiment, no xylem fibers were observed, suggesting that GA does not exclusively promote fiber differentiation and, further, that fiber differentiation might be a secondary effect of GA-triggered xylem expansion.

GA-Mediated Stimulation of Xylem Expansion Does Not Require the Auxin Pathway or Polar Auxin Transport

The GA pathway has been shown to interact with the auxin pathway in the regulation of diverse developmental processes (e.g., Fu and Harberd, 2003; Frigerio et al., 2006), including the regulation of cambial activity (Björklund et al., 2007). We thus examined whether the stimulatory effect of GA on xylem expansion might also be mediated through or in conjunction with auxin signaling or polar auxin transport. To this end, we again employed the inducible secondary growth system based on the

SUC2:CO-GR line. Treatment with the polar auxin transport inhibitor *N*-1-naphthylphthalamic acid did not affect xylem expansion, neither in control plants nor when xylem expansion was induced (see Supplemental Figure 3A online). Likewise, compared with Col-0 controls, xylem expansion was still observed in null mutants of the prototypical auxin efflux carrier, *PIN-FORMED1*, in which polar auxin transport is impaired (Gälweiler et al., 1998). Thus, our data suggest that polar auxin transport is not a limiting factor for xylem expansion.

Next, we analyzed xylem expansion in diverse auxin-related signaling mutants that display a hypocotyl phenotype, corroborating that the respective genes are active in our tissue of interest. None of the genotypes with constitutively reduced (*non-phototropic hypocotyl4* and *transport inhibitor response1*) auxin signaling examined displayed altered xylem expansion or fiber formation, suggesting that auxin signaling is not limiting this process (see Supplemental Figure 3B online).

GA Signaling Acts Locally to Promote Xylem Expansion

An open question that remained was whether GA signaling stimulates xylem expansion locally or whether it functions at the shoot apex to launch the production of the elusive mobile signal. To decide this issue, we performed reciprocal micrografting as described above between *RGA:rga^D-GR* or *della* quadruple plants and their *Ler* control background. In self-graftings of either genotype, xylem expansion proceeded at a similar rate in both scion and stock and was suppressed in the *RGA:rga^D-GR* background as expected if dexamethasone was applied (Figures 5A to 5D). By contrast, in dexamethasone-treated *Ler* onto *RGA:rga^D-GR* or *RGA:rga^D-GR* onto *Ler* grafts, the *Ler* scions or stocks displayed prominent xylem expansion, whereas the *RGA:rga^D-GR* scions or stocks did not (Figures 5A and 5B). The grafting between the *della* quadruple mutant and *Ler* confirmed the local action of GA signaling. In both *Ler* onto *della* and *della* onto *Ler* grafts, the *della* scions or stocks displayed more prominent xylem expansion than their *Ler* counterparts (Figure 5D). A possible explanation for the observation that expansion in the *Ler* stock was smallest when combined with a *della* quadruple mutant scion is that this scion may have very low GA levels due to the feedback regulation of GA homeostasis, thereby limiting the GA supply to the *Ler* stock (see below). In the respective self-grafts, xylem expansion proceeded at a similar rate in both scion and stock (Figures 5C and 5D). Collectively, our results thus suggest that the effects of altered GA signaling on xylem expansion are not graft transmissible. Therefore, GA signaling is not required for the production of the elusive mobile signal and acts locally to promote xylem expansion, downstream of this signal.

GA Is the Mobile Shoot-Derived Signal That Induces Hypocotyl Xylem Expansion

The local role of GA signaling in xylem expansion together with the *gai1-3* secondary growth phenotype supports the idea that GA itself must also act locally to induce xylem expansion. Moreover, the documented induction of GA biosynthesis in the shoot upon flowering (Eriksson et al., 2006) suggested that GA

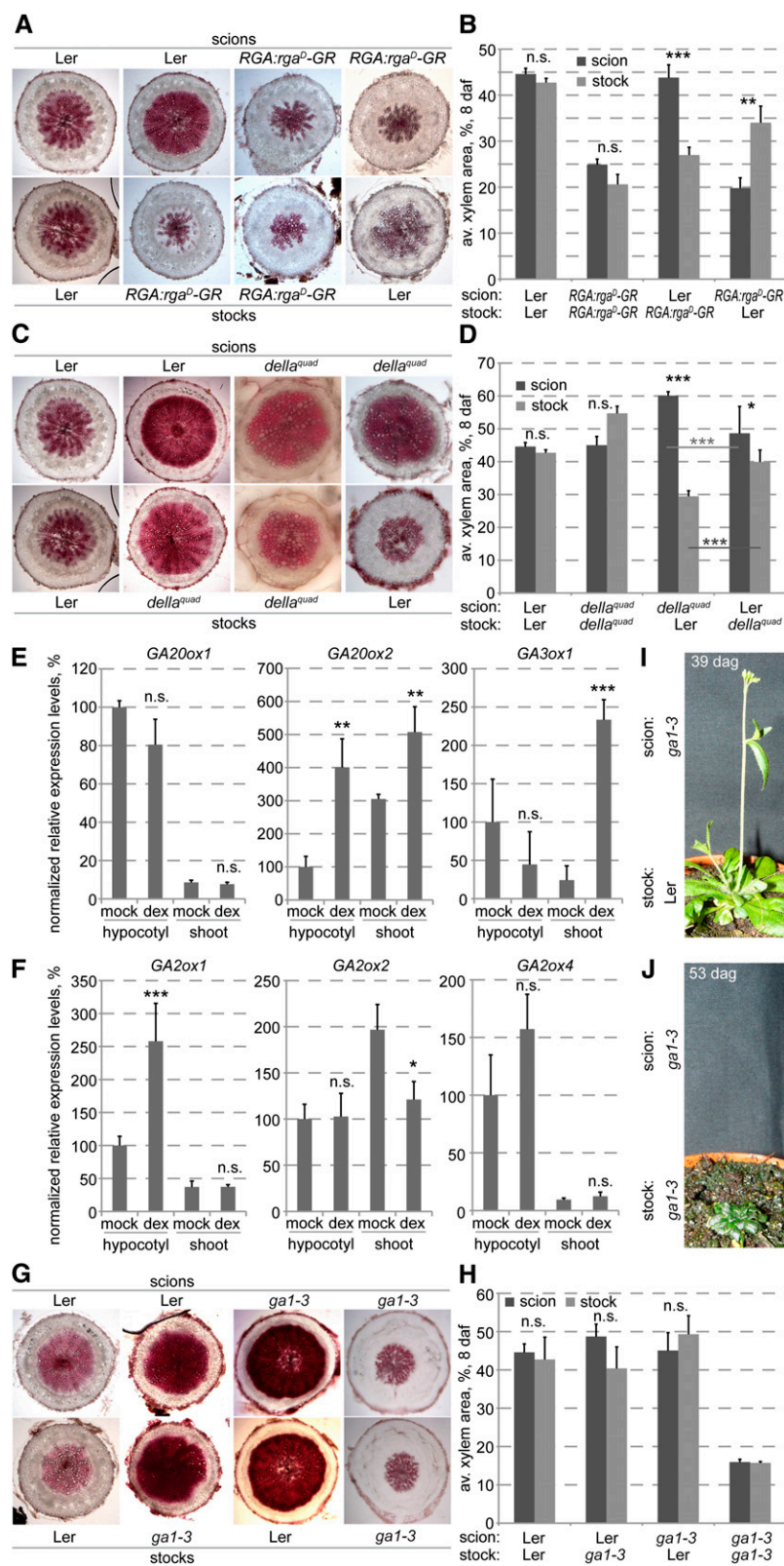


Figure 5. Evidence for GA as the Mobile Shoot-Derived Signal That Triggers Hypocotyl Xylem Expansion upon Flowering.

might also act systemically. We corroborated this result by analysis of the transcript levels of those GA biosynthetic and catabolic enzymes (Hedden and Phillips, 2000) for which we could detect expression in the hypocotyl or shoot. Comparison of the expression of three biosynthetic enzymes in the hypocotyl or shoot (i.e., shoot apical meristem plus cotyledons and the first true leaves) of dexamethasone- versus mock-treated *SUC2:CO-GR* plants revealed a significant increase in *GA20ox2* levels in both hypocotyl and shoot and of *GA3ox1* in the shoot (Figure 5E). Thus, expression of GA biosynthetic enzymes increased in both hypocotyl and shoot (Figure 5E) upon flowering induction. By contrast, of the three catabolic enzyme genes assayed, only *GA2ox1* displayed increased expression in the hypocotyl upon flowering (Figure 5F).

Interestingly, although GA biosynthesis genes were generally induced in both hypocotyl and shoot upon flowering (Figure 5E), increased expression of the gene encoding the last enzymatic step leading to the formation of bioactive GA, *GA3ox1*, was only observed in the shoot. This severalfold increase confirmed that upon flowering induction, bioactive GA mostly accumulates in the shoot (Eriksson et al., 2006). We thus wondered whether GA could be the mobile shoot-derived signal that triggers hypocotyl xylem expansion. To determine whether endogenous GA is mobile, we performed micrografting experiments between the *ga1-3* mutant and the *Ler* background. Technically, these grafts were more demanding because of the severe phenotype of *ga1-3*. For instance, *ga1-3* does not germinate unless GA is supplied externally, and the mutants are stunted and accordingly have short hypocotyls. Since we did not want to interfere with our experiment even by low transient levels of GA, we germinated *ga1-3* seeds by mechanically opening the seed coat (as we did in all experiments using this mutant). We then cut the shoot off at the very top of the hypocotyl to graft a *Ler* scion on top of it. Strikingly, in these plants, the *Ler* scions restored xylem expansion in the *ga1-3* stocks (Figures 5G and 5H). Interestingly, in the reciprocal graftings, the *Ler* stock was also able to gradually rescue the GA-deficient *ga1-3* shoot phenotype, although with a strong delay. That is, the rosette leaves turned from dark to light green, they expanded in size and the inflorescence stem was able to elongate (Figure 5I). No such rescue was observed in self-grafts of the *ga1-3* mutant (Figure 5J). In summary, our results

therefore suggest that in the wild type, GA can move through the plant and is the mobile shoot-derived signal that triggers xylem expansion in the hypocotyl.

DISCUSSION

In our study, we aimed to determine the cause for the acceleration of secondary growth and the shift toward xylogenesis observed in *Arabidopsis* hypocotyls. A previous study had established that the transition toward this so-called xylem expansion is triggered by flowering and involves an elusive, shoot-derived mobile signal (Sibout et al., 2008). Our results suggest that this mobile signal is GA and that GA signaling and *ER* act locally and downstream of GA to regulate xylem expansion.

Among the accessions examined for their secondary growth vigor corrected for differences in flowering time, *Ler* stood out as the strain with strongest xylem expansion. This does not reflect a head start in this trait, as the onset of xylem expansion in *Ler* still depends on flowering. However, once triggered, it proceeds at an enhanced rate. *Ler* carries a loss-of-function mutation in the *ER* gene and was derived from the original La-0 accession by mutagenesis (Redei, 1962; Torii et al., 1996). As La-0 displays much weaker xylem expansion, comparable to the Col-0 reference accession, the *er* mutation appears to be responsible for the enhanced xylem expansion. *ER* is known to function in the regulation of aerial organ shape and size (van Zanten et al., 2009) and is a commonly used genetic background for *Arabidopsis* research due to its practical, compact growth habit. Thus, while *ER* has been shown to be a positive regulator of elongation growth, it appears to be a negative regulator of xylem expansion. *ER* has also been implicated in modulating various hormone pathways, including auxin, brassinosteroid, ethylene, and GA (van Zanten et al., 2009). Thus, the *er* mutation possibly enhances the xylem expansion phenotype through modification of cambial hormonal signaling, including the GA pathway.

Previous studies have painted a somewhat complex picture of the role of GA signaling in xylem development. Application of GA to decapitated, auxin-depleted *Populus* stems was shown to stimulate cell divisions in the cambial zone (Björklund et al., 2007). However, the identity of cells formed through GA-induced

Figure 5. (continued).

- (A) Examples of scion and stock hypocotyl sections from micrograftings of indicated genotypes, sampled at 8 d after flowering.
 (B) Xylem area quantification of scion and stock hypocotyls obtained from indicated micrograftings, sampled at 8 d after flowering. Error bars are SE.
 (C) Examples of scion and stock hypocotyl sections from micrograftings of indicated genotypes, sampled at 8 d after flowering.
 (D) Xylem area quantification of scion and stock hypocotyls obtained from indicated micrograftings, sampled at 8 d after flowering. Error bars are SE.
 (E) Expression level quantifications of GA biosynthetic genes in *SUC2:CO-GR* transgenic plants (*co-2* mutant background), comparing hypocotyls and shoots of 9-d-old mock-treated samples and samples treated with dexamethasone (dex) from 6 to 8 d after germination to induce flowering and thereby xylem expansion. Averaged relative expression levels normalized with respect to the *EF1* housekeeping gene are indicated. Expression was quantified for all genes in each of three replicates; measurements represent the average of the relative expression with respect to *EF1* from each sample. Error bars are SE.
 (F) Same experiment as (E), for GA catabolic genes. Error bars are SE.
 (G) Examples of scion and stock hypocotyl sections from micrograftings of indicated genotypes, sampled at 8 d after flowering.
 (H) Xylem area quantification of scion and stock hypocotyls obtained from indicated micrograftings, sampled at 8 d after flowering. Error bars are SE.
 (I) Development of a *ga1-3* shoot scion grafted onto a *Ler* root stock.
 (J) Development of a *ga1-3* shoot scion grafted onto a *ga1-3* root stock. n.s., not significant; **P* < 0.05; ***P* < 0.01; ****P* < 0.001.

divisions was reported to be relatively obscure, as they appeared more spherical than the flat, thin-walled cells of the untreated cambium. Furthermore, they failed to differentiate into xylem cells and instead preserved their parenchymatic phenotype. When GA was applied together with auxin, both cambial cell divisions and xylem differentiation was stimulated, leading to the conclusion that GA signaling has a role in inducing cambial cell divisions but functions together with auxin in promoting xylem differentiation. This idea fits with a report of GA concentrations in *Populus* stems, which found only trace amounts in dividing cells of the active cambium, whereas the highest levels were detected in differentiating xylem cells (Israelsson et al., 2005). Consistently, the study also found low expression of GA biosynthetic enzymes and GA signaling genes in the dividing cambial cells and higher expression in both differentiating phloem and xylem cells. By contrast, a recent study indicates that the GA receptors are expressed the highest in the phloem and dividing cambial cells (Mauriat and Moritz, 2009). Thus, it appears that a comprehensive expression analysis of GA biosynthesis and signaling genes across the cambial zone is still missing, and it remains to be determined when and where exactly GA signaling is active in secondary growth. The observed synergism between GA and auxin application in *Populus* was accompanied by an increased auxin concentration in stem tissues. Combined with the finding that GA treatment induced expression of a cambial auxin efflux carrier gene, these results indicated that GA action might promote auxin transport (Björklund et al., 2007). Reciprocally, auxin treatment stimulated expression of GA biosynthesis genes and inhibited expression of genes encoding GA degrading enzymes (Björklund et al., 2007), suggesting a feed forward loop that would promote cambial activity.

In *Arabidopsis*, the GA pathway has been shown to interact with the auxin pathway in the regulation of diverse developmental processes. For instance, auxin signaling in roots has been shown to induce degradation of RGA (Fu and Harberd, 2003), thereby promoting root growth, and it has also been suggested that auxin can induce GA biosynthesis (Frigerio et al., 2006). However, the relationship between GA and auxin also appears to be complex and context specific. In the root meristem for instance, while auxin induces RGA degradation, GA signaling in turn dampens auxin signaling and transport through a multi-step process involving signaling components of another plant hormone, cytokinin (Moubayidin et al., 2010). The direct stimulatory effect of GA observed in our studies does not appear to depend on auxin transport or signaling as suggested by analysis of respective mutants. We would like to clarify, however, that this should be seen in the context of the quantitative xylem expansion trait and should not be extended to the qualitative level. That is to say that the auxin pathway is clearly indispensable for vascular development (Berleth et al., 2000) but does not appear to be limiting for the observed quantitative acceleration in xylogenesis.

On the one hand, our result that GA stimulates xylogenesis directly could reflect a fundamental difference between *Arabidopsis* and *Populus*. On the other hand, it could reflect the fact that unlike in the hypocotyl, analyses of secondary growth in stems are intrinsically complicated by parallel elongation growth (Sibout et al., 2008). For example, this issue is illustrated by another secondary growth trait that has been attributed to GA,

that is, fiber formation. Analyses of transgenic trees overproducing GA and external GA application experiments have shown that GA can stimulate xylem fiber differentiation and elongation (Digby and Wareing, 1966; Eriksson et al., 2000). For instance, *Populus* trees overexpressing a GA biosynthetic gene produced more total fibers than the control trees. However, this was accompanied by a general growth-promoting effect, including an increase in both stem height and diameter. Thus, while these *Populus* produced more total fibers than the control trees, their relative abundance remained approximately constant (Eriksson et al., 2000). In *Arabidopsis* hypocotyls, fiber differentiation occurs coincident with flowering, similar to xylem expansion, however, with a delay compared with the latter. In the *della* quadruple mutants investigated, the constitutive GA signaling led to xylem expansion well before flowering, concomitant with fiber differentiation. Moreover, in the *gid1 a-b-c* genotypes, fibers were absent but could still be detected in the *ga1-3* mutant. This suggests that GA primarily promotes the proliferation of xylem, while the occurrence of fibers appears to be a delayed secondary effect.

Previous studies, which have relied on external application of labeled GA, have suggested that GA can move inside the plant. For instance, applied GA was transported downwards in *Populus* stems and from leaves to the shoot apex in *Arabidopsis* (Eriksson et al., 2006; Björklund et al., 2007), whereas it moved upwards in pea (*Pisum sativum*) stems (Proebsting et al., 1992). The latter study also demonstrated cross-complementation between scions and stocks of one GA biosynthesis mutant and another in pea. The results from our *ga1-3* versus *Ler* micrografting experiments confirm that GA is mobile. They also confirm that endogenous GA is able to move inside the plant in both acro- and basipetal directions because GA production in either the shoot or root was sufficient to compensate for impaired GA biosynthesis in the grafted scion or stock. This finding is underlined by the basically complete phenotypic rescue of *ga1-3* shoot scions by *Ler* root stocks, albeit with a strong temporal delay of over 2 weeks with respect to other grafts. This might mean that the drain of GA by the GA-deficient shoot leads to decreased feedback inhibition of GA biosynthesis in the root, thereby elevating its GA export capacity. However, an alternative explanation would be that the observed rescue results from GA export from the root system that increases with its growth. This would explain the considerable delay in the rescue of the *ga1-3* scions, as well as the eventual, albeit rather weak, xylem expansion observed in *pny pnf* mutants.

Beyond genetically proving the mobility of GA, our grafting experiments also suggest that in the wild-type situation, GA itself is the mobile shoot-derived signal that is transported to the cambial zone of the hypocotyl upon flowering initiation to trigger xylem expansion. This idea is consistent with the described feed-forward upregulation of GA levels at the shoot apex upon flowering induction (Eriksson et al., 2006). In particular, the concentration of GA was shown to increase dramatically already before floral initiation and to stay at constantly high levels thereafter (Eriksson et al., 2006). Combined with the observed upregulation of the last step GA biosynthetic enzyme *GA3ox1* in the shoot, this suggests that it is likely GA that, at least initially, triggers xylem expansion. In an ontogenetic context, this

sequence of events would make sense, as it would increase the water and solute transport capacity of the hypocotyl in anticipation of the substantial height increase associated with inflorescence formation and bolting.

METHODS

Plant Materials and Growth Conditions

Arabidopsis thaliana and other species were grown either in standard tissue culture conditions or in soil as described, in continuous light or in short-day conditions (16-h-dark/8-h-light cycle) depending on the experiment as indicated in the text and figure legends. The *spl3*, *spl4*, *spl9*, *ft-1*, *lfy-1*, *lfy ft* double, *tf1*, *pin1*, *tir1*, *nph4*, *hy5*, *hy5 hyh* double, *pnf pnf* double, *gai-3*, *rga-24 gai-t6 rgl1-1 rgl2-1* quadruple, *gai-1*, and *rga*^{Δ17} mutants, as well as the 35S:MiM156, 35S:MiR156, 35S:MiM172, 35S:MiM172, RGA:rga^D-GR, GAI:gai^D-GR, and SUC2:CO-GR transgenic lines used in this study have been described previously (Kardailsky et al., 1999; Kobayashi et al., 1999; Smith et al., 2004; Sibout et al., 2006; Corbesier et al., 2007; Franco-Zorrilla et al., 2007; Kanrar et al., 2008; Sibout et al., 2008; Todesco et al., 2010). The *gid1a-b-c* triple mutants and their transgenic derivatives containing either a *SUC2:FT* or a *35S:FT* transgene were generated by crossing and selection of the triple mutant in segregating lines by genotype (Willige et al., 2007). *Arabidopsis alpina*, *Aster alpinus*, *Nicotiana benthamiana*, *Solanum lycopersicum*, *Cardamine hirsuta*, *Barbarea verna*, and *Taraxacum officinalis* seeds were either lab stocks or obtained from colleagues or garden centers. Natural *Arabidopsis* accessions correspond to the lines distributed by the Nottingham Arabidopsis Stock Centre (<http://nasc.nott.ac.uk>). Absence of flowering was defined as absence of an inflorescence meristem. Where applicable, flowering time was determined in parallel grown control plants.

GA, Paclobutrazol, N-1-Naphthylphthalamic Acid, and Dexamethasone Treatment

For tissue culture experiments to analyze the effects of paclobutrazol and N-1-naphthylphthalamic acid, *SUC2:CO-GR* plants were grown for 5 d on half-strength Murashige and Skoog media in the presence or absence of 10 μM dexamethasone (Duchefa) to induce flowering before being transferred on plates containing either 10 μM paclobutrazol (Duchefa) or 5 μM N-1-naphthylphthalamic acid (Duchefa) or mock solution. After 14 d, floral meristems appeared in all dexamethasone-induced plants, and hypocotyls were sampled for phenotypic analyses. Induction of soil-grown *SUC2:CO-GR*, *RGA:rga^D-GR*, or *GAI:gai^D-GR* plants was achieved by watering with a 10 μM dexamethasone solution three times per week. For GA treatments, plants were sprayed with a 1 mM GA₃ (Sigma-Aldrich) or mock solution at similar frequency (King et al., 2008).

Micrografting

Grafting between hypocotyls of scions and stocks using 1-mm pieces of silicon tubing was performed in tissue culture as described (Turnbull et al., 2002; Sibout et al., 2008) using young, 6-d-old seedlings grown on plates at 25°C in 16-h light days. Successful grafts were transferred into soil 7 d after grafting and grown under continuous light conditions.

Phenotypic Analyses of Hypocotyl Sections

Maceration of hypocotyls was performed as described (Muñiz et al., 2008), and xylem element abundance was scored by light microscopy, typically classifying at least 200 cells. To determine xylem area, hypocotyls were embedded in 6% agarose (Sigma-Aldrich) immediately after

harvest and sectioned (85 μm thick) using a Leica-VT 1000S vibratome. Sections were stained for lignin with phloroglucinol (Prolabo VWR 26337) to highlight xylem vessels and fibers. Sections were photographed using a Diaplan 3 microscope (Leica) and analyzed using ImageJ software as described (Sibout et al., 2008). All images were taken at high resolution, and measurements were performed at zoom levels that allowed unequivocal identification of the xylem border.

Molecular Biology

Molecular biology procedures were performed according to standard procedures as described (Sibout et al., 2008). For quantitative PCR analyses, hypocotyls and shoots were dissected and quantitative PCR was performed using a Stratagene MX3000P instrument as described (Sibout et al., 2006). Expression levels were normalized with respect to the elongation initiation factor 1 gene (*EF1*). All quantitative PCR experiments were repeated in independent triplicates. The following oligonucleotides were used: *EF1* (AT5G60390), 5'-GGTCACCAAGGCTGCAGT-GAAGAA-3' and 5'-GCTCAAACGCCATCAAAGTTTAAAGAA-3'; *GA2ox1* (AT4G25420), 5'-GGGTATCTTCTTGATGTGATGCTGTCCAAA-3' and 5'-GGTGAACAGCGAGAGCGAGAGGAAA-3'; *GA2ox2* (AT5G51810), 5'-CAAGGAACATAGACCAAGTGAAGTCAGGGTA-3' and 5'-ACGGGA-TATCAAGAGCTGTTTGCATAGA-3'; *GA3ox1* (AT1G15550), 5'-ACCA-GAACAATACCGCCGGTCTACAA-3' and 5'-ATCAGATTGCGGACCC-CAAAGGAA-3'; *GA2ox1* (AT1G78440), 5'-TTGGTGACTCTCTCCAGGT-GATGACAAAG-3' and 5'-GTCAACGAGCGAGTCTCTGAGTCAAT-3'; *GA2ox2* (AT1G30040), 5'-AGGAAGAGGCGAGAAGATGGTGAA-3' and 5'-GGGACCTGAAGAGCATCTCCAACATTAA-3'; *GA2ox4* (AT1G47990), 5'-TACGAAACATGTCTAAACGGCTATCCTCAA-3' and 5'-GGCATA-GAGCATTGACCTACGGAGAAGAAA-3'.

Accession Numbers

Sequence data from this article can be found in the Arabidopsis Genome Initiative or GenBank/EMBL databases under the following accession numbers: *EF1* (AT5G60390), *GA2ox1* (AT4G25420), *GA2ox2* (AT5G51810), *GA3ox1* (AT1G15550), *GA2ox1* (AT1G78440), *GA2ox2* (AT1G30040), *GA2ox4* (AT1G47990), *GAI* (AT1G14920), *RGA* (AT2G01570), and *ER* (AT2G26330).

Supplemental Data

The following materials are available in the online version of this article.

Supplemental Figure 1. Xylem Expansion in Various Inflorescence Specification and Flowering Time Mutants.

Supplemental Figure 2. Xylem Expansion in Mutants of Genes Targeted by the Age-Related Flowering Pathway.

Supplemental Figure 3. Xylem Expansion in Hypocotyls with Impaired Auxin Transport or Signaling.

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Mobile Gibberellin Directly Stimulates *Arabidopsis* Hypocotyl Xylem Expansion

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