the plant journal

SEB Society for Experimental Biology

The Plant Journal (2019) **99,** 717–732 doi: 10.1111/tpj.14356

Arabidopsis and sunflower plants with increased xylem area show enhanced seed yield

Julieta V. Cabello and Raquel L. Chan*

Instituto de Agrobiotecnología del Litoral, Universidad Nacional del Litoral, CONICET, Centro Científico Tecnológico CONICET Santa Fe, Colectora Ruta Nacional N° 168 km. 0, Paraje El Pozo, (3000) Santa Fe, Argentina

Received 31 January 2019; revised 9 April 2019; accepted 17 April 2019; published online 22 April 2019.
*For correspondence (e-mail rchan@fbcb.unl.edu.ar).

SUMMARY

Plant architecture plasticity determines the efficiency at harvesting and plays a major role defining biomass and seed yield. We observed that several previously described transgenic genotypes exhibiting increased seed yield also show wider stems and more vascular bundles than wild-type plants. Here, the relationship between these characteristics and seed yield was investigated. Hanging weight on the main stem of Arabidopsis plants provoked significant stem widening. Such widening was accompanied by an increase in the number of vascular bundles and about 100% of yield increase. In parallel, lignin deposition diminished. Vascular bundle formation started in the upper internode and continued downstream. AUX/LAX carriers were essential for this response. The increase of vascular bundles was reverted 3 weeks after the treatment leading to an enlarged xylem area. *Aux1, lax1,* and *lax3* mutant plants were also able to enlarge their stems after the treatment, whereas *lax2* plants did not. However, none of these mutants exhibited more vascular bundles or seed yield compared with untreated plants. Weight-induced xylem area enhancement and increased seed yield were also observed in sunflower plants. Altogether these results showed a strong correlation between the number of vascular bundles and enhanced seed yield under a long-day photoperiod. Furthermore, changes in the levels of auxin carriers affected both these processes in the same manner, suggesting that there may be an underlying causality.

Keywords: vascular bundles, xylem area, auxin carriers, seed yield, LAX2, mechanical treatment, *Arabidopsis thaliana*, *Helianthus annuus*.

INTRODUCTION

Developmental plasticity allows higher plants to modulate their phenotype depending on environmental conditions. In contrast with animals, plants exhibit a remarkable flexibility in their architecture and growth patterns, due to the continuously active shoot and root meristems and their potential to generate organs after embryogenesis (Wolters and Jurgens, 2009). The environment influences plant growth by modulating hormone levels and signaling. The resulting plant architecture is defined as a three-dimensional organization of the plant body and it has major agronomic importance, strongly influencing the efficiency of harvesting and finally plant yield (Reinhardt and Kuhlemeier, 2002). During crops domestication and improvement, plant architecture was considered to be the main trait for obtaining high-yielding varieties. A paradigmatic example of the importance of plant architecture is the Green Revolution, particularly based on architecture modification, choosing wheat varieties with shorter and sturdier stems. This selection led to dramatic increases in worldwide agricultural productivity (Peng *et al.*, 1999). Architectural and physiological traits, such as wider stems and increased number of vascular bundles, have been observed in rice and Arabidopsis transgenic plants exhibiting high-yielding phenotypes (Zhao *et al.*, 2015; Cabello *et al.*, 2016). Rice plants overexpressing the *PLANT ARCHITECTURE AND YIELD 1* (*PAY1*) gene produced around 38% more grain yield than controls significantly changing their plant architecture; particularly increasing stem width (Zhao *et al.*, 2015). Arabidopsis plants transformed with constructs able to express the sunflower transcription factor (TF) HaHB11 (driven by the *35S* or the *HaHB11* promoter) yielded 50–100% more seeds than controls, depending on the expression level, when grown in standard conditions (Cabello *et al.*, 2016). Such plants exhibited a marked increase in stem width and biomass as well as enhanced flooding and drought tolerance (Cabello *et al.*, 2016, 2017).

Shoot vascular bundles establishment is a complex process in which several hormones are involved; auxins control vascular bundle spacing whereas brassinosteroids modulate bundle number by promoting early procambial divisions (Ibañes *et al.*, 2009). Auxins are essential phytohormones, participating in plant growth control and development. Their transport and distribution along the plant generate several organized patterns including the periodic shoot vascular patterning (Ibañes *et al.*, 2009). The polar transport of auxin from cell to cell is coordinately achieved by efflux and influx transporters encoded by *PIN-FORMED* (*PIN*) and *P-GLYCOPROTEIN* (*PGP*) (Geisler *et al.*, 2005; Petrásek *et al.*, 2006; Cho *et al.*, 2007) and *AUXIN1/LIKE AUX1* (*AUX/LAX*) genes, respectively (Bennett *et al.*, 1996; Swarup *et al.*, 2008). In *Arabidopsis thaliana*, the *AUX/LAX* family is represented by four genes (*AUX1*, *LAX1*, *LAX2*, and *LAX3*) in which products are multimembrane-spanning transmembrane proteins (Péret *et al.*, 2012). These four proteins control periodic vascular patterning and differentiation of xylem cells in plants (Yang *et al.*, 2006; Swarup *et al.*, 2008; Péret *et al.*, 2012; Fàbregas *et al.*, 2015). Among these carriers, AUX1 regulates root gravitropism, lateral root emergence, root hair development, leaf phyllotaxy, and expansion of the apical hook (Marchant *et al.*, 1999, 2002; Swarup *et al.*, 2008) whereas LAX2 regulates vascular patterning in cotyledons (Péret *et al.*, 2012) and leaf venation patterning (Moreno-Piovano *et al.*, 2017). LAX3 has been associated with lateral root (LR) emergence as well as to the development of the apical hook (Swarup *et al.*, 2008) and LAX1 is required for leaf phyllotactic patterning (Sahlin *et al.*, 2009). Despite the important roles exerted by these carriers in Arabidopsis, they are not essential for this species' life; i.e., quadruple *aux1/lax1/lax2/lax3* mutant plants are viable and fertile. Interestingly, this is not the case in *Brachypodium distachyon*, a species in which the loss-of-function *Bdaux1* mutant is dwarf and infertile (van der Schuren *et al.*, 2018).

Aiming at revealing the molecular mechanisms involved in Arabidopsis secondary growth, a creative technique consisting of applying weight to the main stem for a few days at the early developmental stages was used by Ko *et al.* (2004). Such treatment induced the expression of *AUX/LAX* carriers in stem tissues of plants grown in short-day conditions. Briefly, after the treatment, the expression of *LAX2* was highest in intermediate stems, whereas *AUX1* peaked in mature stems, suggesting that these genes were implicated in the induction and development of secondary xylem (Ko *et al.*, 2004). Moreover, the weight added on the main stem induced cambium differentiation, facilitating auxin transport and promoting secondary xylem development (Ko *et al.*, 2004). When considering the formation of vascular bundles or a relationship between these structures, stem width, and high-yielding phenotypes, little or no information is available so far.

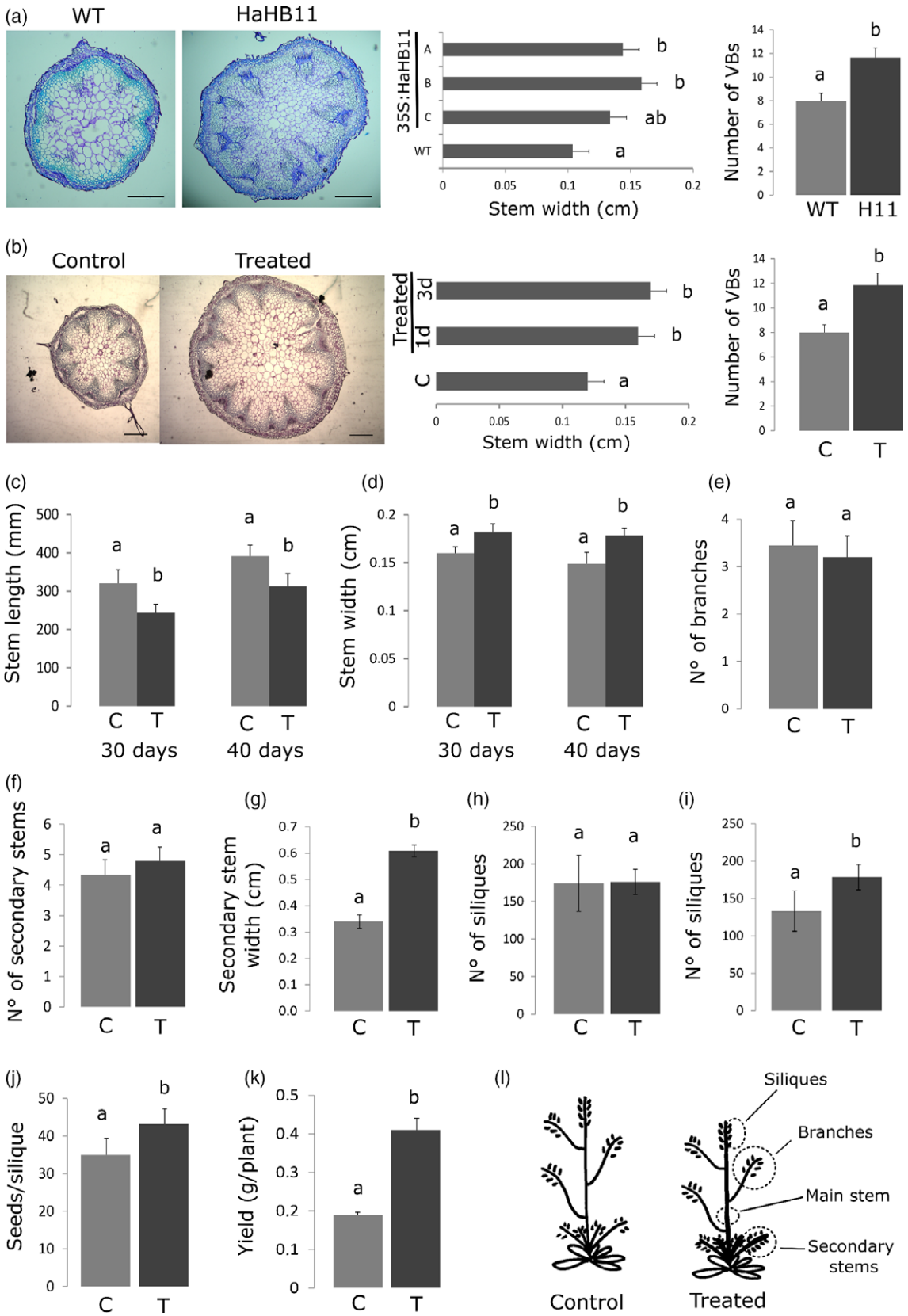
Many research groups worldwide have obtained transgenic or mutant plants exhibiting higher yields than controls (Zhao *et al.*, 2015; Cabello *et al.*, 2017; Wang *et al.*, 2017; Jiang *et al.*, 2018; Lim *et al.*, 2018). However, the evaluation of stems width and the number of vascular bundles in these plants was not investigated or associated with seed yield. The detailed observation of several high-yielding transgenic genotypes obtained in our laboratory led us to investigate this putative relationship. We stated the hypothesis that the inventive technique described by Ko *et al.* (2004) could induce the widening of the stems when applied to plants grown under long-day conditions. Here we report that hanging weight applied during discrete periods of time at particular stages of development provoked an increase of stem width and, transiently, the number of vascular bundles. Such events led to dramatic changes in plant architecture and increased seed yield. This process was controlled by auxin transport and up−down polar distribution. Weight-induced xylem area enhancement and increased seed yield also occurred in sunflower plants.

RESULTS

Transient weight treatment on the main stem triggers a reprogramming of the Arabidopsis vascular system

A detailed observation of Arabidopsis plants transformed with the construct *35S:HaHB11* (able to express a

Figure 1. Plant architecture undergoes significant variations after applying weight to the main stem.
(a) From left to right: illustrative stem cross-sections of the first internode of Arabidopsis WT and transgenic *HaHB11* plants (independent line B) stained with toluidine blue; stem width of the same plants measured with a caliper; number of vascular bundles measured in these sections (three independent lines named A−C; H11 indicates *HaHB11* transgenic plants). Samples were taken from the first internode of 40-day-old plants. For each point, three plants/stems were evaluated. Black bars represent 0.25 mm.
(b) From left to right: stem cross-sections of the first internode of Arabidopsis plants treated for 4 days with weight (1.6 g) and immediately cut; stem width of control and weight-treated (1 and 3 days) plants measured with a caliper; number of vascular bundles measured in stem sections of control and 3-day-treated plants. For each point, three plants/stems were evaluated. Black bars represent 0.04 mm.
(c−k) Architectural parameters assessed in Arabidopsis wild-type plants treated (T) or not (C) with 1.6 g during 2 days. Before and after the treatment, plants were grown under standard conditions.. Stem length (c) and width (d) measures were taken 30 and 40 days after sawing. (e) Number of branches from the main stem. (f) Number of secondary stems/plant. (g) Width of secondary stems. (h) Number of siliques/main stem. (i) Number of siliques/secondary stem. (j) Total siliques. (k) Seed yield evaluated at the end of the life cycle expressed as g/plant. For each point, 16 plants/stems were evaluated.
(l) Illustrative drawing representing the parameters shown in (a−k) forming the architecture of a weight-treated plant compared with its untreated control.
In (a−k) bars represent SE and different letters indicate significant differences between means ($P < 0.01$, Tukey test).



sunflower homeodomain-leucine zipper TF), grown under long-day conditions, indicated that besides the previously informed increased biomass and seed yield (Cabello *et al.*, 2016), a significant widening of stems together with an increase in the number of vascular bundles (Figure 1a). Similarly, transgenic plants overexpressing one of the HaHB11-closest Arabidopsis member (AtHB7), which exhibit increased yield compared with WT (Re *et al.*, 2014), also had wider stems and more vascular bundles (Figure S1). To determine if yield improvement was directly associated with stem width and number of vascular bundles, we looked for a non-transgenic technique able to induce such traits. Previously, studying stems' secondary growth under short-day conditions, it has been reported that the application of particular and limited quantities of weight on the main stem triggered cambium differentiation and lignification (Ko *et al.*, 2004). We decided to test a similar methodology on plants grown under long-day conditions. With this purpose, individual Arabidopsis plants at different developmental stages (exhibiting each one stems having from 4 to 15 cm height) were treated with 1.6 and 2.5 g agar-caps over 1 to 4 days (Figure S2a). After these periods, agar-caps were discarded and plants continued to grow in standard conditions until harvest. Immediately after the hanging weight treatment (from this point called weight treatment), histological cuts were carried out showing a similar increase in the number of vascular bundles as that observed in *HaHB11* plants (Figure 1b). Seed yield positively varied after different combinations of time, weight, and stem height indicating that the methodology was useful to induce stem widening under long-day conditions (Figure S2a). The best yield improvement resulted from applying 1.6 g during 3 days to 4.5 cm stem height plants (Figure S2b).

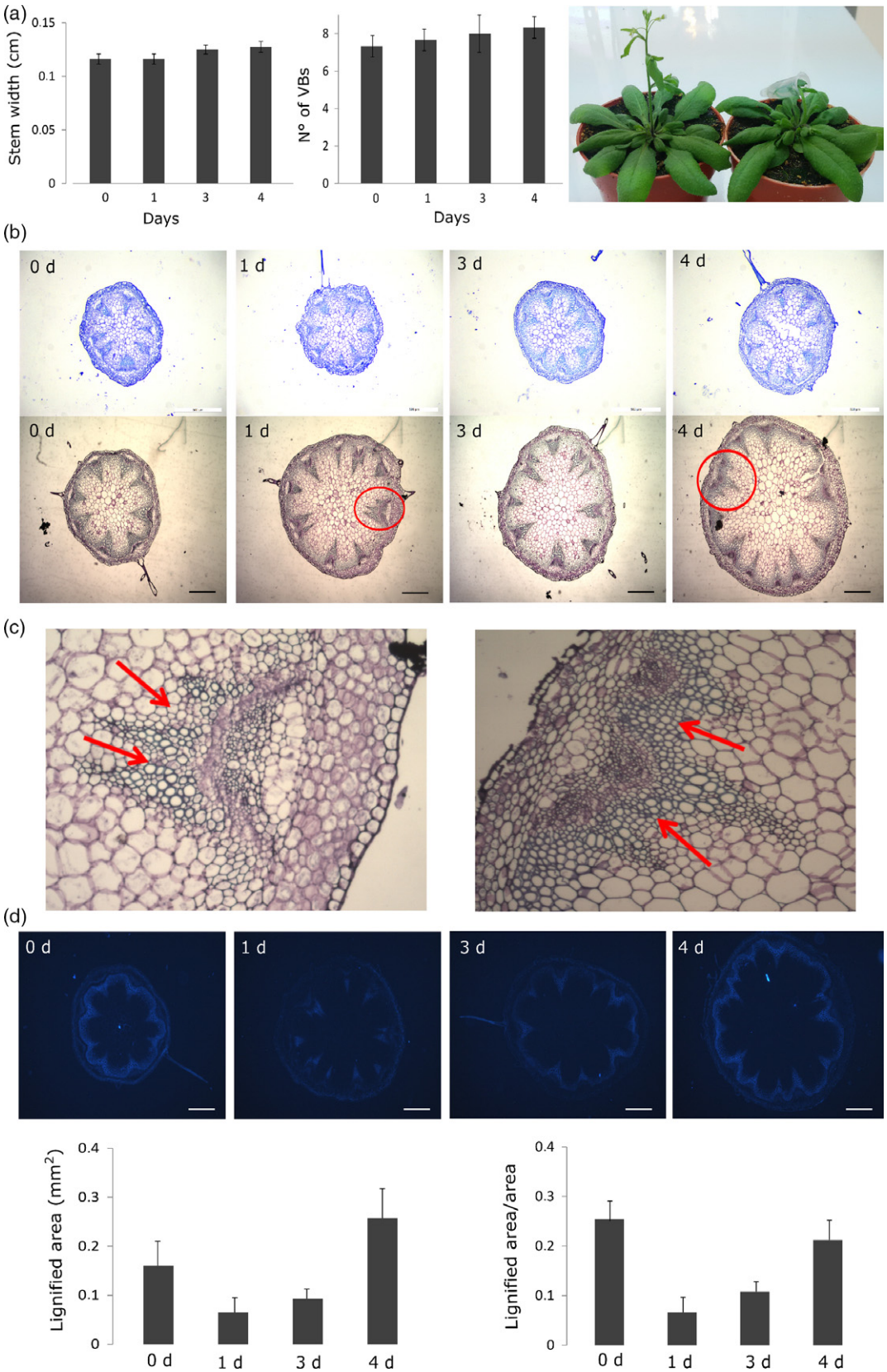
To discard hypoxia (produced by agar-caps) as the factor triggering the described changes, weight on the stems was applied with a different technique, by hanging a piece of wood, and the results were similar (Figure S3a). Moreover, bending stems as the source of the changes was discarded by artificially bending the stems without applying weight and which did not trigger stem widening, increased vascular bundle number or yield (Figure S3b). Considering that Ko *et al.* (2004) carried out experiments with decapitated plants, a third control was performed to test whether the observed effects were the consequence of apical dominance. Decapitated plants were evaluated for seed yield at the end of the life cycle and they did not show significant differences with controls for this trait (Figure S3c). During the same period (24 and 48 h) and until 4 days of treatment, stem width, the number of vascular bundles and stem cross-sections of untreated plants did not significantly vary (Figure 2a,b, upper panel).

Different plant architecture parameters were scored including main stem height and width, number of secondary branches, number of secondary stems, silique number on main and secondary stems, seed number per silique, and seed yield (Figure 1c–k). At 30 and 40 days, stems of treated plants were wider and shorter compared with those of untreated plants and continued to exhibit these characteristics until harvesting. Notably, secondary stems were also wider than controls and the number of siliques per secondary stem was larger. Moreover, not only the number of siliques augmented in treated plants but also the number of seeds per silique (Figure 1i–j). All these altered parameters influenced seed yield that was almost twice in treated plants than in controls (Figure 1k). Conversely, the number of secondary stems, number of siliques on the main stem and number of branches did not present significant differences between treated and untreated plants (Figure 1e,f,h). Altogether, these results indicated that applying weight could generate similar traits as those conferred by *HaHB11* or *AtHB7* as transgenes. Such shared characteristics were stem width and increased vascular bundle number at a particular developmental stage, which seem to correlate with final seed yield.

Weight-induced formation of vascular bundles influences lignin deposition in Arabidopsis

To reveal which signals mediated the observed effect on architectural parameters, lignin deposition and auxin role were assessed during the formation of vascular bundles induced by weight application. Arabidopsis plants with stems of 4–5 cm in height were treated with weight application over 1, 2, 3, and 4 days (Figure 2). After treatment, the first internode was cross-sectioned and stained (Figure 2b(lower panel),c). Untreated plants were used as

Figure 2. The formation of vascular bundles, induced by weight application, exhibit an opposite behavior with lignification.
(a) Stem width and number of vascular bundles (VBs) quantified in cross-sections of the first internode from untreated Arabidopsis plants used as controls. Measurements started when plant (30-day-old) stems reached 4–5 cm and continued 1, 3 and 4 days after that. Right: Illustrative picture of control and weight-treated plants.
(b) Stem cross-sections obtained from the first internode of control plants (upper panel) or weight-treated plants (1.6 g/each; lower panel) over 1, 3 and 4 days.
(c) Amplification of sections shown in (b) (signalled with red circles in the lower panel) illustrating the division of vascular bundles. Red arrows indicate division of bundles from weight-treated plants.
(d) Upper panel: Illustrative picture of lignin content visualized by fluorescence in the same plants shown in (a). Scale bars represent 0.3 mm. Quantification of lignified area of the same cross-sections carried out with ImageJ software. Data are shown as lignified area/stem cross (left) or lignified area/total stem area (right) .
In (a) and (d) asterisks indicate P-value < 0.05.



controls over the same time period as treated ones and no differences were detected in stem width or the number of vascular bundles (Figure 2a, b(upper panel)). Deposited lignin in the xylem was identifiable by lignin autofluorescence (Van de Mortel *et al.*, 2006; Zhang *et al.*, 2012; Gallego-Giraldo *et al.*, 2015), whereas toluidine-blue staining allowed visualization of vascular bundles (Figure 2d). Interestingly, lignin deposition and vascular bundle formation followed almost opposite directions. One day after starting the treatment, lignin deposition diminished and after 4 days the initial condition was recovered (Figure 2d). While lignin deposition decreased, additional vascular bundles appeared by division of the pre-existing ones (Figure 2c). At the end of treatment, treated plants exhibited more vascular bundles than controls and detectable lignin was re-established (Figure 2d). Untreated plants did not show significant changes during this time period.

As several previous studies have shown that auxin stimulates cambial cell growth (Elo *et al.*, 2009; Johnsson and Fischer, 2016; Bhalerao and Fischer, 2017), we investigated a putative role for auxin in the formation of vascular bundles by applying external weight to plants transformed with *DR5:GUS*. *DR5* was reported as a synthetic promoter inducible by the presence of auxin (Ulmasov *et al.*, 1997a, b). Plants were treated for 6, 12, 24, 48 or 72 h and β-glucuronidase (GUS) activity was assessed by histochemistry; after which, stems were visualized on paraffin cross-sections. GUS expression reached a maximum after 12 h, slowly declining after this time. Untreated plants used as controls did not show significant changes during the treatment period. Two days after starting the treatment, GUS expression was clearly detected in vascular bundles of treated plants and then disappeared (Figure S4).

Weight-induced vascular bundle formation started in the upper internode and continued downstream until the first internode in Arabidopsis

In all the above reported assays, vascular bundles were evaluated in the first internode of 4 cm height plants. To determine where and when the formation of weight-induced vascular bundles initiated, plants (same height) were treated with weight for 6, 7, 8, 9, 12, and 18 h. For each time point, the first, second, and third internodes were cross-sectioned every 1 mm and stained with toluidine blue dye. Figures 3(a), S5, S6, S7, and S8 show the obtained results; after 6 h of treatment, the number of vascular bundles in stems of treated or untreated plants was eight, independently of the treatment. At 1 h later (7 h of treatment), the first internode exhibited eight vascular bundles (as control conditions plants do), the second internode augmented to nine, whereas the third internode had 11 vascular bundles (Figure 3b). These observations indicated that vascular bundle formation, induced by weight, starts 7 h after treatment initiation and that this process began at the third internode in the direction up−down (Figure 3a,b). At 9 h of treatment the first internode had already 10 vascular bundles and this number was maintained at least until 12 h (Figure S9).

To investigate the role of auxin in this process, a similar assay was carried out with *DR5:GUS* plants, but samples were harvested at 2, 4, and 6 h. This evaluation started early, aiming to detect the moment of weight-induced vascular bundle formation considering that hormone signaling precedes vascular bundle formation. In agreement with the previous observation, GUS staining was evident after 2 h treatment in the second and third internodes but not in the first one, whereas after 4 h GUS was clearly expressed in all the internodes (Figure 3c). Taken together, these results indicated that additional vascular bundle formation is a process starting at the third internode.

The increase of the number of vascular bundles provoked by weight in Arabidopsis is reverted after 2 weeks, leading to an enlarged xylem area

To date, the vascular bundle process induced by weight was evaluated immediately after the treatments. To test if the effect on stem width and vascular bundles was permanent until plant harvest, cross-sections were cut from first, second, and third internodes of treated and untreated plants at 3 weeks after the treatment. Surprisingly, we observed that the process reverted and the number of vascular bundles in treated plants was equal to those of controls. However, xylem area increased more in treated plants than in the controls (Figure 4a, b(upper panel)). Both treated and untreated plants had wider stems after 3 weeks as expected for normal growth, but the proportion of xylem area/whole stem area was larger in treated plants than in the controls; 25% compared with 20% in untreated plants (Figure 4b). In view of these results, histological cuts (first, second, and third internode) of *HaHB11* plants were performed in 10 cm or 20 cm high plants. Notably, such cuts showed the same plasticity observed in weight-treated plants. In younger plants, the number of vascular bundles was increased, whereas in older plants this difference disappeared leading to the enlarged xylem area observed (Figure S10).

Arabidopsis auxin influx carriers are crucial for additional induced vascular bundle formation

It is already known that auxin influx carriers belonging to AUX/LAX family regulate vascular patterning and differentiation in plants (Fàbregas *et al.*, 2015). To investigate which among these carriers played a role in the induced formation of additional bundles, 4–5 cm stem height mutant plants (*aux1*, *lax1*, *lax2.1*, *lax2.2,* and *lax3*) were treated with weight. Stem diameter was assessed both after 24 and 48 h of treatment. The results indicated that *aux1*, *lax1,* and *lax3* genotypes did not increase their stem

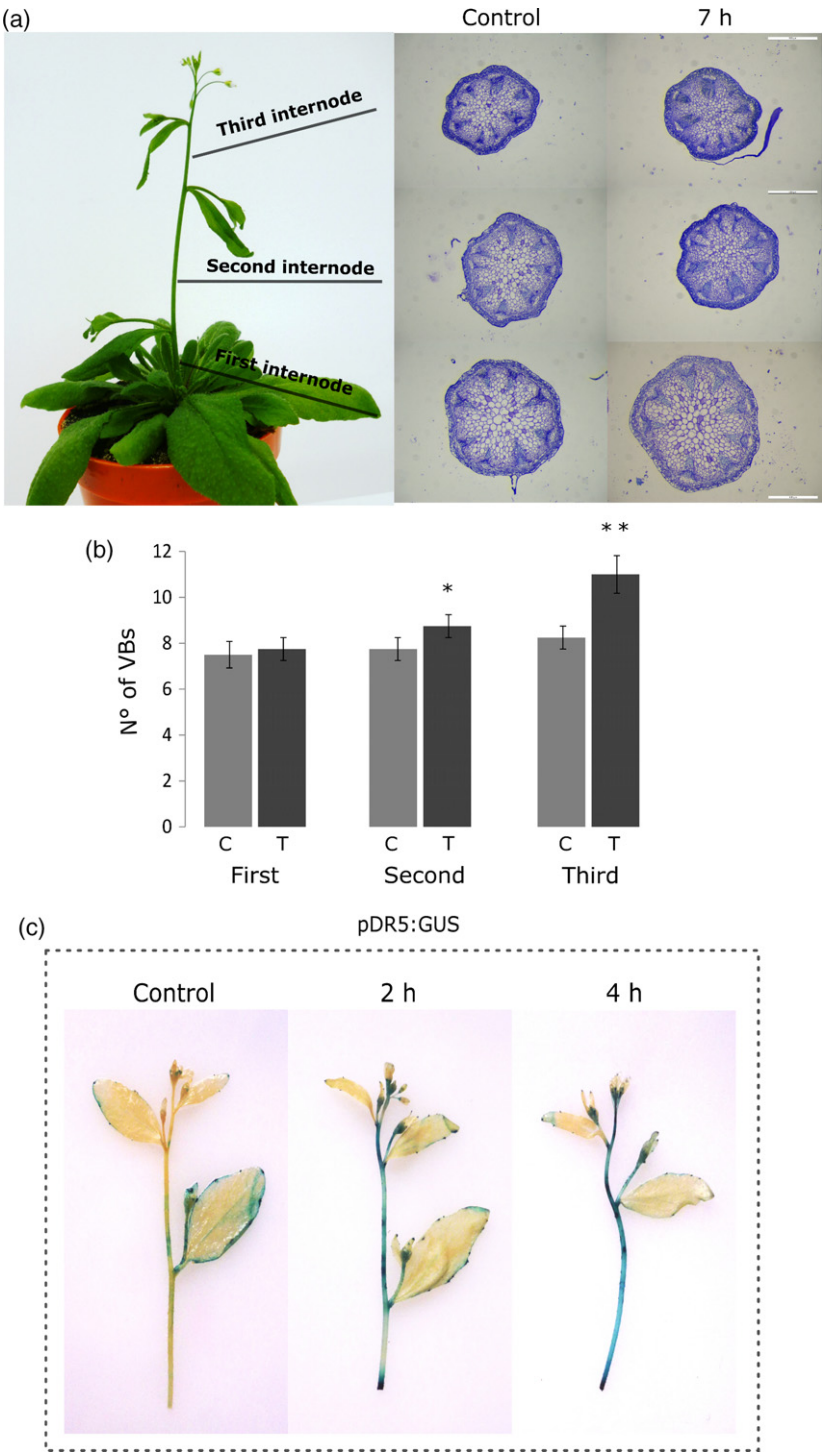


Figure 3. Weight-induced vascular bundle formation starts in the upper internode.
(a) Left: illustrative photograph of Arabidopsis plants signaling first, second and third internodes. Stem cross-sections were obtained from the three internodes of untreated (left) or weight-treated plants (right) and stained with toluidine blue. Scale bars represent 0.5 mm.
(b) Quantification of the number of vascular bundles in each section of Arabidopsis control (C) and weight-treated (T) plants for 7 h with 1.6 g. For each point, four plants/stems were evaluated. Differences were considered significant and indicated with one asterisk when the *P*-value was <0.05 and with two asterisks when the *P*-value was <0.01 (Student's *t*-test).
(c) Illustrative pictures of transgenic Arabidopsis *pDR5:GUS* plants revealed by β-glucuronidase (GUS) histochemistry. From left to right, untreated plants and weight-treated plants (1.6 g) for 2 and 4 h.

width in a similar manner to the controls after 24 h of treatment (Figure 5a). However, after 48 h, all mutants increased their stem width similarly to found in WT plants. The exception was *lax2* mutant (both independent alleles); these plants were insensitive to treatment after 24 or 48 h (Figure 5a), suggesting that the effect of weight is absolutely dependent on *LAX2*. Surprisingly, and although wider stems were clearly visualized after 48 h treatment in *aux1*, *lax1,* and *lax3* mutants by cross-sections performed on the first internode, none of the mutants showed an increased number of vascular bundles (Figure 5b). To investigate which cell type caused stem widening, the areas of cortex, epidermis, vascular bundles, and pith were evaluated in these cross-sections (0, 24 and 48 h after

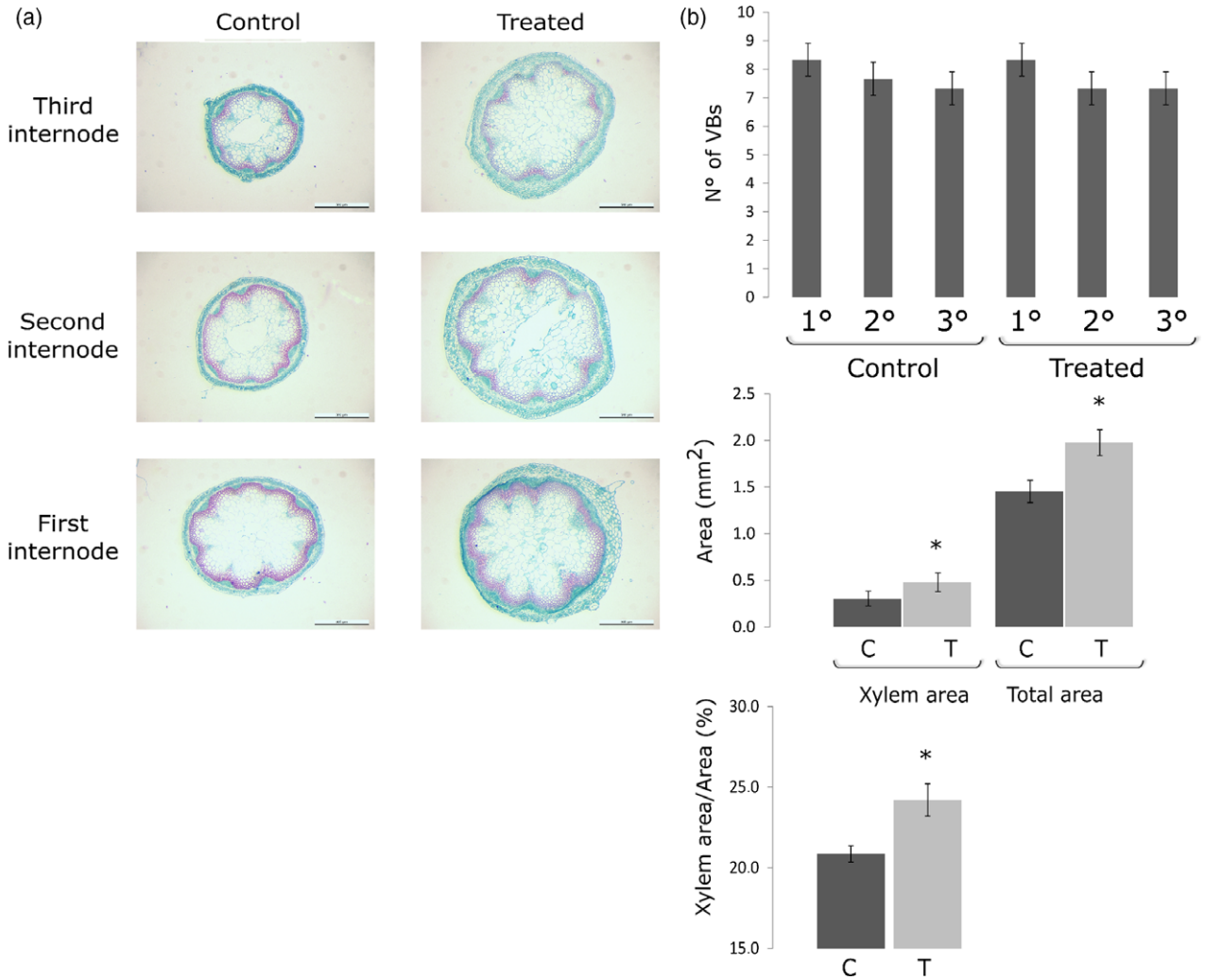


Figure 4. The number of vascular bundles provoked by weight application is restored after 2 weeks, leading to an increased xylem area.
(a) Illustrative pictures of Arabidopsis stem cross-sections stained with Safranin-Fast green dye in untreated or treated plants (1.6 g over 2 days). Cross-sections (1 cm height), taken from the middle of each internode, were stained and photographed 3 weeks after treatment. White bars represent 0.5 mm.
(b) Upper panel: Quantification of vascular bundle number. Middle panel: Xylem and total stem area in the cross-sections of the first internode shown in (a). Lower panel: Xylem area/total stem area ratio. For each point, four plants/stems were evaluated. Differences were considered significant and indicated with asterisks when the *P*-values were <0.05 (Student's *t*-test).

weight treatment). This survey indicated that all these cell types increased, but the increase was similar to that observed in the untreated control plants (Figure S11). Altogether, these results indicated that stem widening and increase in the number of vascular bundles were independent events, even though both effects could be triggered by the same weight treatment. Moreover, it can be said that all auxin carriers analyzed were necessary for the formation of vascular bundles in response to weight.

To determine if seed yield was dependent on both traits (stem widening and number of vascular bundles) or if it resulted from only one of these characteristics, stem diameter, the number of vascular bundles and seed yield were assessed in the mutant genotypes (*aux1*, *lax1*, *lax2.1*, *lax2.2,* and *lax3*) as well as in WT plants treated for 24 or 48 h and in untreated controls (Figure 5a). As expected, WT plants increased for the three parameters, whereas *lax1* and *lax3* mutants increased their stem diameter but not the number of vascular bundles, and *lax2-1* plants did not show any change in any of the parameters (Figure 5a). Interestingly, only the WT plant increased its yield in response to weight application, indicating that stem width as unique trait does not govern seed yield, and was more likely to be associated with an increase in the number of vascular bundles. However, it cannot be ruled out that these mutants had additional effects on seed sets that are independent of the vascular tissues, as the number of genes associated with auxin transport or response has been shown to be pleiotropic.

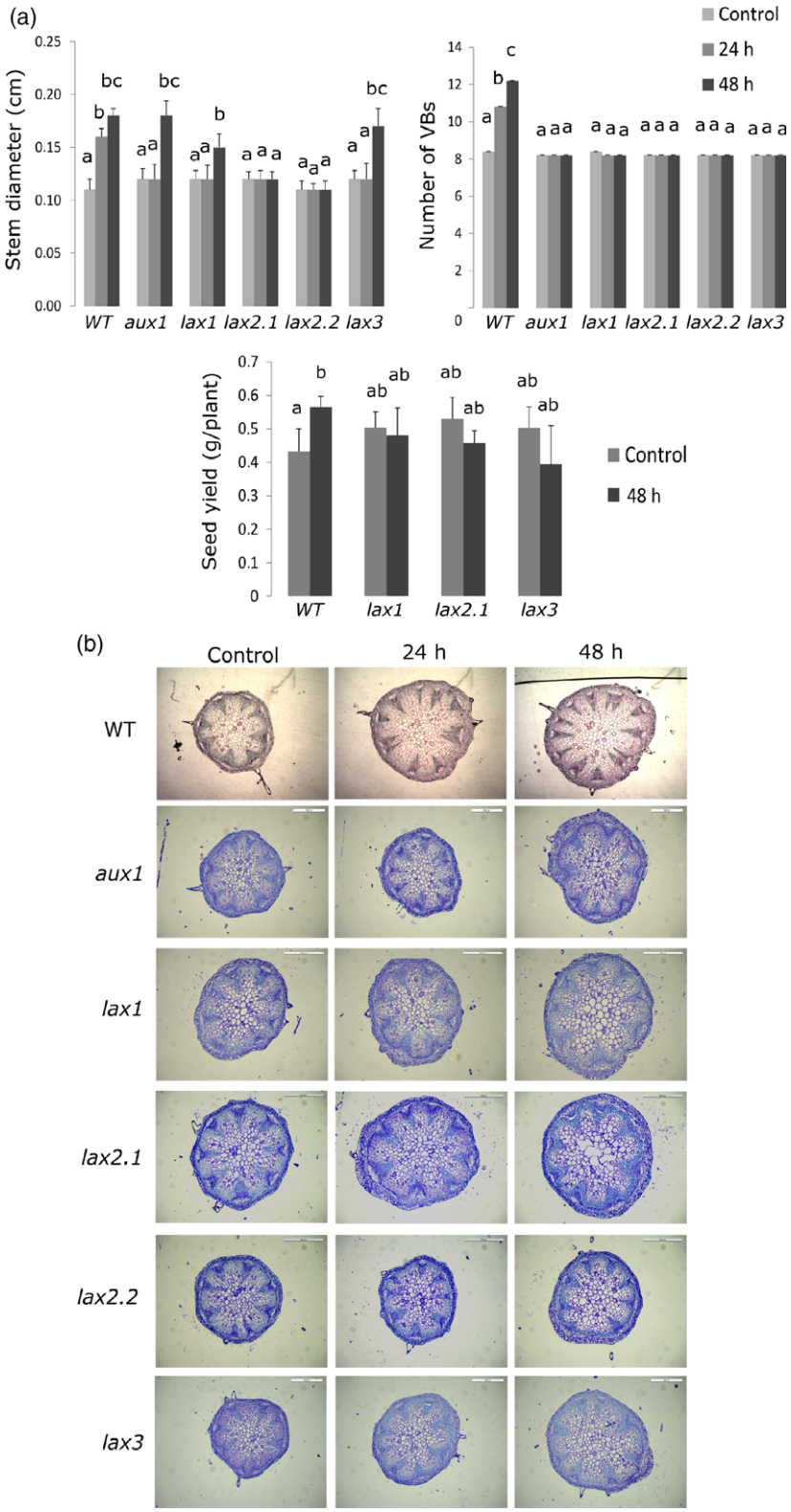


Figure 5. Auxin influx carriers are crucial for weight-induced vascular bundle formation.
(a) Diameter of the first internode, number of vascular bundles and seed yield of Arabidopsis WT and mutant plants untreated or treated 1.6 g weight for 24 or 48 h). Mutant genotypes used were *aux1*, *lax1*, *lax2* and *lax3*. For each point, four plants/stems were evaluated. Bars represent SE and different letters indicate significant differences between means ($P < 0.01$, Tukey test).
(b) Illustrative pictures of Arabidopsis stem cross-sections of the same plants as in (a) stained with toluidine-blue. Scale bars represent 0.5 mm.

To further investigate the role of LAX2 in the formation of vascular bundles, *35S:LAX2* plants (4–5 cm stem height) were cross-sectioned at the first internode. These plants exhibited wider stems, more vascular bundles and also yielded more seeds than the WT. Overexpressors were crossed with *lax2* mutant plants (*lax2* × *35S:LAX2*) and

these resulting plants recovered a WT phenotype regarding weight treatment effect (wider stems, more vascular bundles and increased yield; Figure S12). This latter result indicated that LAX2 is essential for the formation of weight-induced vascular bundles in response to weight treatment, in agreement with the observations carried out for *lax2* mutants (Figure 5a).

Arabidopsis genes encoding auxin influx carriers are transcriptionally regulated by weight treatment in the Arabidopsis shoot inflorescence stem

Due to the role of auxin influx carriers in the formation of weight-induced vascular bundles as described above, we decided to investigate the expression patterns of *AUX/LAX* encoding genes as well as their response after weight treatment. Arabidopsis plants transformed with constructs in which the *GUS* reporter gene expression was driven by *AUX/LAX* promoters were analyzed in standard growth conditions and in plants treated with weight for 24 or 48 h. Figure S13 shows the obtained results: *pAUX1:GUS* plants did not show any detectable expression under normal conditions or after 24 or 48 h of treatment (upper panel). *pLAX1:GUS* expression was noticeable in the vascular tissues of control plants and was induced after treatment; GUS activity was localized on xylem vessels after 48 h of treatment. *pLAX2:GUS* plants did not show any detectable expression under at normal conditions or after 24 h, but GUS activity was slightly induced after 48 h and noticeable on vascular bundles. *pLAX3:GUS* stems showed expression on the vascular tissue and on the interfascicular region in normal conditions, but this expression was significantly diminished after treatment (Figure S13). These results indicated that, although all these carriers were shown as important for stem widening in response to weight, only *LAX1*, *LAX2,* and *LAX3* were detected as responsive to the treatment, suggesting a cooperative, but not essential, function of the other transporters. Quantification of the corresponding transcripts by real-time qPCR indicated similar results (Figure S13b).

Auxin carrier-mediated induction of vascular bundle formation is dependent on the photoperiod

Arabidopsis plants grown under short-day conditions (8 h light/16 h darkness) naturally had wider stems and more vascular bundles compared with plants grown under long-day conditions (Ko *et al.*, 2004). To investigate the role of auxin influx carriers in the formation of vascular bundles and increase of stem width under short-day photoperiod, *aux/lax* mutant plants were grown and assessed under such conditions. Phenological stage instead plant age was taken into account for comparison. Cross-sections of the first internode were taken when the stems were 5 and 10 cm in height and the first internode diameter was evaluated in the same plants/developmental stage. Only the quadruple mutant, *aux1/lax1/lax2/lax3*, and *lax1* and *lax2* single mutants had narrower stems than WT, whereas *aux1* and *lax3* mutants did not differ from the controls, suggesting that not all these carriers were necessary for this process (Figure 6a). Considering vascular bundles, no significant differences between genotypes were detected, indicating that the events occurring under short-day conditions and those provoked by applying weight under long-day photoperiod, were different. Moreover short-day conditions were not able to emulate weight treatment (Figure 6b).

Sunflower plants also increased their vascular bundle number after weight treatment, boosting seed yield

To determine if the mechanism of weight-induced vascular bundles formation was conserved between species, sunflower plants were treated with weight when they were 7 days old (Figure 7a). After treatment, both hypocotyls and epicotyls (3 cm high hypocotyls and 1.5 cm high epicotyls) were cross-sectioned and stained. Figure 7(b) shows that, after the treatment, both organs significantly increased their width, number of vascular bundles and xylem rows compared with organs from untreated plants. Plants were grown until harvest and seed yield evaluated, indicating a 40% increment in treated plants (Figure 7c). These observations indicated that, at least in these two species, Arabidopsis and sunflower, treatment with weight is capable of initiating the formation of vascular bundles and this event positively affected final seed yield.

DISCUSSION

Plant scientists and biotechnologists devote enormous effort to obtain crop varieties with improved behavior in front of broad stressing factors, both biotic and abiotic. Frequently, the evaluated traits of improved varieties include wet and dry biomass, number of grains, seed weight, flower, and root architecture and, particularly, seed yield. However, stem width and morphology have been rarely assessed. Observing in detail several transgenic genotypes obtained by our and other research groups, we noticed that among these, many plants exhibiting high yield also had wider stems. Firstly, we directly associated stem width with yield but the analysis of stem morphology, particularly regarding vascular bundles, evidenced that transport tissues were clearly altered in those transgenic plants. The first question was which of these traits (stem width or number of vascular bundles) was responsible for yield increase. How could we obtain plants with one of these characteristics modified to test the hypothesis? The technique applied by Ko *et al.* (2004) to study secondary growth under short-day conditions was a good candidate for this investigation. Application of discrete weight on the main stem resulted in an increase in stem width, but also in the number of vascular bundles. Without

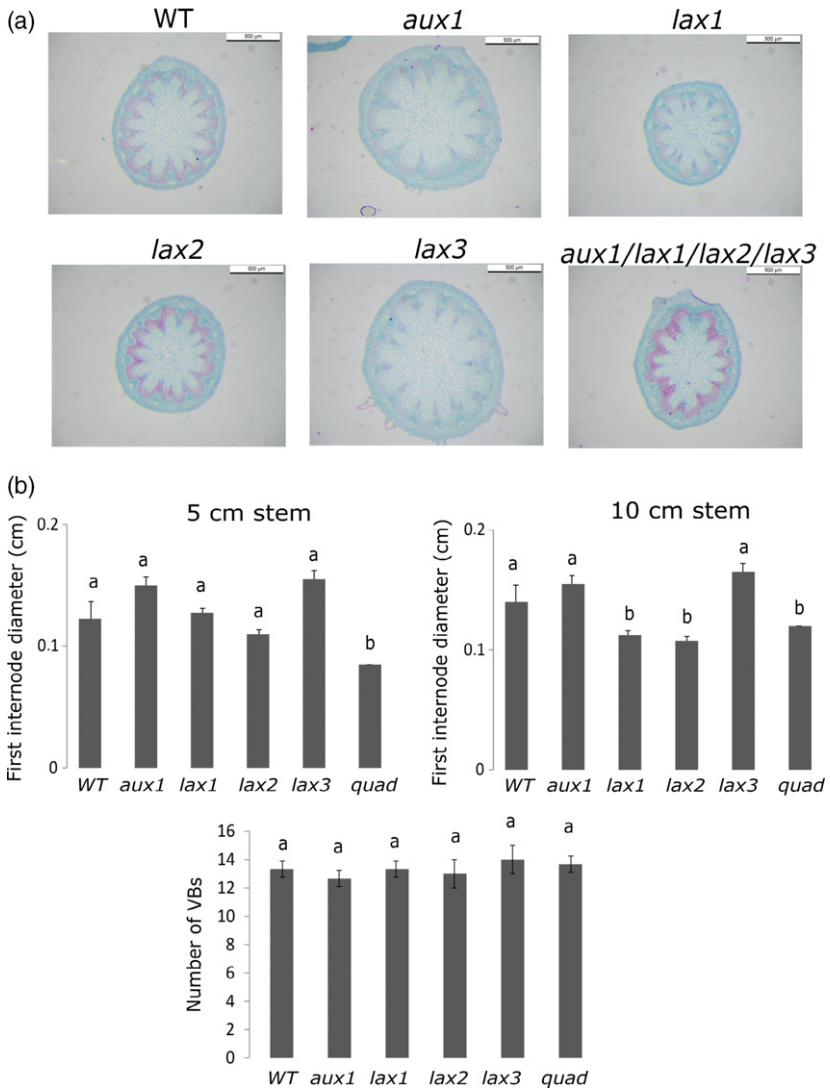


Figure 6. Developmental events related to stem width and formation of vascular bundles under short-day conditions differ from those occurring after weight treatments under long-day photoperiod.

(a) Illustrative photographs of Arabidopsis stem cross-sections of the first internode of WT, *aux1*, *lax1*, *lax2*, *lax3* and *quad* mutant plants when they had 5 cm height. Staining was performed with Safranin-Fast green dye. Scale bars represent 0.5 mm.

(b) First internode diameter and number of vascular bundles of WT and mutant (*aux1*, *lax1*, *lax2*, *lax3* and *quad*) plants at two growing stages: 5 and 10 cm stem height. Plants were grown under a short-day regime. For each point, four plants/stems were evaluated. Bars represent SE and different letters indicate significant differences between means ($P < 0.01$, Tukey test).

transgenic strategies, we achieved high-yielding Arabidopsis plants. Notably, such yield increase was associated not only with wider and shorter stems but also with augmentation of the number of siliques in secondary stems, but not in the main stem.

The response to weight application was clearly fast compared with other plasticity traits provoked by external stimuli. For example, it was informed that grafted (grafting being the process by which plants were cut) phloem reconnected after 3 days and xylem after 7 days (Kümpers and Bishopp, 2015), whereas initiation of weight-induced vascular bundle formation took only 7 h.

The plasticity of plant tissues was also evidenced by restoring the number of vascular bundles 2 weeks after treatment. However, such treatment left a mark, the enlargement of xylem area. It is tempting to suggest that such enlargement of xylem area was related to more efficient water transport. In accordance with this suggestion, transgenic Arabidopsis plants expressing *HaHB4* and yielding more than their controls (Chan and González, 2012) exhibited a clear increase in xylem area but not in the number of vascular bundles (Moreno-Piovano *et al.*, 2017).

The plasticity showed by weight-treated plants also temporally affected lignin deposition. It was notable how visible lignin diminished when vascular bundles and stem width increase occurred and, then, once these events finished, lignin accumulation was equal in treated or untreated plants. A similar scenario was observed applying brassinosteroids (24-epi brassinolide) to the vascular cambium of a vertical stem of the woody plant *Liriodendron tulipifera* that produced the down-regulation of lignin biosynthesis and significant modifications in the cell wall carbohydrates (Jin *et al.*, 2014).

Ko *et al.* (2004) reported that auxins were an integral part of weight signaling, inducing the transition from primary to secondary growth in Arabidopsis. Hence, we

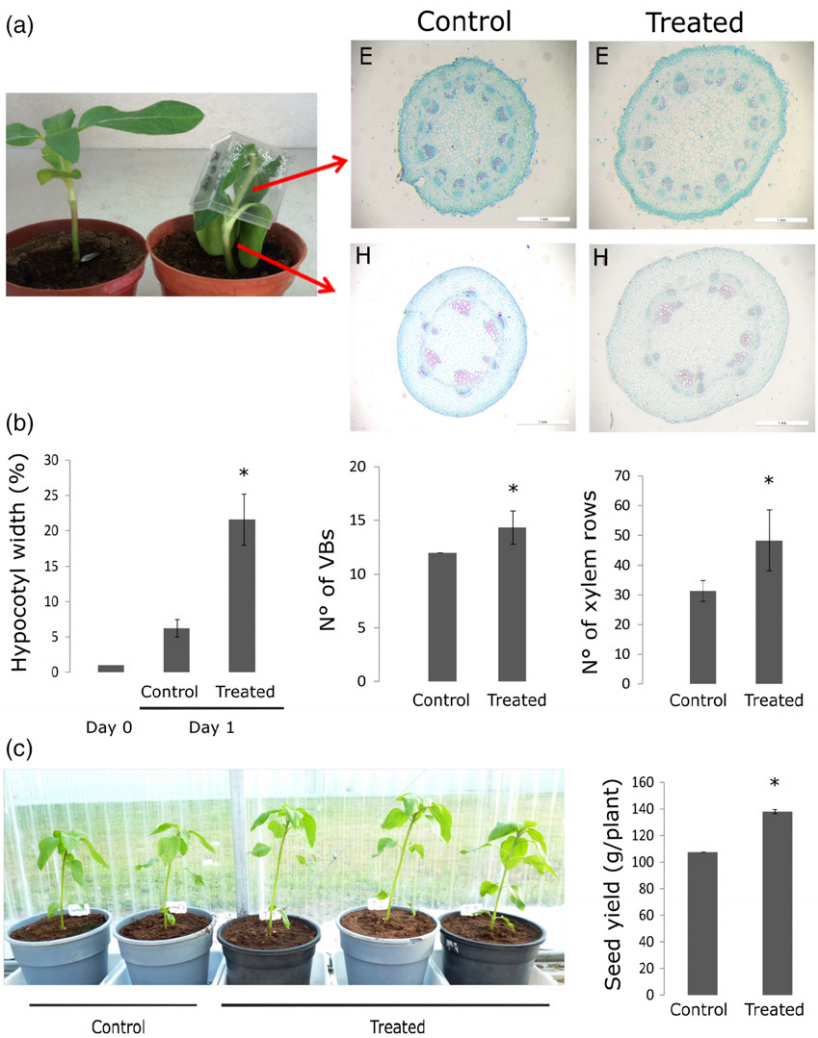


Figure 7. Sunflower plants treated with weight increase the number of vascular bundles and seed yield.
(a) Left: Illustrative picture of sunflower plants untreated or treated with 12 g of weight (acrylic box) during 24 h. Right: Cross-sections of epicotyls (upper panel) and hypocotyls (lower panel) of the same plants. White scale bars represent 1 mm.
(b) Hypocotyl width, numbers of vascular bundles and xylem rows were quantified using ImageJ. Differences were considered significant and indicated with asterisks when *P*-values were <0.05 (Student's *t*-test).
(c) Illustrative picture of sunflower plants treated with weight (12 g) for 3 days and then grown at normal conditions. Seed yield of such plants obtained at harvest and expressed in g/plant. Differences were considered significant and indicated with asterisks when *P*-values were <0.05 (Student's *t*-test).

investigated if such hormones were involved in setting vascular bundles. Weight-treated plants transformed with *DR5:GUS* indicated that auxins are implicated in the increase of the number of vascular bundles in agreement with previous reports in which it was demonstrated that auxins stimulate cambial cell growth (Elo *et al.*, 2009; Johnsson and Fischer, 2016; Bhalerao and Fischer, 2017).

Arabidopsis auxin influx carriers were shown as involved in the process of weight-induced vascular bundles formation. Each one of these carriers was described as implicated in a particular developmental event and all these were regulated during secondary stem development; *AUX1* expression increased in mature compared with immature stems, whereas *LAX2* decreased. Both *LAX1* and *LAX3* were slightly induced during stem maturation (Ko *et al.*, 2004). It was suggested that the photoperiod could change the balance between passive and active influx transport across the cell membrane, passive influx being more relevant under long-day than under short-days conditions (Fàbregas *et al.*, 2015). By combining experimental and theoretical approaches, these authors proposed novel roles for Arabidopsis auxin influx carriers in vascular patterning and differentiation during plant development (Fàbregas *et al.*, 2015).

Coming back to the question of whether stem diameter or vascular bundles had a positive correlation with yield increase, Arabidopsis *AUX/LAX* mutants helped to give a partial response. After 48 h weight treatment *aux1*, *lax1,* and *lax3* increased their stem diameter, but did not generate additional vascular bundles and neither yielded more than the untreated controls. Conversely, the *lax2* mutant did not respond to treatment by any of both traits, indicating that this gene is essential in these developmental events. It would be interesting to determine if *AUX/LAX* carriers have similar functions in other plant species, however such analyses will need the availability of mutants.

Considering the regulation of the expression of Arabidopsis *AUX/LAX* genes studied here with transgenic plants in which their promoters were fused to *GUS*, *AUX1* was not detected in stems whereas *LAX1* increased and

LAX3 decreased in response to weight, particularly in vascular bundles. Notably, *LAX2* that was shown to be essential for weight response, slightly increased in vascular bundles. We cannot rule out that the segment taken as the promoter region is incomplete. *LAX2* levels were also assessed by RT-qPCR and, in accordance with histology observations, transcripts did not show significant differences after the treatment. Due to the results obtained by analysis of *lax2* mutant plants, it is tempting to speculate that there is possible post-transcriptional regulation of this gene. Previous studies informed by other authors indicated that *LAX2* was particularly expressed in vascular tissue during embryogenesis (Péret *et al.*, 2012). Considering the regulation by auxin, *LAX3* and *LAX1* were reported as induced by auxin (Swarup *et al.*, 2008 and Péret *et al.*, 2012), whereas neither *AUX1* nor *LAX2* expression seemed to be altered by this hormone (Péret *et al.*, 2012).

Finally, we demonstrated that weight treatment was also effective on sunflower plants and that, in both species, this treatment influenced seed yield. It is important to note that even when stem width did not result in a good parameter to be associated with yield, the increment in the number of vascular bundles at early stages that led later to an increase in xylem area and improved yield, were accompanied by wider stems. Further work is needed to examine the correlation between seed yield and xylem area in a broader range of plants.

It is important to note that in all the assays shown here, plants were grown under standard conditions and were not subject to any stress. In this regard, Richards and Passioura (1989) informed that in water-deficit stressing conditions, wheat plants tested in the field over 5 years, and exhibiting a reduced diameter of the major xylem vessel in seminal roots, yielded more than those plants with wider vessels (Richards and Passioura, 1989). The authors assigned such a positive effect to better water use after anthesis producing a higher harvest index. Even though it is difficult to compare root xylem vessels from a monocot such as wheat with the stem xylem of a dicot, the key different point between the mentioned work and this one is not the species, but more likely the water-deficit condition of the first species. According to Richards and Passioura when the top soil is dry and the subsoil is wet, wheat plants are able to efficiently use water after anthesis, increasing harvest index, whereas with wider vessels they did not observe significant differences between genotypes. In this regard, it is tempting to speculate that an increase in the number of vascular bundles or xylem area will not produce higher yields under any conditions. *HaHB11* transgenic plants, which originated the present study, exhibited stem narrowing under water-deficit conditions (Cabello *et al.*, 2017). Notably, these stressed plants exhibited a lower yield than the controls, although they demonstrated better survival to drought. Further investigations, including treating plants with weight under stress, will be needed to answer interesting questions about the correlation between stem width, vascular bundles, stress survival and seed yield.

CONCLUSION

We can conclude that an increased number of vascular bundles or xylem area in stems could be indicative of high-yielding lines in different species. These traits are dependent on AUX/LAX carriers and, among these, LAX2 has a key role. At this time, it is difficult to imagine a practical application of these findings to increase crop yield on a large scale, however it is certain that a better understanding of the pathways underlying the control of vascular bundle development and its effect on crop yield would allow a more efficient design of innovative strategies for sustainable agriculture.

EXPERIMENTAL PROCEDURES

Plant material, growth conditions and plant treatments

Arabidopsis thaliana plants (Col-0 ecotype) were grown on Klasmann Substrate No. 1 compost (Klasmann-Deilmann GmbH, Germany) in a growth chamber at 22–24°C under long-day (16/8 h light/dark cycles) or short-day (8/16 h light/dark cycles) conditions, indicated in each figure, with a light intensity of approximately 120 μmol m^{-2} sec^{-1} in 8 × 7 cm pots. Four plants were planted per pot, unless stated differently.

Experiments performed under short-day conditions were performed with one plant per pot in a growth chamber with a photoperiod of 8 h light/16 h dark at 22–24°C until stem length reached 5 or 10 cm. The first internode was cut and imbibed in 70% ethanol for paraffin inclusion as explained below.

The mutant alleles *lax2-1, lax2-2, lax1, lax3, aux1-21,* and *quadruple aux1/lax1/lax2/lax3*, all on the Col-0 background have been previously described (Swarup *et al.*, 2005, 2008; Bainbridge *et al.*, 2008). *Arabidopsis* null mutant lines *lax2-1* (dSpm line) and *lax2-2* (GK_345D11), as well as overexpressor plants bearing either the *HaHB11* cDNA or the *AtHB7* cDNA driven by the 35S cauliflower mosaic virus, have been previously described (Péret *et al.*, 2012; Re *et al.*, 2014; Cabello *et al.*, 2016).

Transgenic plants carrying AUX/LAX promoters fused to *GUS* have been previously described (*ProAUX1:G*US: Marchant *et al.*, 2002; *ProLAX1:GUS* and *ProLAX2:GUS*: Bainbridge *et al.*, 2008 and *ProLAX3:GUS*: Swarup *et al.*, 2008). *DR5:GUS* transgenic plants were obtained from the Arabidopsis Biological Resource Center (ABRC).

Helianthus annuus seeds (HA89 public line) were germinated on wet filter paper for 7 days and then transferred to 8 × 7 cm pots each with equal amounts of Klasmann Substrat No. 1 compost (Klasmann-Deilmann GmbH, Germany) and placed in a growth chamber at 22–24°C under long-day conditions (16/8 h light/dark cycles) with a light intensity of approximately 120 μmol m^{-2} sec^{-1}. Plants were sown one per pot in a 45 cm plastic square tray and then transferred to larger pots (40 litre) and placed in a greenhouse until harvest.

Weight treatments

Weight treatment was carried out essentially as described by Ko *et al.* (2004) with some modifications. Briefly, a cap tube containing 2% agar (1.6 g) was placed on the top of 5 cm high non-

decapitated Arabidopsis inflorescence stems, both for WT or mutant plants. Weight was applied for the periods of time indicated in each figure legend and observations were usually performed immediately after removing the weight, with the exception of the experiment shown in Figure 4 in which observations were performed 2 weeks after treatment. Controls were performed with untreated plants of the same age and height. Each experiment included at least three to six plants for treatment and was repeated at least three times, with similar results.

For sunflowers, weight treatment was applied with 11–12 g acrylic empty boxes when epicotyls reached a height of 1.5 cm and hypocotyls reached 3 cm. For yield evaluation, the treatment was applied for 3 days, whereas for evaluation of the number of vascular bundles and stem width, extension of the treatment was 1–2 days.

Histology and microscopy

Arabidopsis inflorescence sections (1 cm height) were harvested from the middle of the first internode of 5 cm height stems. In all cases, 0.5–1.0 cm length sections were fixed at 24°C for 1 h in a solution containing 3.7% formaldehyde, 5% acetic acid, and 47.5% ethanol, and then dehydrated through a graded series of ethanol solutions (70, 80, 90, 96, and 100%; 30 min each one) followed by 1 h in 100% xylene. The samples were placed into plastic molds and finally embedded with 100% Histoplast (Biopack™, Argentina). Each block was incubated overnight at room temperature to ensure solidification. Transverse stem sections (10 μm thick) were obtained using a Leica microtome (Microtome RM2125, Leica). Cross-sections were mounted on slides coated with 50 mg/ml poly-D-Lys (Sigma Chemical Co., St. Louis, MO, USA) in 10 mM Tris–HCl pH 8.0 and dried for 16 h at 37°C. After removing the paraffin with 100% xylene for 15 min at room temperature, sections were rehydrated using a graded series of ethanol (100, 96, 90, 80, 70, and 50%; 1 min each one) to finish in distilled water. Samples were then stained with 0.1% toluidine blue, rinsed, and mounted on Canadian balsam (Biopack™, Argentina) for microscopic visualization in an Eclipse E200 microscope (Nikon, Tokyo, Japan) equipped with a Nikon Coolpix L810 camera.

Safranin-Fast green dye was used for xylem/phloem differential detection. After removing paraffin with 100% xylene for 15 min at room temperature, sections were imbibed in ethanol (100, 96, and 90%), then the slices were transferred to Safranin (in ethanol 80%) for 4 h. After this step, slices were put in ethanol (90, 96, and 100%) to finally be imbibed on Fast green dye (in ethanol 100%) over several seconds. Slices were mounted on Canadian balsam.

For lignin auto-florescence, paraffin from slices was removed using xylene 100%. Then slices were dehydrated in ethanol 100% and rapidly mounted on Canadian balsam. Lignin was visualized using microscopy by autofluorescence, as previously described, to detect lignin deposition on walls of xylem cells and interfascicular fiber cells (Van de Mortel *et al.*, 2006; Zhang *et al.*, 2012; Gallego-Giraldo *et al.*, 2015).

When the intensity of expression in certain tissues was difficult to visualize on 10 μM cross-sections, photographs were initially taken from whole paraffin inclusions.

Histochemical GUS staining

In situ assays of GUS activity were performed as described by Jefferson *et al.* (1987). Whole plants were immersed in a 1 mM 5-bromo-4-chloro-3-indolyl-β-glucuronic acid solution in 100 mM sodium phosphate pH 7.0 and 0.1% Triton X-100 and, after applying three times vacuum for 5 min, they were incubated at 37°C overnight. Chlorophyll was cleared from green plant tissues by immersing them in 70% ethanol. Paraffin inclusions were performed after chlorophyll was totally cleared.

Plant phenotyping

Different plant architecture parameters were scored on control and weight-treated Arabidopsis plants including: main stem height and width, number of secondary branches, number of secondary stems, silique number on main and on secondary stems, seed number per silique and seed yield. Measurements were performed manually or with the aid of a ruler or gauge. All experiments were performed with 16 plants per treatment and repeated at least four times.

RNA isolation and expression analyses by real-time RT-PCR

Total RNA for real-time RT-PCR was isolated from Arabidopsis stems using TRIzol® reagent (Invitrogen, Carlsbad, CA, USA) according to the manufacturer's instructions. Total RNA (1 μg) was reverse-transcribed using oligo(dT)18 and M-MLV reverse transcriptase II (Promega, Fitchburg, WI, USA).

Quantitative real-time PCR (qPCR) was performed using a Mx3000P Multiplex qPCR system (Stratagene, La Jolla, CA, USA) as described before (Cabello *et al.*, 2016) and using the primers listed in Table S1. Transcript levels were normalized by applying the ΔΔCt method. Actin transcripts (*ACTIN2* and *ACTIN8*) were used as internal standards to normalize differences in template amounts. Three biological replicates, obtained by pooling tissue from three to four individual plants and tested by duplicate, were used to calculate the standard deviation.

Statistical analysis

The evaluation of yield, stem width and number of vascular bundles, shown in Figures 1, 6 and 7, was performed using one-way analysis of variance (ANOVA) considering genotype or treatment as main factors. Significant differences ($P < 0.01$) between means were analyzed using post hoc Tukey comparison. Data shown in Figure 5 were analyzed using a two-way ANOVA considering weight treatment and genotype as factors. When interaction terms were significant ($P < 0.01$), differences between means were analyzed using Tukey comparison and indicated by different letters.

Data shown in Figure 4 represent the three replicates used to calculate the SE. Differences were considered significant and indicated with asterisks when *P*-values were <0.05 (Student's *t*-test).

ACKNOWLEDGEMENTS

The authors would like to thank Dr Federico D. Ariel and Dr Daniel González for critical reading of the manuscript and helpful suggestions. The authors thank Ana Paula Cabello and Karla Martínez Figueredo for precious help in statistical analyses. We thank Dr Ranjan Swarup for kindly providing *aux/lax* single and quadruple mutant as well as *AUX/LAX* promoters fused to *GUS* seeds used in this study by our colleague Dr Javier Moreno.

CONFLICT OF INTEREST

The authors declare no conflict of interest.

AUTHORS' CONTRIBUTIONS

Conceived and designed the experiments: JVC and RLC. Performed the experiments: JVC. Analyzed the data: JVC and RLC. Wrote the paper: JVC and RLC.

SUPPORTING INFORMATION

Additional Supporting Information may be found in the online version of this article.

Figure S1. *35S:AtHB7* plants exhibit increased yield and more vascular bundles than WT.

Figure S2. Combinations of applied weight and stem height on Arabidopsis plants affect seed yield at harvest.

Figure S3. Stem architecture and seed yield resulted from weight application and not from bending or hypoxia.

Figure S4. The number of vascular bundles in 4.5 cm stem height untreated Arabidopsis plants is eight and does not vary along the stem.

Figure S5. The number of vascular bundles in 4.5 cm stem height untreated Arabidopsis plants is eight and does not vary along the stem.

Figure S6. The number of vascular bundles remains constant between internodes and within each internode even after 6 h of weight treatment

Figure S7. The number of vascular bundles increased after 7 h of weight treatment.

Figure S8. The number of vascular bundles continues to increase until 9 h of weight.

Figure S9. The number of vascular bundles was still 10, 12 h after the weight treatment.

Figure S10. *HaHB11* plants show the same plasticity as do weight-treated plants.

Figure S11. AUX/LAX mutants increased the area of their cortex, epidermis, vascular bundles and pith similarly to control plants after 48 h weight treatment.

Figure S12. *LAX2* overexpressors both on Col-0 or *lax2.1* backgrounds increased stem diameter and vascular bundle number after weight treatment.

Figure S13. Genes encoding auxin influx carriers are regulated by weight treatment.

Table S1. Oligonucleotides used for real-time qPCR.

REFERENCES

Bainbridge, K., Guyomarc'h, S., Bayer, E., Swarup, R., Bennett, M., Mandel, T. and Kuhlemeier, C. (2008) Auxin influx carriers stabilize phyllotactic patterning. *Genes Dev*. **22**, 810–823.

Bennett, M.J., Marchant, A., Green, H.G., May, S.T., Ward, S.P., Millner, P.A., Walker, A.R., Schulz, B. and Feldmann, K.A. (1996) Arabidopsis AUX1 gene: a permease-like regulator of root gravitropism. *Science*, **273**, 948–950.

Bhalerao, R.P. and Fischer, U. (2017) Environmental and hormonal control of cambial stem cell dynamics. *J. Exp. Bot.* **68**, 79–87.

Cabello, J.V., Giacomelli, J.I., Piattoni, C.V., Iglesias, A.A. and Chan, R.L. (2016) The sunflower transcription factor HaHB11 improves yield, biomass and tolerance to flooding in transgenic Arabidopsis plants. *J. Biotechnol.* **222**, 73–83.

Cabello, J.V., Giacomelli, J.I., Gómez, M.C. and Chan, R.L. (2017) The sunflower transcription factor HaHB11 confers tolerance to water deficit and salinity to transgenic Arabidopsis and alfalfa plants. *J. Biotechnol.* **257**, 35–46.

Chan, R.L. and González, D.H. (2012) Modified Helianthus annuus transcription factor improves yield. US 2013/0263327.

Cho, M., Lee, S.H. and Cho, H. (2007) P-glycoprotein4 displays auxin efflux transporter-like action in Arabidopsis root hair cells and tobacco cells. *Plant Cell*, **19**, 3930–3943.

Elo, A., Immanen, J., Nieminen, K. and Helariutta, Y. (2009) Stem cell function during plant vascular development. *Sem. Cell. Dev. Biol.* **20**, 1097–1106.

Fàbregas, N., Formosa-Jordan, P., Confraria, A., Siligato, R., Alonso, J.M., Swarup, R., Bennett, M.J., Mähönen, A.P., Caño-Delgado, A.I. and Ibañes, M. (2015) Auxin influx carriers control vascular patterning and xylem differentiation in *Arabidopsis thaliana*. *PLoS Genet.* **11**, e1005183.

Gallego-Giraldo, L., Shadle, G., Shen, H., Barros-Rios, J., Fresquet Corrales, S., Wang, H. and Dixon, R.A. (2015) Combining enhanced biomass density with reduced lignin level for improved forage quality. *Plant Biotechol. J.* **14**, 1–10.

Geisler, M., Blakeslee, J.J., Bouchard, R. *et al.* (2005) Cellular efflux of auxin catalyzed by the Arabidopsis MDR/PGP transporter AtPGP1. *Plant J.* **44**, 179–194.

Ibañes, M., Fàbregas, N., Chory, J. and Caño-Delgado, A.L. (2009) Brassinosteroid signaling and auxin transport are required to establish the periodic pattern of Arabidopsis shoot vascular bundles. *Proc. Natl Acad. Sci. USA* **106**, 13630–13635.

Jefferson, R.A., Kavanagh, T.A. and Bevan, M.W. (1987) GUS fusions: beta-glucuronidase as a sensitive and versatile gene fusion marker in higher plants. *EMBO J.* **6**, 3901–3907.

Jiang, D., Chen, W., Dong, J., Li, J., Yang, F., Wu, Z., Zhou, H., Wang, W. and Zhuang, C. (2018) Overexpression of miR164b-resistant OsNAC2 improves plant architecture and grain yield in rice. *J. Exp. Bot.* **69**, 1533–1543.

Jin, H., Do, J., Shin, S.J., Choi, J.W., Choi, Y.I., Kim, W. and Kwon, M. (2014) Exogenously applied 24-epi brassinolide reduces lignification and alters cell wall carbohydrate biosynthesis in the secondary xylem of *Liriodendron tulipifera*. *Phytochemistry*, **101**, 40–51.

Johnsson, C. and Fischer, U. (2016) Cambial stem cells and their niche. *Plant Sci.* **252**, 239–245.

Ko, J.H., Han, K.H., Park, S. and Yang, J. (2004) Plant body weight-induced secondary growth in Arabidopsis and its transcription phenotype revealed by whole-transcriptome profiling. *Plant Physiol.* **135**, 1069–1083.

Kümpers, B.M. and Bishopp, A. (2015) Plant grafting: making the right connections. *Curr. Biol.* **25**, 11–13.

Lim, S.D., Yim, W.C., Liu, D., Hu, R., Yang, X. and Cushman, J.C. (2018) A Vitis vinifera basic helix-loop-helix transcription factor enhances plant cell size, vegetative biomass, and reproductive yield. *Plant Biotechnol. J.* **16**, 1595–1615.

Marchant, A., Kargul, J., May, S.T., Muller, P., Delbarre, A., Perrot-Rechenmann, C. and Bennett, M.J. (1999) AUX1 regulates root gravitropism in Arabidopsis by facilitating auxin uptake within root apical tissues. *EMBO J.* **18**, 2066–2073.

Marchant, A., Bhalerao, R., Casimiro, I., Eklöf, J., Casero, P.J., Bennett, M. and Sandberg, G. (2002) AUX1 promotes lateral root formation by facilitating indole-3-acetic acid distribution between sink and source tissues in the Arabidopsis seedling. *Plant Cell*, **14**, 589–597.

Moreno-Piovano, G.S., Moreno, J.E., Cabello, J.V., Arce, A.L., Otegui, M.E. and Chan, R.L. (2017) A role for LAX2 in regulating xylem development and lateral-vein symmetry in the leaf. *Ann. Bot.* **120**, 277–590.

Peng, J., Richards, D.E., Hartley, N.M. *et al.* (1999) 'Green revolution' genes encode mutant gibberellin response modulators. *Nature*, **400**, 256–261.

Péret, B., Swarup, K., Ferguson, A. *et al.* (2012) AUX/LAX genes encode a family of auxin influx transporters that perform distinct functions during Arabidopsis development. *Plant Cell*, **24**, 2874–2885.

Petrásek, J., Mravec, J., Bouchard, R. *et al.* (2006) PIN proteins perform a rate-limiting function in cellular auxin efflux. *Science*, **312**, 914–918.

Re, D.A., Capella, M., Bonaventure, G. and Chan, R.L. (2014) Arabidopsis AtHB7 and AtHB12 evolved divergently to fine tune processes associated with growth and responses to water stress. *BMC Plant Biol.* **14**, 150.

Reinhardt, D. and Kuhlemeier, C. (2002) Plant architecture. *EMBO Rep.* **3**, 846–851.

Richards, R.A. and Passioura, J.B. (1989) A breeding program to reduce the diameter of the major xylem vessel in the seminal roots of wheat and its effect on grain yield in rain-fed environments. *Aust. J. Agric. Res.* **40**, 943–950.

Sahlin, P., Söderberg, B. and Jönsson, H. (2009) Regulated transport as a mechanism for pattern generation: capabilities for phyllotaxis and beyond. *J. Theor. Biol.* **258**, 60–70.

van der Schuren, A., Voiniciuc, C., Bragg, J., Ljung, K., Vogel, J., Pauly, M. and Hardtke, C.S. (2018) Broad spectrum developmental role of Brachypodium AUX1. *New Phytol.* **219**, 1216–1223.

Swarup, R., Kramer, E.M., Perry, P., Knox, K., Leyser, H.M., Haseloff, J., Beemster, G.T., Bhalerao, R. and Bennett, M.J. (2005) Root gravitropism requires lateral root cap and epidermal cells for transport and response to a mobile auxin signal. *Nat. Cell Biol.* **7**, 1057–1065.

Swarup, K., Benková, E., Swarup, R. *et al.* (2008) The auxin influx carrier LAX3 promotes lateral root emergence. *Nat. Cell Biol.* **10**, 946–954.

Ulmasov, T., Hagen, G. and Guilfoyle, T.J. (1997a) ARF1, a transcription factor that binds to auxin response elements. *Science*, **276**, 1865–1868.

Ulmasov, T., Murfett, J., Hagen, G. and Guilfoyle, T.J. (1997b) Aux/IAA proteins repress expression of reporter genes containing natural and highly active synthetic auxin response elements. *Plant Cell*, **9**, 1963–1971.

Van de Mortel, J.E., Almar Villanueva, L., Schat, H., Kwekkeboom, J., Coughlan, S., Moerland, P.D., van Themaat, E.V.L., Koornneef, M. and Aarts, M.G.M. (2006) Large expression differences in genes for iron and zinc homeostasis, stress response, and lignin biosynthesis. Distinguish roots of *Arabidopsis thaliana* and the related metal hyperaccumulator *Thlaspi caerulescens*. *Plant Physiol.* **142**, 1127–1147.

Wang, J.L., Tang, M.Q., Chen, S. *et al.* (2017) Down-regulation of BnDA1, whose gene locus is associated with the seeds weight, improves the seeds weight and organ size in *Brassica napus*. *Plant Biotechnol. J.* **15**, 1024–1033.

Wolters, H. and Jurgens, G. (2009) Survival of the flexible: hormonal growth control and adaptation in plant development. *Nat. Rev. Genet.* **10**, 305–317.

Yang, Y.D., Hammes, U.Z., Taylor, C.G., Schachtman, D.P. and Nielsen, E. (2006) High-affinity auxin transport by the AUX1 influx carrier protein. *Curr. Biol.* **16**, 1123–1127.

Zhang, K., Bhuiya, M.W., Rencoret Pazo, J., Miao, Y., Kim, H., Ralph, J. and Liu, C.J. (2012) An engineered monolignol 4-O-methyltransferase depresses lignin biosynthesis and confers novel metabolic capability in Arabidopsis. *Plant Cell*, **24**, 3135–3152.

Zhao, L., Tan, L., Zhu, Z., Xiao, L., Xie, D. and Sun, C. (2015) PAY1 improves plant architecture and enhances grain yield in rice. *Plant J.* **83**, 528–536.