Coordination of auxin-triggered leaf initiation by tomato *LEAFLESS*

Yossi Capua[a,b] and Yuval Eshed[a,1]

[a]Department of Plant and Environmental Sciences, Weizmann Institute of Science, Rehovot, Israel 7610001; and [b]Faculty of Agriculture, The Hebrew University of Jerusalem, Rehovot, Israel 76100

Edited by Richard Scott Poethig, University of Pennsylvania, Philadelphia, PA, and approved February 14, 2017 (received for review October 17, 2016)

Lateral plant organs, particularly leaves, initiate at the flanks of the shoot apical meristem (SAM) following auxin maxima signals; however, little is known about the underlying mechanisms. Here, we show that tomato *leafless* (*lfs*) mutants fail to produce cotyledons and leaves and grow a naked pin while maintaining an active SAM. A similar phenotype was observed among pin-like shoots induced by polar auxin transport inhibitors such as 2,3,5-triiodobenzoic acid (TIBA). Both types of pin-like shoots showed reduced expression of primordia markers as well as abnormal auxin distribution, as evidenced by expression of the auxin reporters *pPIN1:PIN1:GFP* and *DR5:YFP*. Upon auxin microapplication, both *lfs* meristems and TIBA-pin apices activated *DR5:YFP* expression with similar kinetics; however, only *lfs* plants failed to concurrently initiate leaf primordia. We found that *LFS* encodes the single tomato ortholog of *Arabidopsis DORNRONSCHEN* (*DRN*) and *DRN-like* (*DRNL*) genes and is transiently expressed at incipient and young primordia, overlapping with auxin response maxima. *LFS* is rapidly induced by auxin application, implying feed-forward activity between *LFS* and auxin signals. However, driving *LFS* at auxin response maxima sites using the *DR5* promoter fails to fully rescue *lfs* plants, suggesting that additional, auxin-independent regulation is needed. Indeed, extended GCC-box elements upstream of *LFS* drove primordia-specific expression in a LFS-dependent but auxin-independent manner. We thus suggest that *LFS* transiently acts at the site of primordia initiation, where it provides a specific context to auxin response maxima culminating in leaf primordia initiation.

DRN/DRNL | extended GCC-box | lateral organ formation | SAM | TIBA pins

Lateral aerial plant organs form at the flanks of the shoot apical meristem (SAM) in a species-specific order termed phyllotaxis. Phyllotactic patterns tightly correlate with local maxima of the phytohormone auxin at the SAM periphery (1–3). These maxima are achieved by active transport (4). At these sites, auxin is required for leaf initiation, and when polar auxin transport is chemically impaired by polar auxin transport inhibitors (PATI), leaves fail to form (5–7), resulting in a pin-like shoot. When auxin is externally applied onto the meristem of these pins or to the meristem in a wild-type (WT) background, leaf primordia are initiated (5). Genetic analyses have also demonstrated that auxin response maxima, dictated by the activity of the auxin efflux carrier PINFORMED1 (PIN1), can be detected before, and are required for, leaflet initiation in compound leaves (6). However, similar evidence for leaf initiation is lacking (7).

Like other classical hormones, such as cytokinin (CK), florigen, and gibberellin, auxin has many functions, which are largely dictated by its levels and which vary within and among plants. In young leaf primordia, auxin triggers primordia bulging at P_0 by stimulating local cell divisions. At P_1 and P_2, auxin elicits provasculature development (8). Thus, in the same group of cells, auxin cues are differentially translated to cell division (P_0) or to differentiation (P_1) within a 1- to 2-d gap. The mechanisms directing such context-specific responses are poorly understood.

In this work, we investigate leaf initiation at the SAM periphery and show that the tomato *leafless* (*lfs*) mutant, which is disrupted in a single *DRN*/*DRNL* homolog, fails to initiate cotyledons, leaves, and leaflets. *LFS* expression largely overlapped with auxin response maxima during primordia initiation, yet exogenous auxin application did not stimulate leaf initiation when applied to bare *lfs* shoots. We suggest that LFS responds to auxin response maxima and sets the stage for the context-specific response that culminates in leaf initiation. The transient LFS expression, which is terminated shortly after leaf initiation, may prevent rapid differentiation by promoting, among others, CK signals.

Results

The *leafless* Mutants Produce Pin-Like Shoots with Active SAM. Five recessive *lfs* mutants (Fig. 1*A*) were identified in a screen for tomato plants that fail to properly produce leaves; complementation tests demonstrated that they were all allelic. The *lfs* seedlings lacked cotyledons, but after germination, a broad collar-like tissue surrounded the SAM, likely representing the vestigial cotyledons (Fig. 1*A* and Fig. S1*A*). As the seedlings matured, the miniature plant shoot appeared as an elongated pin. Although the meristems at the top of these pins featured a typical smooth and glossy appearance, the immediately adjacent stem tissue underwent rapid differentiation, as was inferred by the presence of large and dense trichomes just below the SAM dome (Fig. 1 *A*–*F*). The bare *lfs* plant SAMs occasionally formed one to three simple leaves, with zero to three leaflets and entire margins with abnormal phyllotaxis (Fig. 1*A*). These leaves hosted axillary buds that carried pin-like shoots in their axils; however, additional pin-like shoots also initiated from areas that corresponded to cryptic axillary buds (Fig. 1*A* and Fig. S1*A*). The naked meristems topping the elongated pin mutant shoots turned into a flower at about the same time as their WT siblings (Fig. S1*A*). The flowers were deformed, with sepals missing or small, petals nearly normal, stamens malformed and

Significance

Plant leaves form at the flanks of the shoot apical meristem in response to cues provided by the phytohormone auxin. Auxin signals determine the sites of leaf initiation and bulging, a process followed by gradual and ongoing differentiation of leaf tissues. We show here that the tomato ethylene response factor-type transcription factor *LEAFLESS* is induced by, and necessary for, auxin-triggered leaf initiation. These mechanisms provide a localized and transient developmental context for a specific morphogenetic output generated by general regulators such as auxin.

Author contributions: Y.C. and Y.E. designed research; Y.C. performed research; and Y.C. and Y.E. wrote the paper.

The authors declare no conflict of interest.

This article is a PNAS Direct Submission.

Data deposition: The expression dataset reported in this paper has been deposited in the Gene Expression Omnibus database (accession no. GSE93540).

[1]To whom correspondence should be addressed. Email: yuval.eshed@weizmann.ac.il.

This article contains supporting information online at www.pnas.org/lookup/suppl/doi:10.1073/pnas.1617146114/-/DCSupplemental.

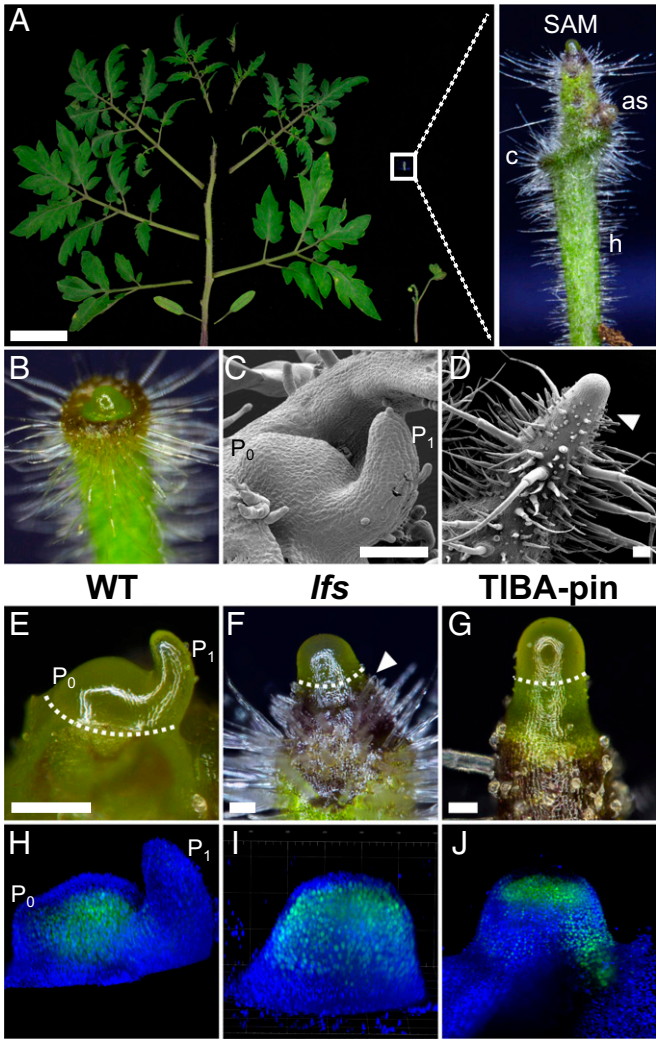

Fig. 1. *LFS* and auxin response maxima direct tomato leaf initiation. (*A*) A 5.5-wk-old WT (*Left*) and *lfs* (*Bottom Right*) tomato. (Scale bar: 5 cm.) (*Inset*) A close-up of the apex of a 15-d-old *lfs* seedling. as, axillary shoot; c, collar-like structure; h, hypocotyl. (*B*) A 9-d-old *lfs* seedling in which the SAM starts to protrude from the collar-like structure surrounding it. (*C* and *D*) SEM images of vegetative WT (*C*) and *lfs* (*D*) apices. (*E*) WT apical meristem bearing one incipient and one visible primordia (P_0 and P_1, respectively). (*F*) *lfs* meristem. (*G*) TIBA-induced pin. Dashed lines in *E*–*G* mark the apex tissue, sampled for gene expression analyses. Note the proximity of trichomes to the SAM in *D* and *F*, marked by the arrowhead. (*H*–*J*) Three-dimensional reconstructions of live *TCS:YFP* apices captured by a light sheet microscope. (*H*) WT. (*I*) *lfs*. (*J*) TIBA pin. (Scale bars in *C*–*G*: 100 μm.)

sterile and with two or more carpels, which rarely gave rise to parthenocarpic fruit (Fig. S1 *B* and *C*). Floral organs were often fused. All of these defects were similar in the five tested alleles. In the WT background, we could not differentiate *lfs*/+ plants from +/+ sibs.

Apices of TIBA-Induced and *lfs* Pins Do Not Express Primordia Markers. Naked pin-like apices can be induced to initiate leaves in tomato by foliar application of 2,3,5-triiodobenzoic acid (TIBA) (9). Such "TIBA-pins" can be obtained by different application regimes (0.1–1 mM, applied two to five times) and are used here as a reference system to assess and compare leaf initiation processes in *lfs* and in WT apices. Following repeated external application of TIBA on young tomato seedlings, plants underwent growth arrest. Soon after the last application, the apical meristem ceased to produce leaves, elongated, and produced a smooth, leaf-less naked pin, somewhat similar to the *lfs* pin (Fig. 1*G*). To determine whether the SAMs of the pin-like shoots retained their function, we monitored the expression of the two-component sensor (TCS) that marks cytokinin signaling (10). In WT, this reporter is expressed in the SAM proper, is excluded from the outermost two cell layers, and is weakly expressed in young leaf primordia (Fig. 1*H*). The same apical expression was maintained in *lfs* but was excluded from L1 in mutant plants (Fig. 1*I*). In TIBA pins, cytokinin expression was weaker and appeared deeper in the meristem, distributed in a lens-shaped pattern (Fig. 1*J*). To determine whether TIBA-treated and *lfs* tomato pins express young organ primordia genes, we examined the expression pattern of the primordia marker *FILAMENTOUS FLOWER* (*FIL*) (11). In WT, *pFIL>>NLS:RFP* signals were regularly detected at the meristem periphery, as leaf primordia initiated and progressed into later stages of leaf development (Fig. 2*A*). In *lfs* plants, we detected, albeit randomly, *pFIL>>RFP* expression at the meristem periphery. This expression was found at abnormal phyllotaxis and was associated with the rare primordia formation (Fig. 2*B*), whereas in TIBA pins, weak, uniform expression was observed around the meristem (Fig. 2*C*). To obtain a better perspective of primordia marker expression in naked pins, we compared the transcriptomes of apical *lfs* and TIBA-pin meristems to that of the WT plant (Fig. 1 *E*–*G*), using cDNA next-generation sequencing (NGS). The expression profiles of the two pins showed significant clustering and were substantially distinct from the WT profile (Fig. S1*D*). Of the ∼35,000 tomato genes, 256 showed fivefold higher expression levels in WT relative to *lfs* apices (Dataset S1, Table S1). These genes included several known primordia initiation and patterning markers (Table 1), such as six of the nine tomato *YABBY* genes (12), *ASYMMETRIC LEAVES2* (*AS2*) (13), *WOX1* (14), and *PETROSELINUM* (*PTS*) (15). The levels of these markers were

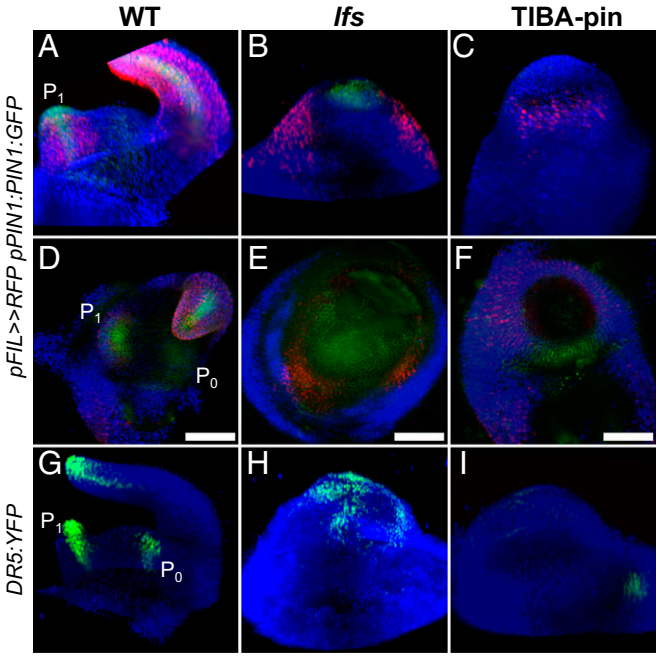

Fig. 2. Auxin distribution is impaired in *lfs* and in TIBA pins. (*A*–*F*) Expression profiles in *pFIL>>NLS:RFP pPIN1:PIN1:GFP* apices, as captured by a light sheet microscope. Similar analyses by confocal imaging are presented in Fig. S2. (*A*–*C*) Three-dimensional reconstructed projections. (*D*–*F*) Top view, obtained using maximum-intensity projection. (*G*–*I*) Three-dimensional reconstruction of *DR5:YFP* apices. Note that the patchy pattern in *lfs* (*H*) does not correspond with the discrete primordia. (Scale bars in *D*–*F*: 100 μm.)

Table 1. Leaf primordia marker expression in WT, *lfs*, and TIBA-pin apices

Gene	Annotation	WT*	*lfs**	TIBA pins*
Solyc03g044300	AP2	168	15	44
Solyc11g008830	AS2	175	0	0
Solyc03g006900	Crinckler	1,550	62	580
Solyc06g072480	PTS	3,516	76	243
Solyc06g069240	TCP18	140	1	16
Solyc03g118770	WOX1	130	4	4
Solyc01g091010	YABBY	7,743	39	174
Solyc06g073920	YABBY	413	8	17
Solyc07g008180	YABBY	1,708	53	68
Solyc08g079100	YABBY	3,674	6	12
Solyc11g071810	YABBY	217	9	22
Solyc12g009580	YABBY	189	20	254

*Expression after dseq normalization ($n = 2$).

similarly low in TIBA pins, suggesting that, much like auxin, *LFS* acts at a very early stage of primordia initiation.

Auxin Signaling Is Impaired in *lfs* Apices. A hallmark of primordia initiation is the preceding formation of auxin response maxima (3). Therefore, expression distribution of the auxin markers *DR5:YFP* and *pPIN1:PIN1:GFP* in WT, *lfs*, and TIBA pins was examined (Fig. 2 and Fig. S2). In WT, expression of the *DR5:YFP* marker was clearly observed at the incipient primordia (P_0), as well as in distinct foci at the tips of the young leaf and leaflet primordia. In each primordium, the expression domain looked like an inverted cone, with its wide end at the tip of the primordia and the narrow region close to its base (Fig. 2*G* and Movie S1). In both *lfs* and TIBA pins, expression was abnormal; in *lfs* apices, expression was recorded in a large, dispersed, nonuniform patch over the meristem (Fig. 2*H*). Marker expression size, shape, and location varied between individuals and sampling times and did not seem to correspond with the rarely formed primordia. In contrast, *DR5:YFP* expression in the TIBA pins was very weak (Fig. 2*I*) and, when detected, seemed to uniformly cover the meristem (Fig. S2*F*), indicating weak residual auxin activity. WT *pPIN1:PIN1:GFP* expression was seen in the membranes of all of the cells of the meristem epidermis, but was enriched at the sites of the incipient and young primordia (Fig. 2 *A* and *D*). In contrast, in both *lfs* and TIBA pins, expression was overall weaker and lacked a discrete maxima (Fig. 2 *B*, *C*, *E*, and *F*). Taken together, the two sources of pin-like apices showed a similar absence of primordia markers, including auxin response maxima.

***LFS*, but Not Auxin, Inhibits Differentiation at the Apex Periphery.** The two types of pin-like shoots failed to initiate leaf primordia. However, their stems, just below the SAM dome, were markedly different; the stem of the naked TIBA pin was glossy and lacked trichomes, whereas many trichomes covered the basal domain of the *lfs* apex (Fig. 1 *D* and *F*, arrowheads). In WT, all stem parts just below the apex lacked trichomes (Fig. 1*C*), suggesting that the *lfs* apex periphery undergoes rapid differentiation. To identify processes associated with this precocious differentiation, we searched for genes that were modified (more than twofold) in *lfs* apices but were not or only mildly modified in TIBA pins (Dataset S1, Table S2). Of 909 genes matching this criterion, 13 are involved in different aspects of CK signaling (Dataset S1, Table S2, highlighted in yellow), including three *CYTOKININ OXIDASEs* (*CKXs*) and five *type A RESPONSE REGULATOR* (*RR*). Other members of these gene groups showed similar trends (Dataset S1, Table S3), indicating reduced CK levels and responses in *lfs* apices. Thus, rapid differentiation of *lfs* compared with TIBA pins can be attributed, at least partly, to altered CK signaling.

***lfs* Meristems Fail to Initiate Leaf Primordia Following Auxin Microapplication.** Previous studies have demonstrated that microapplication of auxin is sufficient to initiate leaf primordia outgrowths in PATI-induced pins (5, 16). The loss of normal expression of auxin signaling and transport markers may indicate loss of auxin sensitivity in *lfs* or failure to respond to auxin. To test whether auxin could still initiate leaf primordia in *lfs* apices, a lanolin paste with 10–100 mM indoleacetic acid (IAA) was applied to its apices. IAA-treated *lfs* pins did not generate any leaf primordia even after 2 wk and seemed morphologically unaffected by the treatment. In contrast, in TIBA pins, leaf primordia initiated at and beyond the application site, typically surrounding the meristem like a sleeve (Fig. 3 *A* and *B*). Despite the morphological differences, *DR5* expression markedly increased and displayed similar dynamics (Fig. S3 *A*–*C*) in both *lfs DR5:YFP* plants and TIBA-pin controls following auxin treatment (Fig. 3 *C*–*F*). Furthermore, application of 20 mM IAA to the hypocotyl of 10-d-old plants induced bending in *lfs* seedlings, as it did in WT plants (14/14 and 16/16 plants, respectively; Fig. 3*G*), demonstrating that the *lfs* mutant is still auxin-sensitive. To test if inhibited auxin signaling at the organ primordia can indeed inhibit leaf initiation, proteolysis-resistant *AUX/IAA* genes were expressed at the primordia domain under the *FIL* promoter. To this end, IAA7 and IAA26, two genes up-regulated in *lfs*, and ENTIRE/IAA9, a gene that is not up-regulated (Dataset S1, Table S4, highlighted), were mutated at their proteolysis-defining domain, DII (17, 18). Transactivation of the three genes with the *pFIL* driver resulted in various degrees of primordial arrest, peaking at formation of pin-like shoots (Fig. 3*H* and Fig. S3 *D* and *E*). Primordial expression of the same mutated genes in *Arabidopsis* under the *pANT* promoter also inhibited leaf formation, resulting, in extreme cases, in pin-like shoots (Fig. S3 *F* and *G*).

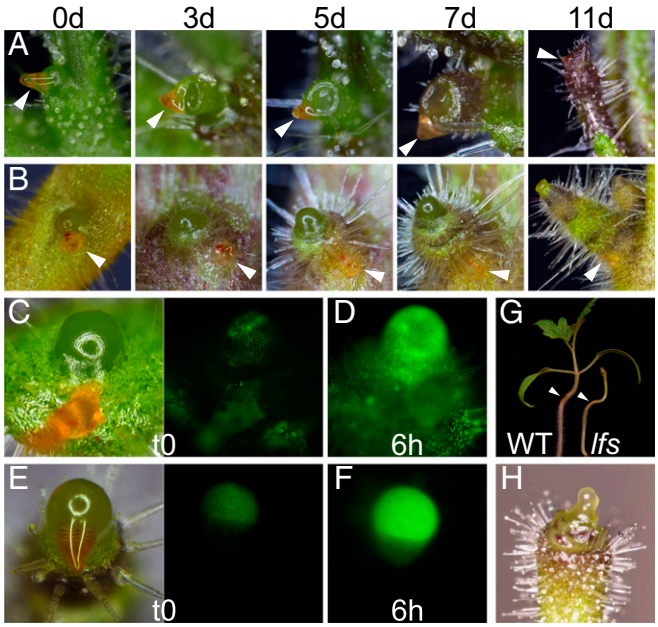

Fig. 3. Auxin-induced leaf formation is *LFS*-dependent. (*A*) TIBA pin. (*B*) *lfs* at the indicated time points after microapplication of 20 mM IAA. Arrowheads show the site of application (orange paste). (*C*–*F*) Response of *DR5:YFP* plants to 50 mM IAA, 6 h after application. (*C* and *D*) TIBA pins, imaged at 300 ms exposure. (*E* and *F*) *lfs* meristems imaged at 1,500 ms exposure. (*G*) WT and *lfs* plant bending in response to application of 20 mM IAA on the hypocotyl (marked by arrowheads); images were taken 2 d after application. (*H*) A *pFIL>>IAA26-mDII* seedling with no cotyledons and rudimentary leaves.

LFS Is the Single Tomato Ortholog of the _Arabidopsis DRN_ and _DRNL_ Genes. To determine which gene encodes *LFS*, a mapping population of 214 F_2 plants from a cross between *Solanum pimpinellifolium* and an M82 line heterozygous for *lfs* was used. Informative recombinants mapped the gene to a 440-kbp region on chromosome 5. The region included a candidate gene, *Solyc05g013540*, which was sequenced from available mutant alleles. Two *lfs* alleles, *e2187* and *e1710*, had single-base-pair changes that caused an early nonsense mutation and a missense mutation within the conserved AP2 domain of the protein, respectively (Fig. 4*A*). In three other alleles, amplification of the genomic region of *Solyc05g013540* failed, most likely due to genomic rearrangements resulting from their generation using fast neutrons. These data, combined with the partial complementation of *lfs* obtained by expressing its WT cDNA under its endogenous promoter (Fig. S4), indicate that *Solyc05g013540* encodes *LFS*.

LFS encodes for a single AP2-domain transcription factor, a member of the large ethylene response factor (ERF) family. Cladistic analysis (Fig. 4*B* and *SI Appendix*) showed that LFS is the single tomato ortholog of the *Arabidopsis* DORNRONSCHEN (DRN) and DRN-like (DRNL), showing a closer relation to DRNL than to DRN. PUCHI, the closest family member among all ERFs, clustered as an outgroup. In *Arabidopsis*, *drn-1* mutants develop normally, but exhibit a low incidence of defects in cotyledon development (19). *drnl-2* plants exhibit a similarly low frequency of cotyledon defects, as well as impaired anther development (20). Progenies of *drn-1 drnl-2*/+ parents featured a short hypocotyl, topped by a bare tip, surrounded by what may be vestigial cotyledons (Fig. 4*C*). However, unlike in tomato (Fig. 4*D*), these plants did not support a viable SAM throughout their lives, and thus organ initiation could not be assessed. Notably, when the weaker allele *drnl-1* is used to generate double-mutant *drn drnl* plants, their phenotype is much weaker and most plants have rather normal shoots (19, 21). Both tomato *lfs* and *Arabidopsis drn drnl* seeds had a short shelf life, and their germination rate decreased with time, leading to non-Mendelian segregation ratios. Ectopic expression of either *LFS* or *DRNL* under the *FIL* promoter in tomato caused epinasty in cotyledons and leaves (Fig. S4*D*), similar to the effects of *DRNL* overexpression in tobacco (22). These results suggest that at the protein level the tomato and *Arabidopsis* gene products have similar potentials. Like *LFS*, *DRN* and *DRNL* have also been implicated in cotyledon initiation (23, 24) and in auxin signaling (25).

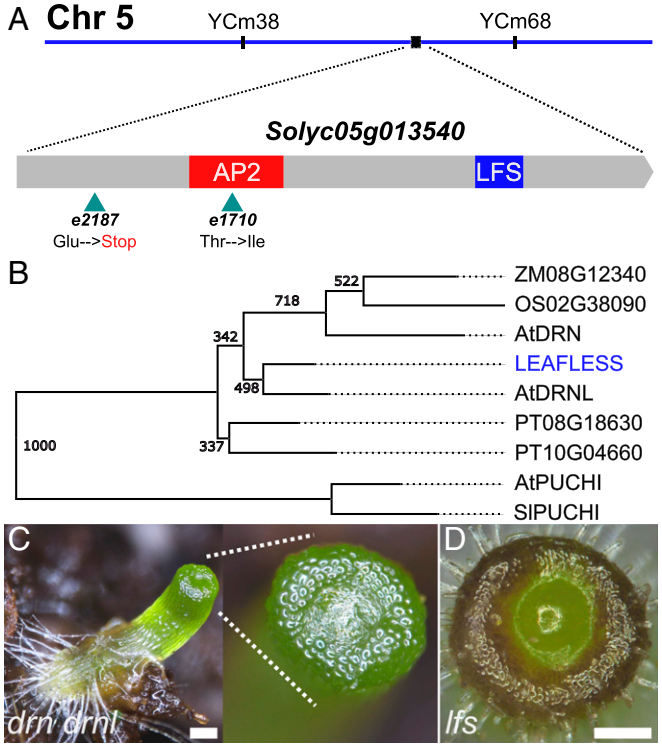

Fig. 4. *LFS* is the single tomato ortholog of *DRN* and *DRNL*. (*A*) Recombination-based mapping placed *LFS* between the PCR markers YCm34 and YCm68 (Dataset S1, Table S5). Two identified lesions in *Solyc05g013540* are indicated by arrowheads. The AP2 box and a LFS-specific box are color-coded (*SI Appendix*). (*B*) Phylogenetic relationship of closest LFS homologs in select mono- and dicotyledonous plants. Nodes show bootstrap values. (*C*) *Arabidopsis drn-1 drnl-2* whole seedling. (*Inset*) The SAM. (*D*) Top view of a tomato *lfs* plant. (Scale bars in *C* and *D*: 250 μM.)

LFS Expression Is Transient in the Incipient Leaf Primordia and Overlaps with _DR5_ Expression. To assess the role of *LFS* in leaf initiation, its expression pattern in the shoot apex was characterized by RNA in situ hybridization (Fig. 5 *A* and *B*). *LFS* mRNA was first detected at primordia anlagen (P_0), several cell layers below the epidermis. In P_1 primordia, it was expressed only in the cell layer beneath the epidermis. Later, its expression was seen at the tips of $P_{1–2}$ leaf primordia, at sites of initiating leaflet primordia, as well as in axillary buds. At all stages, the expression domain had an inverted cone shape, which was similar to that of the *DR5* expression domain (Figs. 2*G* and 5*C* and Movie S1). Detailed comparison of the two expression domains, performed using serial sections (Fig. S5 *A* and *B*), demonstrated a significant overlap between the *LFS* and *DR5* expression domains, with both showing early, subepidermal expression at the P_0 primordia, although the *DR5 expression* domain was slightly broader and included one to two outer cell layers. The spatial and temporal *LFS* expression pattern was congruent with reports in *Arabidopsis* (22) and maize (26), where expression of *LFS* orthologs precedes the formation of newly formed leaves (P_0) and is transiently maintained until primordia initiation ($P_{1–2}$).

LFS Expression Is Induced by Auxin. To understand the relations between primordia formation, *LFS* expression, and auxin signaling, we examined *LFS* RNA distribution in the absence of primordia. In *lfs* apices, a large patch of *LFS* expression was evident (Fig. 5*D*), similar to the expression of *DR5-YFP* in *lfs* apices (Fig. 2*G*), and differing from the defined domains observed in a typical phyllotaxis. In TIBA pins, *LFS* was expressed in a weak ring-like pattern, primarily at the L2 layer of the two to three most distal apex cells (Fig. 5*E*). In agreement, *LFS* expression levels, as determined by NGS, were lower in TIBA pins compared with *lfs* apices (TIBA pins: 460; WT: 709; *lfs*: 3,459) (Dataset S1, Table S2), further demonstrating the differences between the two kinds of pins and suggesting complex control on *LFS* expression by polar auxin transport and by itself.

To characterize the temporal hierarchy between *LFS* and auxin signaling, we compared the dynamics of *LFS* and *DR5:YFP* activation following microapplication of IAA onto TIBA pins (Fig. 5 *F–I*), as measured by quantitative RT-PCR (qRT-PCR). Up-regulation of both genes began after 1 h and continued to increase at a similar rate, albeit at a two orders of magnitude difference, for at least 6 h (Fig. 5*F*). Indeed, 6 h after IAA application, strong expression of *LFS* RNA was detected at the central region of the pin, adjacent to one of the sides, likely where IAA was applied (Fig. 5*H*). Similarly, *DR5:YFP* TIBA pins showed strong *YFP* RNA expression in the entire meristem in response to IAA (Fig. 5*I*), consistent with the fluorescent signals (Fig. 3 *E* and *F* and Fig. S3 *A–C*). These results suggest that auxin response maxima at incipient leaf primordia promote *LFS* expression, which then subsequently facilitates auxin-induced primordia initiation.

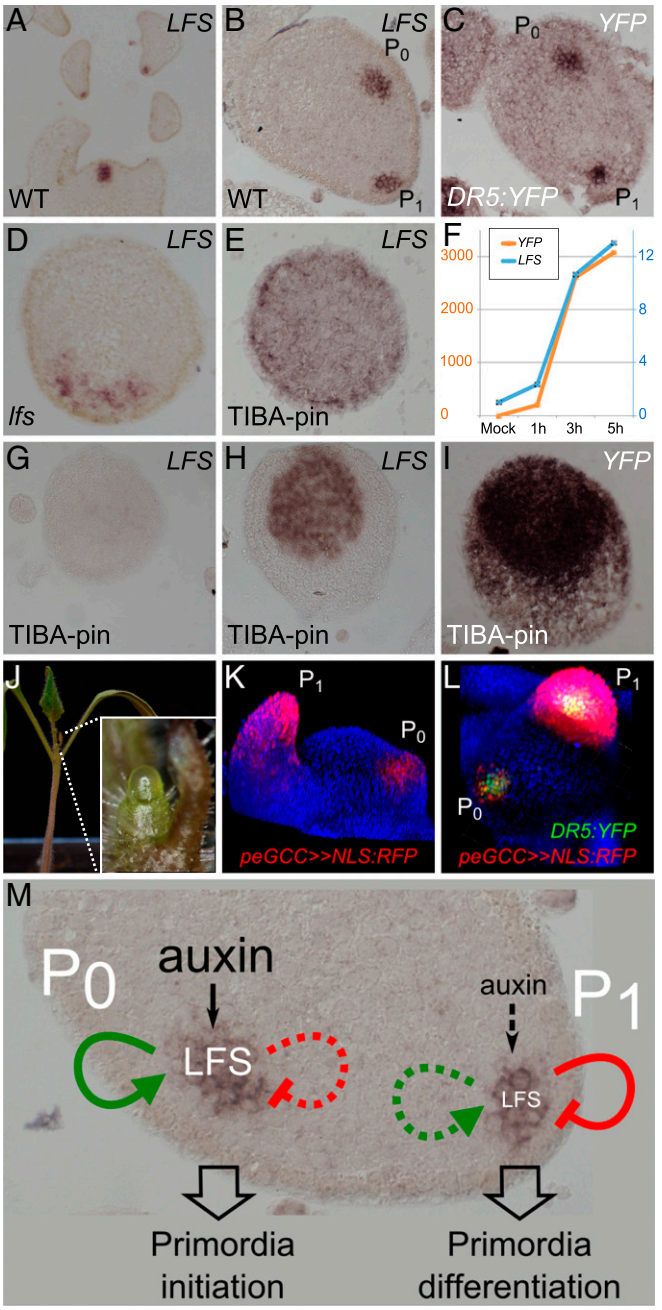

Fig. 5. Complex regulation of *LFS* expression in leaf primordia. (*A–E*) In situ hybridization of *LFS* or *YFP* probes on WT, *DR5:YFP*, and *lfs* apices (genotypes are indicated at the *Bottom Left* of each panel; probes are at the *Top Right*). All sections were stained overnight, except *E*, which was stained for 4 d. (*F*) Relative quantification of *LFS* and *YFP* expression in response to auxin microapplication to TIBA pins. (*G–I*) In situ hybridization of *LFS* or *YFP* probes on *DR5:YFP* TIBA pins in response to microapplication of 20 mM IAA at t0 (*G*) and after 6 h (*H* and *I*). Probes in *G–I* were stained for 16 h. (*J*) A *lfs pDR5>>LFS* plant. (*Inset*) The pin-like apex. (*K*) Tomato apex with *peGCC>>NLS:RFP* marking young leaf primordia. (*L*) *peGCC>>NLS:RFP DR5:YFP*, viewed from the top. (*M*) A model for transient LFS action. Green arrows indicate GCC-dependent activation, and red arrows indicate GCC-independent inhibition.

Extended GCC-Box Elements Are Sufficient for Primordia Expression. If auxin is the primary driver of LFS expression, we set out to assess whether *lfs* can be rescued by LFS expression driven by a *DR5* promoter. *pDR5>>LFS lfs* plants showed only partial rescue of the *lfs* defects, as manifested by normal cotyledons and simpler first two leaves than usual, alongside a meristem that ceased to generate leaves and eventually flowered on top of an elongated pin (Fig. 5*J*). We thus searched for additional elements that could regulate *LFS* expression.

DRN can positively regulate a reporter comprising GCC-box elements (27), and *DRNL* positively regulates *STY* gene expression through GCC-box elements in their promoters (28). The upstream regions of both *LFS* and *DRNL* include conserved and extended GCC boxes (eGCC; 25 and 3.1 kbp upstream of *LFS* and 845 bp upstream of *DRNL*), suggesting that both genes may control their own expression. A synthetic *peGCC* promoter, comprising six repeats of the extended GCC boxes of *LFS* and *DRNL* promoter regions (Fig. S5*C* and Dataset S1, Table S5), effectively drove *NLS:RFP* expression in incipient and young leaf primordia (Fig. 5 *K* and *L*). Unlike *DR5:YFP*, the *peGCC* reporter did not show expression levels above those measured in the background in *lfs* apices (Fig. S5*D*), suggesting that it is positively regulated by LFS. In TIBA pins, too, the reporter was not detected (Fig. S5*E*), suggesting that low LFS levels in these pins (Fig. 5 *E* and *G*) were insufficient to trigger its expression. The high *LFS* expression levels in *lfs* compared with WT (3,459 and 709 normalized counts, respectively; Dataset S1, Table S2) suggest that other mechanisms contribute to *LFS* down-regulation after the initial activation, and these are likely to be eGCC-independent (Fig. 5*M*).

Discussion

The tight association between auxin expression maxima and initiating primordia and restoration of primordia formation in both PATI-treated shoots and mutants lacking auxin transport components, such as *pin* and *pid* mutants (2), by external application of auxin, implicates the role of auxin in primordia initiation. Mathematical models have further supported the suggested role of auxin maxima, dictated by auxin transporter activity, as a mechanism for achieving ordered primordia initiation patterns (3). But how auxin, a broad-acting hormone, specifies primordia cells, has remained an enigma. The results of this and previous studies (3, 5, 21) provide a framework (Fig. 5*M*) for understanding the specification process. Briefly, we suggest that auxin response maxima define the primordia initiation site and activate *LFS* expression. LFS then licenses incipient primordia cells to divide and bulge in response to auxin signals, while inhibiting differentiation of the bulging primordia and the underneath stem tissue. Later down-regulation of LFS in the expanding primordia allows for spatially defined differentiation of the various leaf domains, for example, auxin-induced provasculature formation.

Auxin acts at the SAM periphery by removing AUX/IAA suppressors that inhibit factors such as *MONOPTEROS* (29). In PATI-induced pins, auxin-induced primordia initiation is not restricted to the radial dimension of the meristem but rather only to a certain distance from the SAM summit (5). Thus, a ring domain surrounding the meristem periphery is adequate for auxin-induced primordia initiation and coincides with the LFS-expressing domain in TIBA-induced tomato pins (Fig. 5*E*). Of note, although auxin failed to stimulate primordia initiation in *lfs* (Fig. 3*B*) and auxin transport and distribution were abnormal (Fig. 2), the hormone still successfully induced molecular responses at the shoot apex as well as shoot bending at the hypocotyl (Fig. 3 *C–G*).

Sassi et al. (30) showed that chemical or genetic perturbation of cortical microtubules restores some primordia initiation in *pin1* mutants as well as in naphtylphtalamic acid (NPA)-treated *Arabidopsis* apices, suggesting that auxin is not the only factor directing organ formation and that, although it is sufficient for initiation, it might not be necessary. Indeed, in *Arabidopsis pin1* and *pid* mutants, abolishment of lateral organ initiation is restricted to the bolting stem, whereas rosette leaves develop quite normally (4, 7, 31), questioning the unique contribution of auxin regulatory mechanisms to primordia initiation. Similarly, when

flowers are formed on *lfs* shoots, they produce nearly all organs. Thus, additional mechanisms may contribute to primordia initiation, even if these are not required by theoretical models.

We suggest that primordia initiation is induced by auxin maxima and requires LFS activity, which both enhances and later moderates auxin responses. *LFS* expression peaks at incipient primordia, where it allows auxin to specify leaf initiation while transiently preventing differentiation. LFS positively regulates itself, presumably through an eGCC-box element that can largely mimic the primordia-specific LFS expression. However, LFS also negatively regulates itself through a yet-unknown mechanism, as evident by the high *LFS* transcript levels in *lfs* meristems. Such transient expression is common in plants with diverse leaves, such as *Arabidopsis*, tomato, and maize (20, 26), suggesting that LFS defines a unique step in the conversion of SAM cells into an organ. Genetically, LFS promotes CK biosynthesis/signaling (Dataset S1, Table S2), thereby maintaining a hormonal balance at the meristem periphery that delays differentiation. This is consistent with activities of DRN and DRNL in tissue culture assays, the overexpression of which can substitute for cytokinin supplementation in shoot regeneration assay (23). As leaves are believed to evolve from branched meristem systems (reviewed in ref. 32), LFS expression may define a SAM program change and not necessarily activation of a leaf program.

Materials and Methods

Light-sheet imaging was carried out using a Zeiss Z1 microscope and processed by ZEN and ARIVIS. Apices were dissected under water and embedded in 1.5% low-melting-point agarose column before imaging. Detailed information on plant materials, imaging methods, RNA expression analyses, and cladistics is described in *SI Materials and Methods*.

ACKNOWLEDGMENTS. We thank Naomi Ori, John Alvarez, Idan Efroni, and Chihiro Furumizu for their helpful discussions; members of the Y.E. laboratory for their valuable input; and John Chandler, Hadas Ben-Gera, Chris Kuhlemeier, and Marcus Heisler for materials. We also acknowledge the technical assistance of the following Weizmann Institute units and staff: The Electron Microscopy Unit, Yoseph Addadi from the de Picciotto-Lesser Cell Observatory of the Moross Integrated Cancer Center, Gilgi Friedlander from the Israel National Center for Personalized Medicine, and Ziva Amsellem and Shilo Rosenwasser. This work was supported by Israel Science Foundation Research Grants 1552/13 and 1788/14.

1. Heisler MG, et al. (2005) Patterns of auxin transport and gene expression during primordium development revealed by live imaging of the Arabidopsis inflorescence meristem. *Curr Biol* 15(21):1899–1911.
2. Reinhardt D, et al. (2003) Regulation of phyllotaxis by polar auxin transport. *Nature* 426(6964):255–260.
3. Kuhlemeier C (2007) Phyllotaxis. *Trends Plant Sci* 12(4):143–150.
4. Okada K, Ueda J, Komaki MK, Bell CJ, Shimura Y (1991) Requirement of the auxin polar transport system in early stages of Arabidopsis floral bud formation. *Plant Cell* 3(7):677–684.
5. Reinhardt D, Mandel T, Kuhlemeier C (2000) Auxin regulates the initiation and radial position of plant lateral organs. *Plant Cell* 12(4):507–518.
6. Barkoulas M, Hay A, Kougioumoutzi E, Tsiantis M (2008) A developmental framework for dissected leaf formation in the Arabidopsis relative Cardamine hirsuta. *Nat Genet* 40(9):1136–1141.
7. Guenot B, et al. (2012) Pin1-independent leaf initiation in Arabidopsis. *Plant Physiol* 159(4):1501–1510.
8. Scarpella E, Barkoulas M, Tsiantis M (2010) Control of leaf and vein development by auxin. *Cold Spring Harb Perspect Biol* 2(1):a001511.
9. Gorter C (1949) The influence of 2, 3, 5-triiodobenzoic acid on the growing points of tomatoes. *Proc Kon Ned Akad Wet*:1185–1193.
10. Steiner E, et al. (2016) The putative O-linked N-acetylglucosamine transferase SPINDLY inhibits class I TCP proteolysis to promote sensitivity to cytokinin. *Plant Physiol* 171(2):1485–1494.
11. Lifschitz E, et al. (2006) The tomato FT ortholog triggers systemic signals that regulate growth and flowering and substitute for diverse environmental stimuli. *Proc Natl Acad Sci USA* 103(16):6398–6403.
12. Sarojam R, et al. (2010) Differentiating Arabidopsis shoots from leaves by combined YABBY activities. *Plant Cell* 22(7):2113–2130.
13. Iwakawa H, et al. (2002) The ASYMMETRIC LEAVES2 gene of Arabidopsis thaliana, required for formation of a symmetric flat leaf lamina, encodes a member of a novel family of proteins characterized by cysteine repeats and a leucine zipper. *Plant Cell Physiol* 43(5):467–478.
14. Nakata M, et al. (2012) Roles of the middle domain-specific WUSCHEL-RELATED HOMEOBOX genes in early development of leaves in Arabidopsis. *Plant Cell* 24(2): 519–535.
15. Kimura S, Koenig D, Kang J, Yoong FY, Sinha N (2008) Natural variation in leaf morphology results from mutation of a novel KNOX gene. *Curr Biol* 18(9):672–677.
16. Stieger PA, Reinhardt D, Kuhlemeier C (2002) The auxin influx carrier is essential for correct leaf positioning. *Plant J* 32(4):509–517.
17. Ramos JA, Zenser N, Leyser O, Callis J (2001) Rapid degradation of auxin/indoleacetic acid proteins requires conserved amino acids of domain II and is proteasome dependent. *Plant Cell* 13(10):2349–2360.
18. Ben-Gera H, et al. (2012) ENTIRE and GOBLET promote leaflet development in tomato by modulating auxin response. *Plant J* 70(6):903–915.
19. Chandler JW, Cole M, Flier A, Grewe B, Werr W (2007) The AP2 transcription factors DORNROSCHEN and DORNROSCHEN-LIKE redundantly control Arabidopsis embryo patterning via interaction with PHAVOLUTA. *Development* 134(9):1653–1662.
20. Nag A, Yang Y, Jack T (2007) DORNROSCHEN-LIKE, an AP2 gene, is necessary for stamen emergence in Arabidopsis. *Plant Mol Biol* 65(3):219–232.
21. Chandler JW, Jacobs B, Cole M, Comelli P, Werr W (2011) DORNRÖSCHEN-LIKE expression marks Arabidopsis floral organ founder cells and precedes auxin response maxima. *Plant Mol Biol* 76(1-2):171–185.
22. Marsch-Martinez N, et al. (2006) BOLITA, an Arabidopsis AP2/ERF-like transcription factor that affects cell expansion and proliferation/differentiation pathways. *Plant Mol Biol* 62(6):825–843.
23. Ikeda Y, Banno H, Niu Q-W, Howell SH, Chua N-H (2006) The ENHANCER OF SHOOT REGENERATION 2 gene in Arabidopsis regulates CUP-SHAPED COTYLEDON 1 at the transcriptional level and controls cotyledon development. *Plant Cell Physiol* 47(11): 1443–1456.
24. Chandler JW, Cole M, Jacobs B, Comelli P, Werr W (2011) Genetic integration of DORNRÖSCHEN and DORNRÖSCHEN-LIKE reveals hierarchical interactions in auxin signalling and patterning of the Arabidopsis apical embryo. *Plant Mol Biol* 75(3): 223–236.
25. Cole M, et al. (2009) DORNROSCHEN is a direct target of the auxin response factor MONOPTEROS in the Arabidopsis embryo. *Development* 136(10):1643–1651.
26. Zimmermann R, Werr W (2007) Transcription of the putative maize orthologue of the Arabidopsis DORNROSCHEN gene marks early asymmetry in the proembryo and during leaf initiation in the shoot apical meristem. *Gene Expr Patterns* 7(1-2):158–164.
27. Matsuo N, Banno H (2008) The Arabidopsis transcription factor ESR1 induces in vitro shoot regeneration through transcriptional activation. *Plant Physiol Biochem* 46(12): 1045–1050.
28. Eklund DM, et al. (2011) Expression of Arabidopsis SHORT INTERNODES/STYLISH family genes in auxin biosynthesis zones of aerial organs is dependent on a GCC box-like regulatory element. *Plant Physiol* 157(4):2069–2080.
29. Chandler JW, Werr W (2015) Cytokinin-auxin crosstalk in cell type specification. *Trends Plant Sci* 20(5):291–300.
30. Sassi M, et al. (2014) An auxin-mediated shift toward growth isotropy promotes organ formation at the shoot meristem in Arabidopsis. *Curr Biol* 24(19):2335–2342.
31. Christensen SK, Dagenais N, Chory J, Weigel D (2000) Regulation of auxin response by the protein kinase PINOID. *Cell* 100(4):469–478.
32. Kenrick P, Crane PR (1997) *The Origin and Early Diversification of Land Plants: A Cladistic Study* (Smithsonian Institution Press, Washington, DC).
33. Menda N, Semel Y, Peled D, Eshed Y, Zamir D (2004) In silico screening of a saturated mutation library of tomato. *Plant J* 38(5):861–872.
34. Park SJ, Jiang K, Schatz MC, Lippman ZB (2012) Rate of meristem maturation determines inflorescence architecture in tomato. *Proc Natl Acad Sci USA* 109(2):639–644.
35. Edgar R, Domrachev M, Lash AE (2002) Gene Expression Omnibus: NCBI gene expression and hybridization array data repository. *Nucleic Acids Res* 30(1):207–210.
36. Thompson JD, Gibson TJ, Plewniak F, Jeanmougin F, Higgins DG (1997) The CLUSTAL_X windows interface: Flexible strategies for multiple sequence alignment aided by quality analysis tools. *Nucleic Acids Res* 25(24):4876–4882.
37. Bailey TL, et al. (2009) MEME SUITE: Tools for motif discovery and searching. *Nucleic Acids Res* 37(Web Server issue):W202–W208.