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The Gibberellin Pathway Mediates KNOTTED1-Type Homeobox Function in Plants with Different Body Plans

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Summary

Background: The shoot apical meristem (SAM) is an indeterminate structure that gives rise to the aerial parts of higher plants. Leaves arise from the differentiation of cells at the flanks of the SAM. Current evidence suggests that the precise regulation of KNOTTED1-like homeobox (KNOX) transcription factors is central to the acquisition of leaf versus meristem identity in a wide spectrum of plant species. Factors required to repress *KNOX* gene expression in leaves have recently been identified. Additional factors such as the CHD3 chromatin remodeling factor PICKLE (PKL) act to restrict meristematic activity in *Arabidopsis* leaves without repressing *KNOX* gene expression. Less is known regarding downstream targets of KNOX function. Recent evidence, however, has suggested that growth regulators may mediate KNOX activity in a variety of plant species.

Results: Here we show that reduced activity of the gibberellin (GA) growth regulator pathway promotes meristematic activity, both in the natural context of KNOX function in the SAM and upon ectopic KNOX expression in *Arabidopsis* leaves. We show that constitutive signaling through the GA pathway is detrimental to meristem maintenance. Furthermore, we provide evidence that one of the functions of the KNOX protein SHOOTMERISTEMLESS (STM) is to exclude transcription of the GA-biosynthesis gene *AtGA20ox1* from the SAM. We also demonstrate that *AtGA20ox1* transcript is reduced in the *pkl* mutant in a KNOX-independent manner. Moreover, we show a similar interaction between KNOX proteins and GA-biosynthesis gene expression in the tomato leaf and implicate this interaction in regulation of the dissected leaf form.

Conclusions: We suggest that repression of GA activity by KNOX transcription factors is a key component of meristem function. Transfer of the KNOX/GA regulatory module from the meristem to the leaf may have contributed to the generation of the diverse leaf morphologies observed in higher plants.

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Introduction

In *Arabidopsis*, two *KNOX* genes, *STM* and *KNAT1* (*KNOTTED1-LIKE* in *ARABIDOPSIS THALIANA1*), are expressed in partially overlapping domains of the SAM. Transcripts for both genes, however, are excluded from incipient leaf primordia, leading to the hypothesis that KNOX gene products are required for meristem function, whereas their absence is required for leaf initiation [1, 2]. Genetic evidence supports this hypothesis; loss-of-function mutations in *STM* result in a failure to initiate or maintain a SAM [2]. Double mutant analysis between weak *stm* alleles and the *KNAT1* loss of function mutation *brevipedicellus* (*bp*) demonstrates that *KNAT1* assumes a redundant role with *STM* in the SAM [3]. Conversely, ectopic expression of *KNAT1* results in the formation of meristems on leaves and the alteration of leaf shape [1, 4]. Ectopic expression of *STM* results in meristem formation on the adaxial surface of the cotyledons and growth arrest [5].

Recent data suggest that certain *KNOX* misexpression phenotypes may be mediated by growth regulators. Ectopic expression of *KNOX* genes from a number of species results in reduced GA levels [6, 7]. In tobacco the KNOX protein NTH15 directly represses transcription of *Ntc12*, a gene encoding a GA 20-oxidase required for GA biosynthesis [8]. Also, plants with increased cytokinin levels are partially phenocopied by *KNOX* misexpression, and cytokinin levels increase in response to regulated misexpression of the maize *KNOX* gene *KN1* in tobacco leaves [9–11].

In contrast to the simple leaves of species such as *Arabidopsis* and tobacco, the dissected leaves of tomato plants express *KNOX* genes. This expression pattern, combined with the increased leaf dissection obtained by overexpression of *KNOX* genes in tomato, has led to the suggestion that differential regulation of *KNOX* genes is involved in the generation of dissected leaf morphology [12, 13]. This idea has been reinforced by a recent study demonstrating that *KNOX* expression early in leaf development correlates with the generation of a complex leaf body plan across a broad spectrum of vascular plants [14].

Here we show that a reduction in GA signaling or biosynthesis promotes *KNOX*-dependent ectopic meristematic activity in *Arabidopsis* leaves. Conversely, elevated GA signaling antagonizes *KNOX* function both upon ectopic expression in leaves and in the natural domain of *KNOX* expression in the SAM. We also dem-

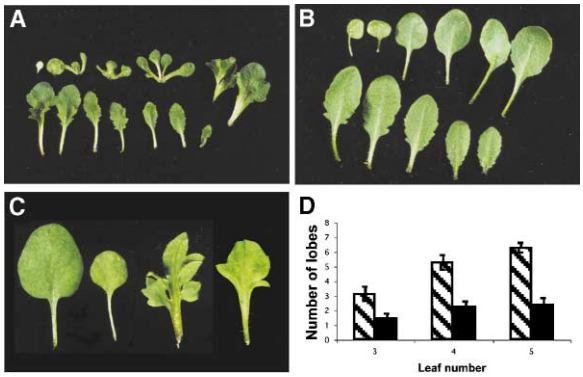

Figure 1. GA Suppresses *KNOX* Misexpression Phenotypes

35S:KN1-GR plants after (A) application of DEX and (B) coapplication of DEX and GA. Treatments were applied when the first leaf pair was visible. (C) Leaves representing F3 progeny of a cross between *35S:KNAT1* and *spy-1*. From left to right: Wild-type, *spy-1*, *35S:KNAT1*, and *spy-1;35S:KNAT1*. (D) The number of lobes per leaf is decreased in *spy-1;35S:KNAT1* (black bars) compared with *35S:KNAT1* (striped bars). The leaf number refers to the order of leaf initiation, and error bars represent standard error.

onstrate that KNOX transcription factors repress expression of the GA-biosynthesis gene *AtGA20ox1* and thus promotes a low GA regime favorable for meristematic activity. Additionally, we show that the PICKLE (PKL) gene product [15, 16] acts independently of KNOX transcription factors to regulate *AtGA20ox1* expression. Thus, regulation of GA biosynthesis emerges as a focal point for regulation of determinate versus indeterminate cell fate in the *Arabidopsis* shoot.

We also show that *KNOX* overexpression represses *LeGA20ox1* transcription in the tomato leaf and, conversely, that exogenous GA application or constitutive GA signaling results in decreased leaf dissection in wild-type and *KNOX*-overexpressing mutants. In tomato, therefore, the regulation of GA biosynthesis by KNOX homeodomain proteins has been reconfigured to function within the leaf, where it regulates the level of leaf dissection.

Results and Discussion

GA Suppresses KNOX Misexpression Phenotypes in *Arabidopsis* Leaves

In order to determine whether *Arabidopsis* leaf phenotypes that are associated with ectopic homeobox expression are mediated through a reduction in GA levels, we used an inducible fusion between KNOTTED1 (KN1) and the rat glucocorticoid receptor (GR) expressed under the control of the *CaMV 35S* promoter [17]. Upon induction with dexamethazone (DEX), these plants exhibit highly lobed leaves (Figure 1A), similar to the phenotypes conditioned by both *35S:KN1* and *35S:KNAT1* transgenes [1, 4]. Plants simultaneously exposed to both DEX and GA showed either complete or partial suppression of this phenotype (Figure 1B). Suppression of the *35S:KN1-GR* leaf phenotype by exogenous GA suggests that *KN1* misexpression represses GA biosynthesis rather than GA signaling and that the repression of GA biosynthesis is an early response. We examined GA/*KNOX* interactions genetically by determining the consequences of *KNAT1* misexpression in a *spindly* (*spy*) mutant background that confers constitutive GA signaling [18]. When *35S:KNAT1* plants, which exhibit highly lobed leaves [4], are homozygous for the *spy-1* mutation, both the number and depth of lobes is suppressed (Figures 1C and 1D).

Blocking GA Signaling Enhances the *as1* Mutation

Given that exogenous GA and elevated GA signaling suppress the effects of *KNOX* misexpression, we examined the effects of reduced GA signaling and biosynthesis on phenotypes conditioned by the *asymmetric leaves1* (*as1*) mutation [19, 20]. The *AS1* locus encodes a MYB transcription factor that negatively regulates the *KNOX* genes *KNAT1, KNAT2*, and *KNAT6* in *Arabidopsis* leaves [19–21]. The *as1-1* allele conditions phenotypes resembling weak *35S:KNAT1* overexpression [20]. Therefore, if repression of GA biosynthesis is instrumental to *KNOX* function, we would expect to enhance the phenotype of *as1-1* by further inhibiting GA signaling and/or biosynthesis. To test this hypothesis, we analyzed double mutants between *as1-1* and *ga insensitive* (*gai*), a mutation in which GA signaling is suppressed [22]. The number of lobes per leaf is significantly increased in *as1-1;gai-1* double mutants (Figures 2A–2C), so that the leaves are more similar to those of *35S:KNAT1* plants. The sinuses of all lobes in *as1-1;gai-1* leaves show an indented notch, as found infrequently in *as1-1* leaves (Figures 2E and 2F). These notches arise as a consequence of small meristematic cells in the sinus that do not expand uniformly in concert with surrounding cells [20]. Additionally, the *as1-1;gai-1* plants exhibit phenotypes described only for a strong *as1* allele [19]; these phenotypes include formation of callus tissue on both the adaxial and abaxial surfaces of double mutant leaves (Figure 2J). Reducing GA biosynthesis with the inhibitor paclobutrazol (PAC) also enhances the *as1-1* mutant phenotype. Ectopic shoot meristems form on the adaxial surface of the leaf margin (Figure 2G), leaf-like outgrowths form in similar positions (Figure 2H), and callus tissue forms in the sinuses of lobes (Figure 2I). These results concur with previously published observations that mutants with reduced GA levels or a block in GA signaling show an increased ability to regenerate shoot meristems from culture [23]. The degree of lobing throughout each leaf of PAC-treated plants is also dramatically enhanced, so that these leaves closely resemble those of *35S:KNAT1* plants (Figure 2D).

KNOX Misexpression Represses *AtGA20ox1* Expression in *Arabidopsis* Leaves

To establish a mechanistic link between *KNOX* function and GA homeostasis, we analyzed mRNA expression of the GA-biosynthesis genes *AtGA20ox1* and *AtGA3ox1*. The enzymes encoded by these genes catalyze the final steps in GA biosynthesis and represent control points in the GA-biosynthesis pathway [24–26]. *AtGA20ox1* is one of at least four *GA20ox* genes in *Arabidopsis* and is believed to contribute the majority of GA 20-oxidase activity during vegetative development [25]. Repression of *AtGA20ox1* transcript levels in response to ectopic

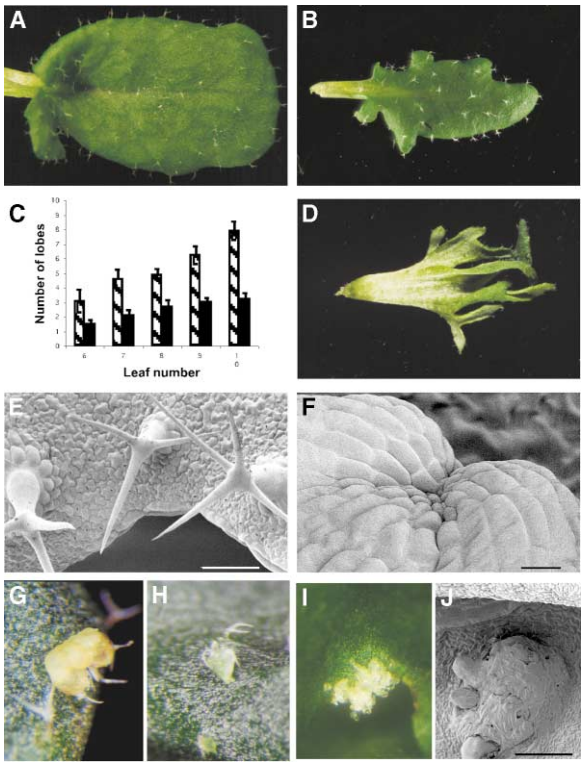

Figure 2. Inhibition of GA Signaling or GA Biosynthesis Enhances *KNOX* Misexpression Phenotypes

Leaves of (A) *as1-1* and (B) *gai-1;as1-1*. (C) Number of lobes per leaf is increased in *gai-1;as1-1* (striped bars) compared with *as1-1* (black bars). The leaf number refers to the order of leaf initiation, and error bars represent standard error. (D) *as1-1* leaf grown on PAC. Scanning electron micrographs of (E) Wild-type sinus, bar = 1 mm; and (F) *gai-1;as1-1* sinus showing notch, bar = 25 μm. (G–I) *as1-1* leaves grown on PAC showing (G) ectopic meristem; (H) ectopic leaves; and (I) callus tissue in the sinus of a lobe. (J) Scanning electron micrograph of *gai-1;as1-1* leaf showing callus tissue, bar = 200 μm.

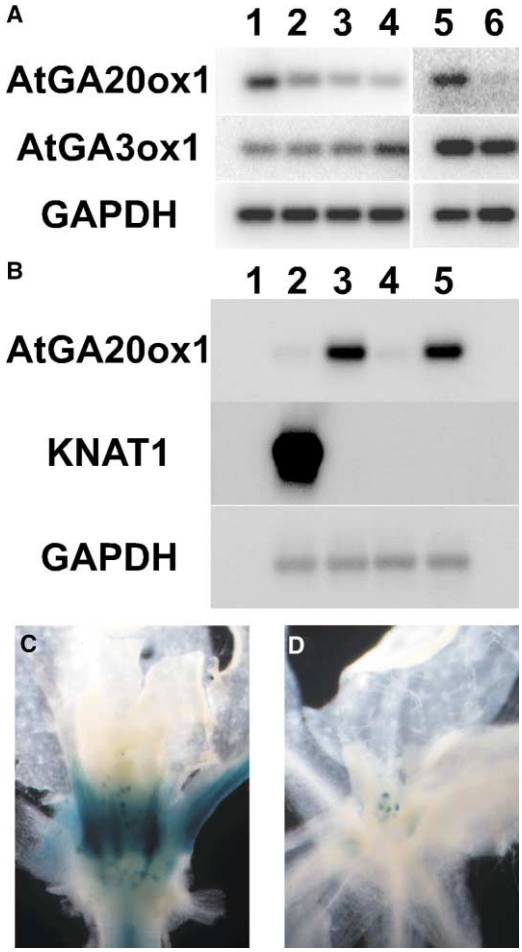

Figure 3. Expression Analysis of *AtGA20ox1*

(A) RT-PCR gel blot analysis of *AtGA20ox1* and *AtGA3ox1* expression in whole seedlings of No-O (lane 1), *35S:KNAT1* (lane 2), *as1-1* (lane 3), *pkl-15* (lane 4), and *35S:KN1-GR* treated without (lane 5) or with (lane 6) DEX. Tissue of *35S:KN1-GR* plants was harvested 30 min after DEX treatment. No PCR product was detected without cDNA in the reaction. (B) RT-PCR gel blot analysis of *AtGA20ox1* and *KNAT1* expression in the following vegetative leaf samples: control amplification with no cDNA included in PCR (lane 1), *35S:KNAT1* (lane 2), No-O (lane 3), *pkl-15* (lane 4), and Ler (lane 5). Control amplification and hybridization of *GAPDH* indicates that equal amounts of cDNA are present in each sample. No PCR product was detected when RT was not included in cDNA synthesis. *AtGA20ox1:GUS* expression in wild-type (C) and *as1-1* (D).

KNOX protein is seen in both *35S:KNAT1* and *35S:KN1-GR* plants (Figures 3A and 3B). This concurs with the finding that a 20-oxidase gene, *Ntc12*, is repressed by the KNOX protein NTH15 in tobacco [8]. Repression of *AtGA20ox1* transcript is detected within 30 min of induction of KN1 activity by DEX in *35S:KN1-GR* plants (Figure 3A). A similar rapid repression of *AtGA20ox1* transcript is seen upon DEX induction in *35S:STM-GR* plants (our unpublished data; *35S:STM-GR* was a gift of R. Sablowski). These results demonstrate that repression of GA-biosynthesis gene expression is an early response to ectopic KNOX activity.

AtGA20ox1 Expression Is Reduced in the *pickle* Mutation in a KNOX-Independent Manner

AtGA20ox1 transcript is also reduced in both *as1* and *pkl* mutants (Figures 3A and 3B). The reduction seen in *as1* suggests that repression of *AtGA20ox1* transcription is a consequence of the misexpression of *KNOX* genes in the leaf. AS1 is not required for the initial downregulation of *KNOX* genes during leaf initiation but acts to exclude *KNOX* genes from leaves after initiation [20]. This is consistent with the expression of *AtGA20ox1:GUS* in the youngest leaf primordia in *as1* and the marked repression seen in later leaves compared with wild-type (Figures 3C and 3D). The *pkl* mutant, however, does not express *KNOX* genes ectopically (Figure 3B), but it does enhance the *as1* phenotype so that ectopic meristems and stipules are formed in the sinuses of leaves [20]. The reduction of *AtGA20ox1* expression in *pkl* suggests that PKL acts via *AtGA20ox1* in a KNOX-independent manner. No repression of *AtGA3ox1* transcript is detected in *as1*, *pkl*, *35S:KNAT1*, or *35S:KN1-GR* plants (Figure 3A), suggesting that *AtGA20ox1* is a specific target in the GA-biosynthesis pathway for KNOX and PKL proteins. PKL is a member of the CHD class of

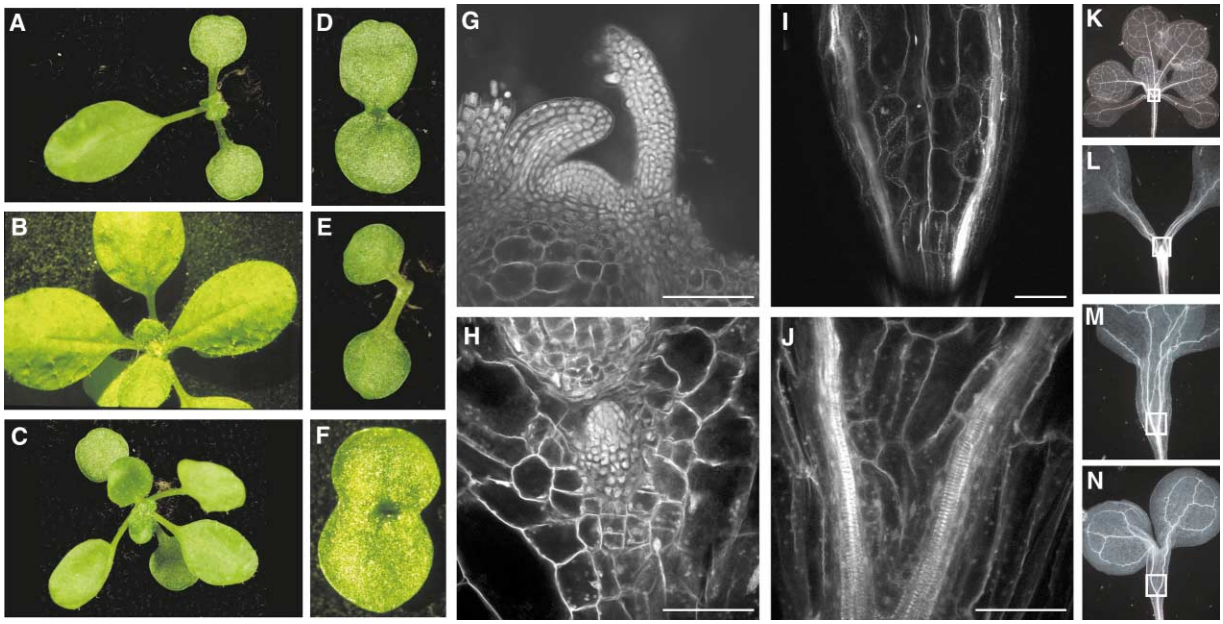

Figure 4. *spy* Enhances the Phenotype of *stm*

Aerial view of 20-day-old plants of the following genotypes: (A) *stm-2*, (B) *spy-5*, (C) Ler, (D) *stm-2;spy-5*, (E) *stm-1*, and (F) *stm-1;spy-5*. (G–J) Shoot apices of 10-day-old seedlings visualized by confocal laser scanning microscopy (CLSM). (G) Ler SAM with two leaves visible, (H) *stm-2* with meristem in cotyledon axil, (I) *stm-2;spy-5* with large, vacuolate cells at the junction of the cotyledon and hypocotyl vasculature, and (J) *stm-11* with large, vacuolate cells similar to (I). The scale bars represent 50 μm. (K–N) Cleared seedlings with box indicating region shown in CLSM: (K) Ler, (L) *stm-2*, (M) *stm-2;spy-5*, and (N) *stm-11*.

chromatin remodeling factors and has been implicated in restricting meristematic activity [15, 16]. Our results suggest that repression of *AtGA20ox1* represents a point of convergence in the control of meristem activity by KNOX and PKL proteins. It will be interesting to determine whether the accessibility of regulatory regions of KNOX target genes (such as *AtGA20ox1*) involves regulation of chromatin structure by proteins such as PKL [20, 27].

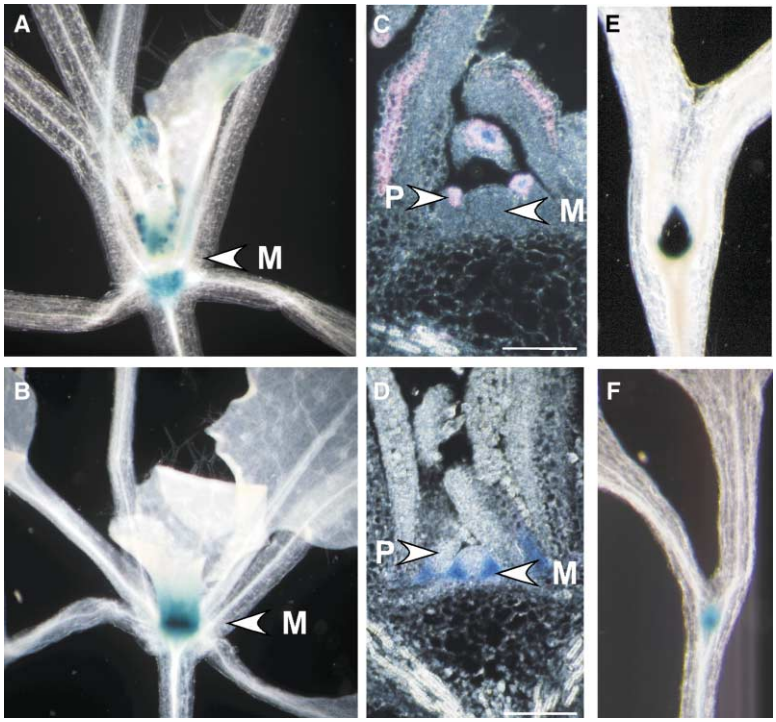

Figure 5. *AtGA20ox1:GUS* and *STM:GUS* Are Expressed in Mutually Exclusive Domains of the Shoot Apex

(A) *AtGA20ox1:GUS* is expressed in wild-type seedlings in young leaf primordia and the top of the hypocotyl and is excluded from the SAM. (B) *STM:GUS* is expressed in wild-type seedlings in the SAM and is excluded from leaf primordia. (C) Longitudinal section of *AtGA20ox1:GUS* expression in the wild-type. (D) Longitudinal section of *STM:GUS* expression in the wild-type. (E) *AtGA20ox1:GUS* is expressed in *stm-11* seedlings at the junction of the cotyledon and hypocotyl vasculature. (F) *KNAT1:GUS* is similarly expressed in the apex of *stm-11* seedlings. M indicates the meristem; P indicates the leaf primordium. Scale bars represent 50 μm.

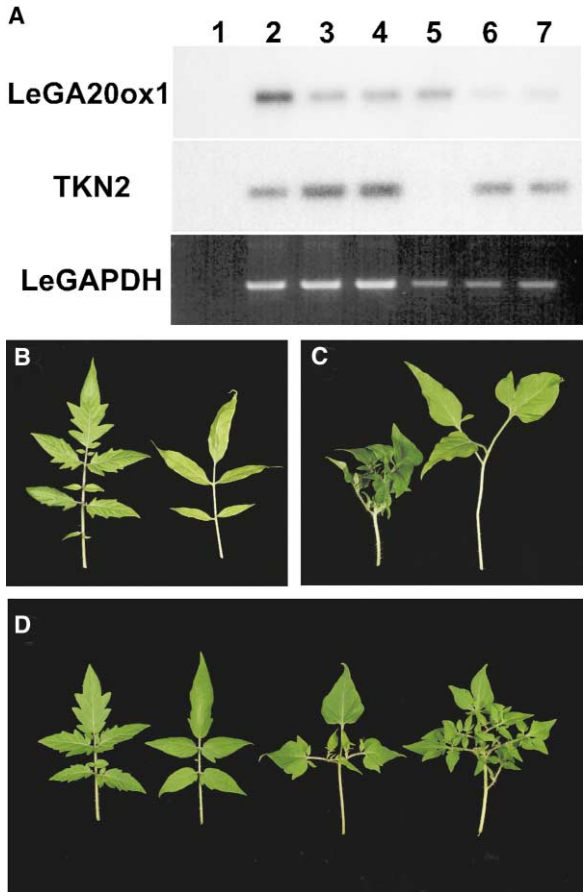

Figure 6. KNOX/GA Interaction Regulates Tomato Leaf Dissection

(A) RT-PCR gel blot analysis of the expression levels of *LeGA20ox1* and *TKN2* in the following leaf samples: control amplification with no cDNA included in PCR (lane 1), wild-type (*Ailsa craig*; lane 2), *Me* (lane 3), and *Cu* (lane 4). In lanes 5–7, a one-tenth dilution of wild-type, *Me*, and *Cu* cDNA was used as a template in the PCR. Control amplification of *LeGAPDH* indicates that equal amounts of cDNA are present in each sample. No PCR product was detected when RT was not included in cDNA synthesis. GA application suppresses the level of leaf dissection: (B) wild-type leaf −GA (left) and +GA (right); (C) *Me*/+ leaf −GA (left) and +GA (right). (D) *pro* suppresses the increased leaf dissection of *Me*/+, from left to right, WT, *pro*, *Me*/+; *pro* and *Me*/+ leaves.

The *stm-2* Phenotype Is Enhanced by the Constitutive GA Signaling Mutant *spindly*

We have shown that *KNOX* misexpression phenotypes in the *Arabidopsis* leaf are partially mediated by reduced activity of the GA pathway. To determine whether this finding pertains to *KNOX* function in the SAM, we examined the effects of constitutive GA signaling (as conditioned by the *spy-5* allele) on the weak *stm-2* allele [28, 29]. *stm-2* lacks an embryonic SAM but initiates meristems that have reduced organogenic potential and give rise to aberrant leaves and shoots (Figure 4A). Examination of *stm-2*;*spy-5* double mutants revealed that constitutive GA signaling is detrimental to meristem function (Figures 4A–4D). *stm-2;spy-5* seedlings fail to recover any organogenic ability and never produce leaves or a shoot (Figure 4D).

Confocal microscopy of 10-day-old seedlings shows that this defect in organogenesis is due to a loss of meristem activity. *spy-5* and wild-type seedlings initiate a SAM of similar dimensions at a position above the junction of the cotyledon and hypocotyl vasculature (Figure 4G). In contrast, *stm-2* initiates a smaller meristem in the cotyledon axil (Figure 4H). *stm-2;spy-5* seedlings lack any evidence of organized meristems (Figure 4I). Small, brightly staining cells typical of meristematic activity are completely absent from the apex of these double mutants. Rather, the cells in this region are large and vacuolate, characteristic of the differentiation that occurs in the strong *stm-11* allele (Figure 4J).

In addition to a loss of meristem activity, the cotyledons fail to separate in *stm-2;spy-5* double mutants (as described for *stm-1* [28] and *stm-11* [30]). This fusion along the cotyledon petioles is significantly enhanced in the double mutants in comparison to *stm-2* individuals and is greater than that seen in *stm-11* individuals (Figures 4K–4N) (the length of petiole fusion is as follows: *stm-2*, 0.50 ± 0.04 mm; *stm-2;spy-5*, 2.02 ± 0.07 mm; *stm-11*, 1.76 ± 0.08 mm). Double mutants of the strong *stm-1* allele with *spy-5* show dramatic fusion of the cotyledon petioles; this fusion extends up the cotyledon lamina (Figure 4F). Previous work has demonstrated that GA promotes differentiation by inducing longitudinal cell expansion and rearrangement of cellulose microfibrils and the cytoskeleton [31]. In contrast, KNOX proteins are believed to suppress differentiation [32]. Given that KNOX proteins repress GA biosynthesis, it is possible that inappropriate GA-induced cell differentiation contributes to the *stm* mutant phenotype and the enhanced *stm;spy-5* double mutant phenotype.

AtGA20ox1:GUS Expression Is Altered in the *stm* Mutant

Analysis of reporter fusions between β-glucuronidase (GUS) and promoter sequences of *AtGA20ox1* and *STM* reveal contrasting patterns of expression in the shoot apex. *AtGA20ox1:GUS* confers a specific expression pattern in young leaf primordia and the top of the hypocotyl (Figure 5A). In longitudinal section, *AtGA20ox1:GUS* expression is seen in young leaf primordia and is absent from the SAM (Figure 5C). In contrast, *STM:GUS* is expressed in the SAM and excluded from incipient and emerging leaf primordia (Figures 5B and 5D).

In order to assess the significance of this mutual exclusion, we analyzed the *AtGA20ox1:GUS* reporter in a *stm* mutant background. If STM is required to repress *AtGA20ox1* transcription, then we would expect to see elevated levels of GUS expression in an altered domain in the *stm* mutant. In wild-type seedlings, *AtGA20ox1:GUS* expression is clearly excluded from the SAM (Figures 5A and 5C), whereas in *stm* seedlings intense expression is observed deep in the apex (Figure 5E). Levels of *AtGA20ox1:GUS* expression are significantly higher in *stm* than in the wild-type; less than 3 hr of incubation with the chromogenic substrate is required to detect staining in the mutant, whereas overnight incubation is required to detect staining in the wild-type.

These results are consistent with STM being a repres-

Table 1. GA Application and Constitutive GA Signaling Reduce the Level of Dissection of Wild-Type and *Me* Leaves.

	Number of Leaflets			
	Order of Leaf Dissection			
Genotype	First	Second	Third	Intercalary
Wild type −GA[1]	5.0 ± 0	1.6 ± 0.4	0	5.2 ± 0.8
Wild type +GA	4.8 ± 0	0	0	0
Me/+ − GA	6.0 ± 1	30.5 ± 18.5	5.0 ± 0	0
Me/+ + GA	3.4 ± 0.7	0.4 ± 0.2	0	0
Wild type[2]	7.3 ± 0.3	0	0	N.D.
pro	5.8 ± 0.5	0	0	N.D.
Me/+	8.7 ± 1.7	32.3 ± 1.5	57.3 ± 19.6	N.D.
Me/+;pro	5.8 ± 0.6	6.0 ± 2.0	8.0 ± 3.4	N.D.

N.D. not determined.
[1] Data for fourth leaf of 3-week-old plants.
[2] Data for fifth leaf of mature plants.

sor of *AtGA20ox1*. An alternative explanation would be that the altered domain of *AtGA20ox1:GUS* expression in *stm* reflects the fact that the meristem has been consumed by differentiation processes. These possibilities are not mutually exclusive. However, *KNAT1:GUS* expression is observed in a similar position to *AtGA20ox1:GUS* in *stm* apices (Figure 5F), indicating that not all apical cells in *stm* seedlings are differentiated. Furthermore, our demonstration that constitutive GA signaling is detrimental to STM activity (Figure 4) provides a physiological rationale for the proposed STM-mediated exclusion of GA biosynthesis from the meristem.

The expression pattern of *AtGA20ox1:GUS* is unaltered in the *KNAT1* loss-of-function mutant *bp* (our unpublished data), probably because of the redundant function of *KNAT1* with *STM* in the SAM [3]. Alternatively, it could indicate different binding affinities of STM and KNAT1 for *AtGA20ox1* or partially divergent functions for these two KNOX transcription factors in the SAM. Future experiments, including analysis of the biochemical properties of different KNOX proteins, will help resolve this point.

The KNOX/GA Interaction Functions in the Dissected Leaves of Tomato

Our results suggest that GA partially mediates the function of KNOX proteins in meristems, the natural domain of *KNOX* expression, and in leaves upon ectopic expression. We investigated whether a similar interaction between KNOX proteins and GA regulates leaf shape in tomato, a species in which *KNOX* genes are expressed in leaves, which are naturally dissected in this species. To this end we used the dominant mutations *Mouse ears* (*Me*) and *Curl* (*Cu*) that both condition misexpression of *Tkn2*, a tomato *KNOX* gene, and result in increased leaf dissection [12, 33]. RT-PCR gel blot analysis demonstrates that overexpression of *TKn2* in *Me* and *Cu* correlates with reduced transcript levels for *LeGA20ox1* (Figure 6A). Application of GA suppressed the degree of dissection in wild-type, *Me*, and *Cu* leaves (Figures 6B and 6C, Table 1, and our unpublished data), suggesting that this *KNOX*-induced repression of a GA-biosynthesis gene is likely to mediate the increased leaf dissection in these mutants. To verify this result, we used the constitutive GA signaling mutant *procera* (*pro*) [34]. *pro* has

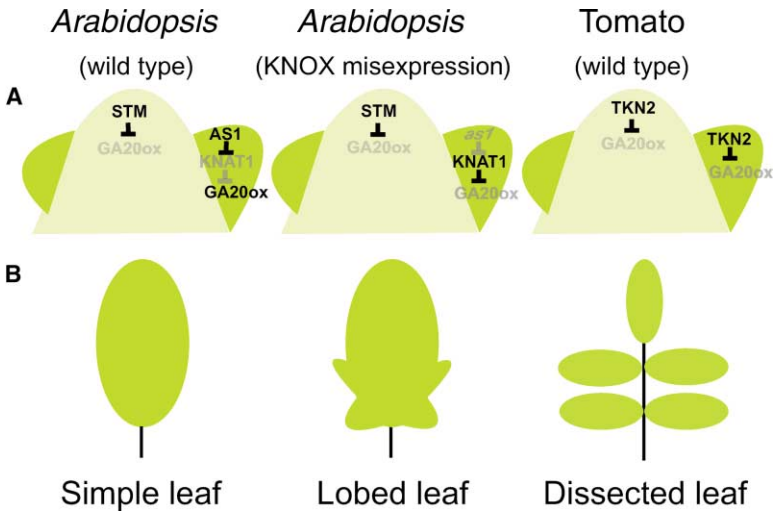

Figure 7. Model Depicting How the Gibberellin Pathway Partially Mediates KNOX Function in Plants with Different Leaf Shapes

(A) STM expression in the SAM represses *GA20ox1* transcription. KNAT1 expression is excluded from the wild-type *Arabidopsis* leaf so that *GA20ox1* is expressed. KNAT1 misexpression in the *Arabidopsis* leaf and TKN2 expression in the wild-type tomato leaf represses *GA20ox1* expression.

(B) This results in a simple wild-type *Arabidopsis* leaf, a lobed KNOX-misexpressing *Arabidopsis* leaf, and a dissected wild-type tomato leaf, respectively. KNOX proteins will regulate additional unknown processes (possibly cytokinin biosynthesis) in order to promote indeterminacy; these are not included in the model.

more entire leaves with fewer leaflets and thus phenocopies wild-type plants supplied with GA (Figures 6B and 6D). The increased dissection phenotype of *Me* is suppressed in the *pro* background (Figure 6D and Table 1). These results suggest that KNOX proteins repress GA biosynthesis and that this interaction is pivotal in regulating the level of dissection of tomato leaves. Furthermore, these results provide a mechanistic explanation for previous observations that tomato leaf shape can be modified by the application of exogenous growth regulators [35].

It is noteworthy that the regulation of GA biosynthesis is likely to be only one of the functions of KNOX proteins. This is suggested by the fact that exogenous application of GA, *spy* [18], *repressor of ga1-3* [36], *gai* [37, 38], and other mutations that render GA signaling constitutively active do not condition obvious meristem defects, nor do GA-biosynthesis mutants phenocopy *KNOX* misexpression phenotypes. The nature of other processes regulated by KNOX proteins is unknown. However, similarities between *isopentenyl transferase* and *KNOX* overexpression phenotypes and increases in cytokinin levels after *KNOX* misexpression suggest that increased cytokinin biosynthesis is a likely downstream effect of *KNOX* action [9, 11]. Thus, KNOX homeodomain proteins may act as general orchestrators of growth regulator homeostasis in the shoot apex by activating cytokinin biosynthesis, simultaneously repressing GA biosynthesis, and promoting meristem activity.

Conclusions

Our work suggests that the regulatory module defined by KNOX transcription factors and GA is part of the developmental program of the shoot apical meristem. STM acts, perhaps redundantly with KNAT1, in the SAM to repress transcription of *AtGA20ox1* (Figure 7A). KNOX misexpression in the leaves of *Arabidopsis* acts to repress *AtGA20ox1* transcription in the leaf and contributes to a lobed leaf form (Figure 7B). Our results suggest that, at least in tomato, this module functions within leaves to control the level of leaf dissection (Figure 7B). This provides an example of how the redeployment of an existing regulatory module through evolution could result in morphological innovation.

Experimental Procedures

Plant Material

as1-1 [39], *spy-1* [18], *spy-5* [40], *stm-2* [28, 29], *stm-1* [41], and *gai-1* [22] were obtained from the *Arabidopsis* Biological Resource Center. Backgrounds and accession numbers are as follows: *as1-1*, accession number CS3374, background Col; *spy-1*, accession number CS6266, background Col; *spy-5*, accession number CS8094, background Ler; *stm-2*, accession number CS8137, background Ler; *stm-1*, accession number CS8154, background Ler; *gai-1*, accession number CS63, background Ler. *stm-11* is in the Ler background [30]. *pkl-15* was previously described as *gym-5* and is in the Ler background [15]. *35S:KNAT1* transgenic line 3B is in the No-O background [4]. *35S:KN1-GR* transgenic line 46-10 is in the Col background. This is a C-terminal fusion of the *KN1* cDNA with the steroid binding domain of the rat glucocorticoid receptor from pBI-ΔGR [17]. *STM:GUS* and *AtGA20ox1:GUS* transgenic lines are in the Col background. The *STM:GUS* fusion has 3.5 kb of promoter sequence that is sufficient to drive expression in the peripheral zone but not in the central zone of the SAM. The *AtGA20ox1:GUS* fusion has a 2708 bp genomic fragment from −1516 from the start of translation to base 27 of the third exon. *Ailsa Craig*, *Me*, *Cu*, and *procera* were obtained from the Tomato Genetics Resource Center, University of California, Davis. Accession numbers are as follows: *Ailsa Craig*, accession number LA2838A; *Me*, accession number LA3081; *Cu*, accession number LA3059; *pro*, accession number LA2838. All mutants are in the *Ailsa Craig* background.

Plants were grown in a greenhouse for long days or in a growth chamber for short days as indicated. Long days were 16 hr light and 8 hr dark; short days were 16 hr dark and 8 hr light. Day temperature was 20°C, and night temperature was 18°C in both short and long days.

Construction of Double Mutants

In a cross between *spy-1* and *35S:KNAT1*, F2 individuals that showed suppressed lobing were selfed. When *35S:KNAT1* was hemizygous in the F2, the F3 progeny segregated into 1/4 *spy-1* unlobed phenotype and 3/4 mildly lobed double mutant phenotype. Homozygous *as1-1* and *gai-1* individuals segregating in an F2 population of a cross between *gai-1* and *as1-1* were selfed. *gai-1* individuals' F3 progeny that segregated *gai-1;as1-1* double mutants were scored compared to F3 progeny of *as1-1* individuals. Homozygous *spy-5* individuals segregating in F2 populations of crosses between *spy-5* and either *stm-2* or *stm-1* were selfed. F3 progeny of *spy-5* individuals that segregated *spy-5;stm* double mutants were scored compared to segregating *stm-2* and *stm-1* families. The introduction of *stm-11* into the Col ecotype via the cross to *AtGA20ox1:GUS* partially suppresses the *stm* phenotype. *AtGA20ox1:GUS* expression was analyzed in *stm* individuals of both weak and strong phenotypic classes. F2 phenotypes were scored in a segregating population from a cross between *Me* and *pro*. Double mutants were selfed to confirm the *Me*+ genotype.

Chemical Treatments

Dexamethazone (Sigma) was dissolved in water to 1 mM stock and applied with a brush at 1 μM with 0.2% silwet surfactant. GA_3 (Sigma) was dissolved in ethanol to 100 mM, diluted with water to 100 μM, and applied by being sprayed with 0.2% silwet surfactant. In all experiments, control plants were treated with the same concentration of surfactant in water. *as1-1* and Col plants were germinated on MS media containing B5 vitamins, and then treatment plants were transferred to the same media containing 10^{-5} M paclobutrazol. Plants were transferred from media to soil for analysis of expanded leaf phenotypes.

SEM Analysis

SEM analysis was carried out as previously described [42].

RT-PCR Gel Blot Analysis

One microgram of total RNA that had been extracted with RNeasy (Qiagen) was used for cDNA synthesis with an oligo (dT) primer and Superscript RT (Invitrogen). PCR primers specific for *AtGA20ox1*, *AtGA3ox1*, *KNAT1*, *GAPDH*, *LeGA20ox1*, *TKN2*, and *LeGAPDH* amplified products of 359, 217, 779, 542, 330, 350, and 500 bp, respectively. These products were detected by Southern hybridization with gene-specific probes. Twenty-one PCR cycles were performed with *Arabidopsis* cDNA, and 30 cycles were performed with tomato cDNA. Experiments were conducted at least three times with equivalent results. Primers were as follows: AtGA20ox1-F, GCCGTAAGT TTCGTAACAACATCTCC; AtGA20ox1-R, GAGAGAGGCATATCAA AGAAGCGG; AtGA3ox1-F, AACAGATGTGTTTAGAGGCCATCCC; AtGA3ox1-R, AGTTCTACATGCATGACCGATTTGG; KNAT1-F, TGA CAACAGCACCACTCCTC; KNAT1-R, GTTTCCCCTCCGCTGTTATT; GAPDH-F, CACTTGAAGGGTGGTGCCAAG; GAPDH-R, CCTGTTGT CGCCAACGAAGTC; LeGA20ox1-F, GTCTTTGCATGCAATTATAATG; LeGA20ox1-R, GAACGTATCCATATAACGATGA [43]; TKN2-F, TAA GCAGGAGTTCATGAAGAAG; TKN2-R, TACATGCACACAAGTAATA TGC; LeGAPDH-F, TGGAATCAGGAACCCTGAAG; and LeGAPDH-R, GATCGACAACGGAGACATCA.

Confocal Laser Scanning Microscopy

Confocal laser scanning microscopy was carried out as described [44].

Tissue Clearing

Tissue clearing was carried out as described [45] and viewed under darkfield microscopy.

GUS Staining

Plants were grown under short-day conditions. GUS staining was carried out, and 7 μm tissue sections were prepared as previously described [46]. Potassium ferricyanide (10 mM) and potassium ferrocyanide (10 mM) were used to limit GUS diffusion. All samples were viewed under darkfield microscopy.

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