Plant Molecular Biology (2019) 99:449–460
https://doi.org/10.1007/s11103-019-00829-7

LMI1-like and *KNOX1* genes coordinately regulate plant leaf development in dicotyledons

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Received: 17 June 2018 / Accepted: 21 January 2019 / Published online: 28 January 2019

Abstract

Key message **This report reveals that the *LMI1-like* and *KNOX1* genes coordinately control the leaf development and different combinations of those genes which produce diverse leaf shapes including broad, lobed and compound leaves.**

Abstract *Class I KNOTTED1-like homeobox* (*KNOX1*) genes are involved in compound leaf development and are repressed by the ASYMMETRIC LEAVES1 (AS1)–AS2 complex. Cotton plants have a variety of leaf shapes, including broad leaves and lobed leaves. *GhOKRA*, a *LATE MERISTEM IDENTITY 1 (LMI1)-like* gene, controls the development of an okra leaf shape. We cloned the corresponding cotton homologs of *Arabidopsis thaliana AS1* and *AS2* and seven *KNOX1* genes. Through virus-induced gene silencing technology, we found that either *GhAS1* or *GhAS2*-silenced cotton plants showed a great change in leaf shape from okra leaves to trifoliolate dissected leaves. In the shoot tips of these plants, the expression of the cotton ortholog of *Knotted in A. thaliana 1* (*KNAT1*), *GhKNOTTED1-LIKE2/3/4* (*GhKNL2/3/4*), was increased. However, *GhKNOX1*s-silenced plants maintained the wild-type okra leaves. A novel dissected-like leaf in *A. thaliana* was further generated by crossing plants constitutively expressing *GhOKRA* with either *as1-101* or *as2-101* mutant plants. The dissected-like leaves showed two different leaf vein patterns. This report reveals that the *LMI1-like* and *KNOX1* genes coordinately control leaf development, and different combinations of these genes produce diverse leaf shapes including broad leaves, lobed leaves and compound leaves. This is the first report on the artificial generation of compound leaves from simple leaves in cotton.

Keywords Leaf development · *LMI1-like* · *KNOX1* · Compound leaves

Abbreviations

AS1	*ASYMMETRIC LEAVES1*
AS2	*ASYMMETRIC LEAVES2*
KNOX1	*Class I KNOTTED1-like homeobox*
CUC	*CUP-SHAPED COTYLEDON*
BP	*KNAT1/BREVIPEDICELLUS*
KNAT	*Knotted in A. thaliana*
Ler	*Landsberg erecta*
LMI1	*LATE MERISTEM IDENTITY1*
ML	Maximum likelihood
RCO	*REDUCED COMPLEXITY*
SAM	Shoot apical meristem
STM	*SHOOT MERISTEMLESS*
TL	*Tendril-less*
VIGS	Virus induced gene silencing
WT	Wild-type

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Electronic supplementary material The online version of this article (https://doi.org/10.1007/s11103-019-00829-7) contains supplementary material, which is available to authorized users.

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Introduction

Plant leaves are initiated from the peripheral region of the shoot apical meristem (SAM), and display great variations in shape and size. They are traditionally classified into two major morphogenetic classes: undivided simple leaves, and compound leaves. Simple leaves have a single lamina that can sometimes be elaborated with less-pronounced incisions such as serrations or lobes on the laminal margin,

for example, unlobed leaves in *Arabidopsis* and broad or lobed leaves in cotton (Fig. 1a–c). Compound leaves or dissected leaves have numerous individual leaflets on a rachis that arises at a node, for example, ternate compound leaves of soybean and pinnately compound leaves of tomato (Fig. 1d, e). The same factors are involved in the formation of serrations and leaflets, and include auxin activity maxima and *CUP-SHAPED COTYLEDON* (*CUC*) genes (Blein and Laufs 2008; Kougioumoutzi 2008; Bilsborough et al. 2011; Kasprzewska et al. 2015). Many compound leaf mutants in *Cardamine hirsuta* and *Medicago truncatula* have defective separation between the adjacent leaflets leading to the conversion of dissected leaves into lobed simple leaves (Peng et al. 2011; Vlad et al. 2014). Lobed simple leaves are essentially an intermediate shape between serrations and dissected leaves. However, whether gene mutations can cause lobed leaves to become compound leaves requires further investigation.

Cotton plants have a variety of leaf shapes, including broad leaves, as in TM-1, and lobed leaves, as in Okra (Fig. 1b, c). We have found that an *HD-ZIP I* transcription factor (*GhOKRA*) controls the formation of deep lobes in cotton (*Gossypium*) (Chang et al. 2016), which has been independently confirmed by two other groups (Andres et al. 2014, 2016; Zhu et al. 2016). The mutant of *GhOKRA* in TM-1 results in the production of broad leaves. Compound leaves are non-existent in cotton. The ε clade of *HD-ZIP I* transcription factors were conserved to regulate leaf shape in many plants (Hofer et al. 2009; Vlad et al. 2014). The *GhOKRA* homolog, *LATE MERISTEM IDENTITY1* (*LMI1*), was first reported in *Arabidopsis thaliana* as a floral regulator and was found to influence leaf morphogenesis (Saddic et al. 2006). Other *GhOKRA* homologs, such as *REDUCED COMPLEXITY* (*RCO*) in *C. hirsuta* and *Tendril-less* (*TL*) in pea, which are also *LMI1-like* genes, are emerging as regulators of lateral organ genesis in compound leaves by affecting lateral organ formation (Hofer et al. 2009; Vlad et al. 2014). The function of these *LMI1-like* genes appears to be similar, such as blade growth-repression in compound leaves and lobed simple leaves (Vlad et al. 2014; Andres et al. 2016).

Class I *KNOTTED1*-like homeobox (*KNOX1*) transcription factors are involved in the maintenance of indeterminate cell fate in the SAMs and developing primordia of complex leaves (Bharathan et al. 2002; Hake et al. 2004; Uchida et al. 2010). The *KNOX1* genes are expressed in SAM to maintain the indeterminate nature of meristem cells, and are down-regulated at the position where leaf primordia initiate (Lincoln et al. 1994; Hay and Tsiantis 2010; Sluis and Hake 2015). At the site of leaf initiation, *KNOX1* genes are downregulated, and auxin signaling occurs. Auxin regulates *KNOX* expression (Hay et al. 2006), and *KNOX* modulates many genes in auxin signaling (Bolduc et al. 2012); thus, they have regulatory interactions. The expression of *KNOX1* genes is essential in the developing primordia for leaflet formation in compound leaf plants. However, in simple leaf plants, the *KNOX1* genes are absent from leaf primordia (Parnis 1996; Hay and Tsiantis 2006; Kougioumoutzi 2008; Efroni and Lifschitz 2010). Compound leaf development requires organogenic activity during primary morphogenesis and leaflet formation. The *KNOX1* genes can be classified into three subclasses; *SHOOT MERISTEMLESS* (*STM*)-like, *Knotted in A. thaliana* (*KNAT*)*2*/*6*-like and *KNAT1*/*BREVIPEDICELLUS*(*BP*)-like. In *A. thaliana, ASYMMETRIC LEAVES1* (*AS1*) and *AS2* repress the activity of *KNAT1* and *KNAT2* in leaves (Byrne et al. 2000, 2002). Both *as1* and *as2* mutants have abnormal lobed leaves with ectopic expression of *BP* and *KNAT2* (Byrne et al. 2000, 2002). The MYB transcription factors, which are encoded by AS1, interact with the *LATERAL ORGAN BOUNDARIES (LOB)* domain protein, AS2, and work together as the AS1–AS2 complex. This complex binds *KNOX* loci, resulting in the recruitment of chromatin remodeling factors, such as HDA6, a histone deacetylase (Luo et al. 2012; Lodha et al. 2013). Both BTB ankyrin genes, BLADE ON PETIOLE1 (BOP1) and BOP2, can activate AS2 directly, but also can repress *KNOX* independently (Khan et al. 2014). Some simple leaves develop from complex primordia through

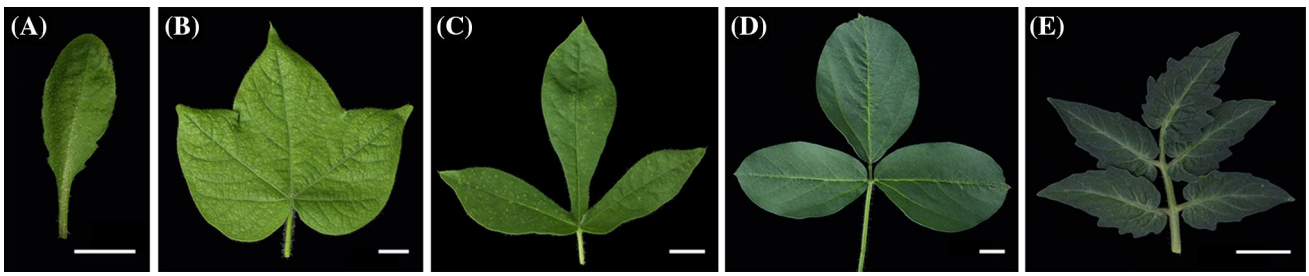


Fig. 1 A variety of leaf shapes in dicotyledons. **a** *A. thaliana*, Col-0, **b** *G. hirsutum* acc. TM-1, **c** *G. hirsutum* acc. Okra cotton, **d** *Glycine max*, Williams 82, and **e** *Lycopersicon esculentum*, Micro-Tom. Scale bars = 1 cm. Okra cotton was used in VIGS assays for investigating the effect of silencing *GhAS1, GhAS2, KNOX1* and *GhOKRA* genes. TM-1 cotton was used in *GhAS1* and *GhAS2* silencing VIGS assays. Transgenic *A. thaliana* (Col-0) constitutively expressed *GhOKRA* resulted in the production of a lobed leaf

secondary morphogenesis, as in *Lepidium oleraceum*, which has *KNOX1* expression in leaf primordia to produce marginal outgrowths (Bharathan et al. 2002). In addition, ectopic expression of *KNAT1* in *A. thaliana* transforms simple leaves into lobed leaves, resulting in an increase in leaf complexity (Chuck et al. 1996). In *Cardamine hirsute*, which has dissected leaves comprising leaflets, a *KNOX1* paralogous gene, Ch*BP*, is concurrently regulated by the microRNA164A (MIR164A)/ChCUP-SHAPED COTYLEDON (*ChCUC*) module and ChASYMMETRIC LEAVES1 (*ChAS1*). This gene does not occur in *A. thaliana*, a relative of *Cardamine hirsute*, which has simple leaves (Rastsomssich et al. 2015). However, it is still unclear whether *KNOX1* genes regulate lobe development in simple leaves. Organogenic activity and leaf marginal structure development are known to dictate the final leaf shape; however, there is little information on the role of *KNOX1* genes in combination with *LMI1-like* genes in the formation of different leaf shapes.

To determine whether the *LMI1-like* gene regulates leaf shape in collaboration with *KNOX1* genes, we silenced the *KNOX1* genes and the homologous genes of *AS1* and *AS2* in wild-type (WT) okra leaf cotton, and found that different expression models of the *LMI1-like* gene and *KNOX1* genes control the formation of broad, lobed and compound leaves. We also generated dissected-like leaves in *A. thaliana*, which further confirms that formation of these leaf shapes is controlled by *LMI1-like* gene and *KNOX1* genes. The present research provides new insights into the formation of different leaf marginal structures, including unlobed leaves, lobed leaves, and leaflets.

Materials and methods

Plant materials

TM-1 with broad leaves is a standard genetic line of Upland cotton (Kohel et al. 1970). Okra cotton with okra leaves, provided by the Institute of Cotton Research of CAAS named as Super Okra, is a *Gossypium hirsutum* accession. The *A. thaliana* mutants used in this study, *as1-101* and *as2*-101, were produced with a *Landsberg erecta* (Ler) background. *as1-101* and *as2-101* seeds were kindly provided by Lin Xu and Hai Huang (Institute of Plant Physiology & Ecology, SIBS, CAS) (Yue et al. 2000; Sun et al. 2002; Xu et al. 2002). Transgenic *A. thaliana* constitutively expressing *GhOKRA* plants were generated previously in a Col-0 background (Chang et al. 2016). The T1 progeny of transgenic *A. thaliana* with lobed leaves was crossed with *as1-101* and *as2-101* mutant plants. The leaves of the F_1 progeny had more lobes than that observed in constitutively expressing *GhOKRA A. thaliana* plants. There were many different leaf phenotypes in the F_2 populations of these two crosses, such as wild-type, *as1-101* or *as2-101*, and constitutively expressed *GhOKRA A. thaliana* leaves, as well as some novel leaf types. All materials were grown in the green houses of Nanjing Agriculture University following normal practices.

Phylogenetic analyses of *KNOX* genes in cotton

The genes in *Gossypium raimondii* are highly homologous with those in *G. hirsutum*. Since *G. hirsutum* has two sub-genomes, and some homologous have not been annotated in the *G. hirsutum* acc. TM-1 genome, so we analyzed KNOX genes in diploid *G. raimondii* for next work. *G. raimondii* genome sequences were downloaded from the Phytozome database (http://www.phytozome.net) (Paterson et al. 2012). *Arabidopsis thaliana KNOX* protein sequences were downloaded from the Arabidopsis Information Resource website (TAIR) (http://www.arabidopsis.org). All cotton proteins were screened for potential *KNOX* genes using HMMER software version 3.0 and the Pfam database (Zhang and Wood 2003; Finn et al. 2011, 2016). KNOX protein sequences from *G. raimondii* were aligned with the homologous proteins from *A. thaliana*. We used the Maximum likelihood (ML) method to construct a phylogenetic tree in MEGA 6.06 (http://www.megasoftware.net). The bootstrap test of phylogeny was performed with 1000 replications (Tamura et al. 2013).

Cloning of *GhAS1, GhAS2* and *KNOX1* genes

Homologs of *AS1* and *AS2* in *A. thaliana* were identified using HMMER software version 3.0 and the Pfam database to screen all TM-1 proteins (Zhang and Wood 2003; Finn et al. 2011, 2016). We cloned the coding sequences (CDSs) of *GhAS1, GhAS2* and some *KNOX1* genes from shoot tips of okra cotton using the genome sequence of TM-1 (Zhang et al. 2015). The primers used are listed in Table S1. We used ExTaq DNA Polymerase (TaKaRa, Japan) for PCR. Amplification products were cloned into the pMD19-T vector (TaKaRa, Japan) for sequencing by the Nanjing Jinsite Biotech. Co. Ltd. All of the genes mentioned above were cloned from okra cotton, with separation of the A and the D sub-genomes. Based on the genome sequences of *G. raimondii* and *G. arboreum* and the sub-genome sequences in *G. hirsutum*, we differentiated between the A and the D sub-genome homologs (Table S2). Both the A and the D sub-genome homologs in *G. hirsutum* are highly homologous with those in *G. raimondii*. Only a few SNPs exist between them. A silencing construct developed from the A or the D sub-genome would silence two sub-genome homeologs. We therefore chose only one sub-genome for further analysis due to the high sequence similarity of the A and the D sub-genomes.

Virus-induced gene silencing (VIGS) assay

We amplified fragments around 300-bp long from the 3′ ends of *GhAS1, GhAS2* and seven *KNOX1* genes (*GhKNL2-GhKNL8*) (Table S2). A ClonExpress II One Step Cloning Kit (C112-01, Vazyme Biotech Co., Ltd) was used to recombine the fragments into *EcoR*I-*Xba*I-digested pTRV2 for VIGS assay. All primers used for VIGS vector construction are listed in Table S1. The VIGS assay was carried out according to methods described previously (Liu et al. 2002). Cotton seedlings were grown in a growth chamber (21–25 °C) under long days light cycle (16 h:8 h, light:dark). The *Agrobacterium* mixture was infiltrated into the abaxial side of cotyledons of 8-day-old cotton seedings by needleless syringes. More than 15 Okra cotton individuals with okra leaves were infiltrated with pTRV2-*GhAS1* or pTRV2-*GhAS2*. Around 15 more TM-1 individuals with broad leaves were infiltrated with pTRV2-*GhAS1* or pTRV2-*GhAS2*. More than 15 Okra cotton individuals with okra leaves were infiltrated with mix of pTRV2-*GhKNLs* (*GhKNL2-GhKNL8)*. We mixed different *KNOX1* genes together in VIGS assays to inhibit functional complementation between paralogous genes as follows; *GhKNL2* and *GhKNL3* with *GhKNL4, GhKNL5* with *GhKNL6*, and *GhKNL7* with *GhKNL8*. These *KNOX1* gene combinations were chosen based on relationships represented by phylogenetic tree (Fig. 2). *KNOX1* genes can be classified into three subclasses. We chose these genes belong to the same subclass as a combination. pTRV2 was used as a negative control. Three independent tests were carried out for all VIGS assays.

Quantitative real time PCR (qPCR) analysis

To detect the expression of silenced genes (*GhOKRA* and *GhKNL2/3/4)*, the shoot tips (approximately 6 mm) of Okra cotton individuals infiltrated with either pTRV2-*GhAS1* or pTRV2-*GhAS2* at the same growth stage were harvested by wiping off the same number of leaves when the leaf shape had changed. We used the Biospin Plant Total RNA Extraction Kit (BioFlux, cat: BSC65S1) to extract total RNA from the shoot tips. First-strand cDNA was generated according to the manufacturer's instructions using TransScript® One-Step gDNA Removal and cDNA Synthesis SuperMix (TransGen Biotec Co., Ltd., cat: AT311) kits. The cotton *Histone 3* gene (His3, GenBank accession number: AF024716) was used as a reference gene. The qPCR primer sequences are listed in Table S1. They are universal for both the A and the D sub-genome homologs. So, we can detect all the two sub-genome homeologs. qPCR products were quantified according to the manufacturer's instructions using the ABI 7500 Real Time System (Applied Biosystems, USA) and the light cycler fast start DNA Master SYBR Green I kit (Roche, Basel, Switzerland).

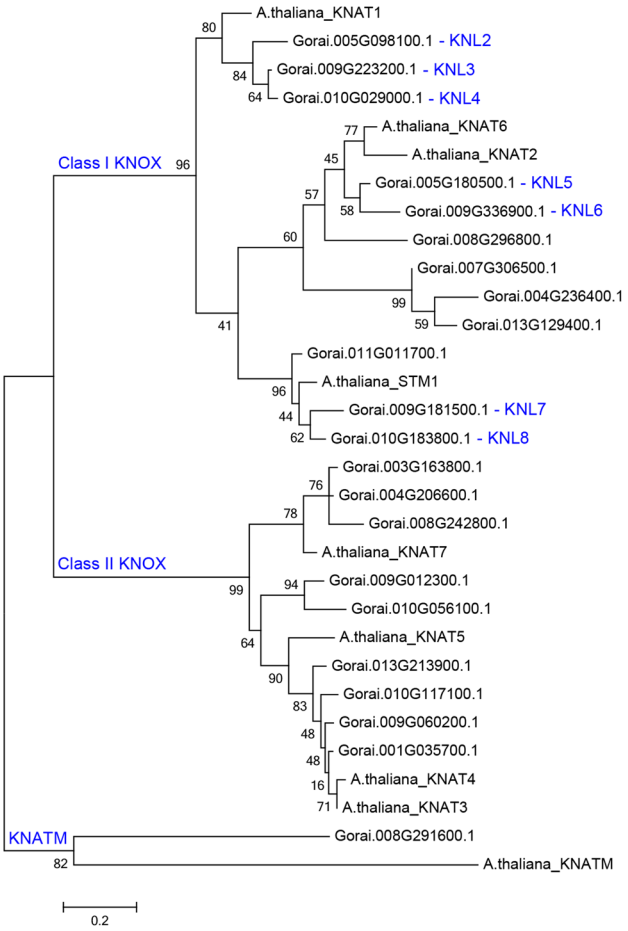


Fig. 2 Phylogenetic tree of *KNOX* genes of *Arabidopsis thaliana* and *Gossypium raimondii*. A maximum-likelihood tree was generated by aligning KNOX protein sequences of *G. raimondii* and *A. thaliana. G. raimondii* KNOX proteins were classified into three clades; class I KNOX, class II KNOX and KNATM. Seven *KNOX1* genes were selected and named *GhKNL2-GhKNL8* in this study. Phylogenetic analysis was carried out using the ML method with 1000 resampling replicates

Results

Plants silenced in either *GhAS1* or *GhAS2* produce compound leaves

To explore the relationship between an *HD-ZIP I transcription factor (GhOKRA)* and other genes in modulating leaf development, we cloned the corresponding cotton homologs of *ASYMMETRIC LEAVES1 (AS1), AS2* and seven *KNOX1* genes that regulate leaf development in *A. thaliana* (Table S2). Based on known *KNOX* gene domains, 22 *KNOX* genes were identified in the *G. raimondii* genome (Fig. 2). Of them, seven cotton class I *KNOX* genes, named *GhKNL2* (*KNOTTED1-LIKE*) to *GhKNL8*, that have close relationships with the corresponding

homologs in *A. thaliana* were cloned from the wild-type (WT) okra leaf cotton (Fig. 2) (Gong et al. 2014).

There was only one corresponding homolog of either *A. thaliana AS1* or *AS2* in each sub-genome in cotton. To our knowledge, there are no reports on how these two genes regulate leaf development in cotton. In the *GhAS1* and *GhAS2* virus induced gene silencing (VIGS) assays, the leaves of all okra leaf plants infiltrated with either pTRV2-*GhAS1* or pTRV2-*GhAS2* became split from the petiolar sinus and dissected 3 weeks after infiltration (Fig. 3a). Leaf blades at the base of the leaf vein disappeared. Three individual petiolulate leaflets arose at a new distinct node. Each leaflet had a long petiole (Fig. 3a, e). The deeply lobed leaves in the okra leaf cotton were replaced by ternate compound leaves; a phenomenon that has not been observed before in cotton. qPCR analysis showed that the expression of *GhAS1* and *GhAS2* was lower in pTRV2-*GhAS1* and pTRV2-*GhAS2*-silenced lines compared to controls (Fig. 3b). It is clear that the lobed simple leaves of okra cotton were transformed into compound leaves when either *GhAS1* or *GhAS2* expression was suppressed. These results demonstrate that *GhAS1* and *GhAS2* regulate the depth of leaf lobes in cotton. *GhAS1* may be involved in leaf adaxial/abaxial polarity in cotton, since individual plants infiltrated with pTRV2-*GhAS1* exhibited rumpled and curled leaves (Fig. 3a).

We used VIGS to explore how *GhOKRA* modulates leaf development. One month after infiltration with pTRV2 fused with *KNOX1* genes (*GhKNL2*-*GhKNL8*), the WT okra leaf cotton retained its okra leaf shape (Fig. 4i–l); however, *KNOX1* gene-silenced plants grew slowly (Fig. 4a–d), leaves in their shoot apex became compact and the distance between nodes was shorter than in controls (Fig. 4e–h, m). From these experiments, we could not reveal the roles of *KNOX1* in lobe development in cotton.

GhOKRA is necessary for compound leaf formation

GhOKRA controlled okra leaf development in the WT cotton. Ectopic expression of the wild cotton *GhOKRA* gene under the control of the 35S promoter (Pro35S::*GhOKRA*) led to a lobed leaf type rather than compound leaves in *A. thaliana* (Chang et al. 2016). To explore whether *GhAS1* and *GhAS2* regulate the depth of leaf lobes in collaboration with the *GhOKRA* gene, we silenced either *GhAS1* or *GhAS2* in both WT okra leaf cotton (Okra cotton) and mutant broad leaf cotton (TM-1). All *GhAS1* or *GhAS2*-silenced WT okra cotton plants had larger lobe depths than controls, suggesting that the functions of *GhAS1* and *GhAS2* are the same in leaf lobe development. *AS1* and *AS2* promote leaf adaxial-abaxial polarity specification and repress *KNOX* gene expression by forming AS1–AS2 protein complexes in *A. thaliana* (Fu et al. 2007; Guo et al. 2008). Therefore, we suppose that the AS1–AS2 complex might influence leaf lobe development as an upstream regulator of *GhOKRA*. All *GhAS1* or *GhAS2*-silencing in broad leaf TM-1 plants caused no change in the leaf lobes, although the second and third leaves of *GhAS1*-silenced plants were seriously curled (Fig. S1). The broad leaf TM-1 had a mutated non-functional *Ghokra* genotype which led to a different result compared with Okra cotton after silencing of *GhAS1* or *GhAS2*. These results suggest that *GhOKRA* is necessary for compound leaf formation.

GhKNL2/3/4 expression was elevated in either *GhAS1* or *GhAS2*-silenced plants

To demonstrate whether the AS1–AS2 complex negatively regulates *GhOKRA* expression, the transcript levels of *GhOKRA* in either *GhAS1* or *GhAS2*-silenced individuals were detected. There was no difference in the expression levels of *GhOKRA* in either *GhAS1* or *GhAS2*-silenced plants compared to the negative control (Fig. 3d), confirming that the AS1–AS2 complex does not regulate *GhOKRA*. The AS1–AS2 protein complex can repress *Knotted in A. thaliana 1 (KNAT1)* and *KNAT2* activity in leaves (Guo et al. 2008). When *KNAT1* was overexpressed in *A. thaliana*, the leaves showed ectopic stipules (Chuck et al. 1996). The transcript levels of *GhKNL2/3/4*, which are homologs of *KNAT1*, were thus analyzed in the shoot tips of either *GhAS1* or *GhAS2*-silenced plants. Compared with the negative control, the expression levels of all three homologs were elevated in the either *GhAS1* or *GhAS2*-silencing plants (Fig. 3c), with a greater increase observed in the *GhAS2*-silenced plants. These results suggest that *GhAS1* and *GhAS2* repress the expression of *KNOX1* in leaf primordia of cotton and this repression might be responsible for the change from lobed simple leaves to compound leaves in the *GhAS1* or *GhAS2*-silenced plants.

Co-expression of *GhLMI1-like* and *KNOX1* genes produces compound-like leaves in *A. thaliana*

In *A. thaliana*, the *GhOKRA* homolog was secondarily lost through duplication, leading to the evolution of a simple leaf (Vlad et al. 2014). Previously, we generated constitutively expressing *GhOKRA A. thaliana* plants with a lobed leaf (Chang et al. 2016). To determine whether coordination of the *LATE MERISTEM IDENTITY 1 (LMI1)-like* and *KNOX1* genes influences the formation of different leaf shapes, we crossed heterozygous constitutively expressing *GhOKRA A. thaliana* plants with homozygous *as1-101* and *as2-101* mutant plants in a *Landsberg erecta* (Ler) background (Yue et al. 2000; Sun et al. 2002; Xu et al. 2002). As compared to the WT type *A. thaliana* (Fig. 5a), the leaves of *as1-101* plants were heart-shaped and the leaf edges were slightly curled down with no lobes (Fig. 5b), while the mutant *as2-101* rosette leaves were broad (Fig. 5c). F_1

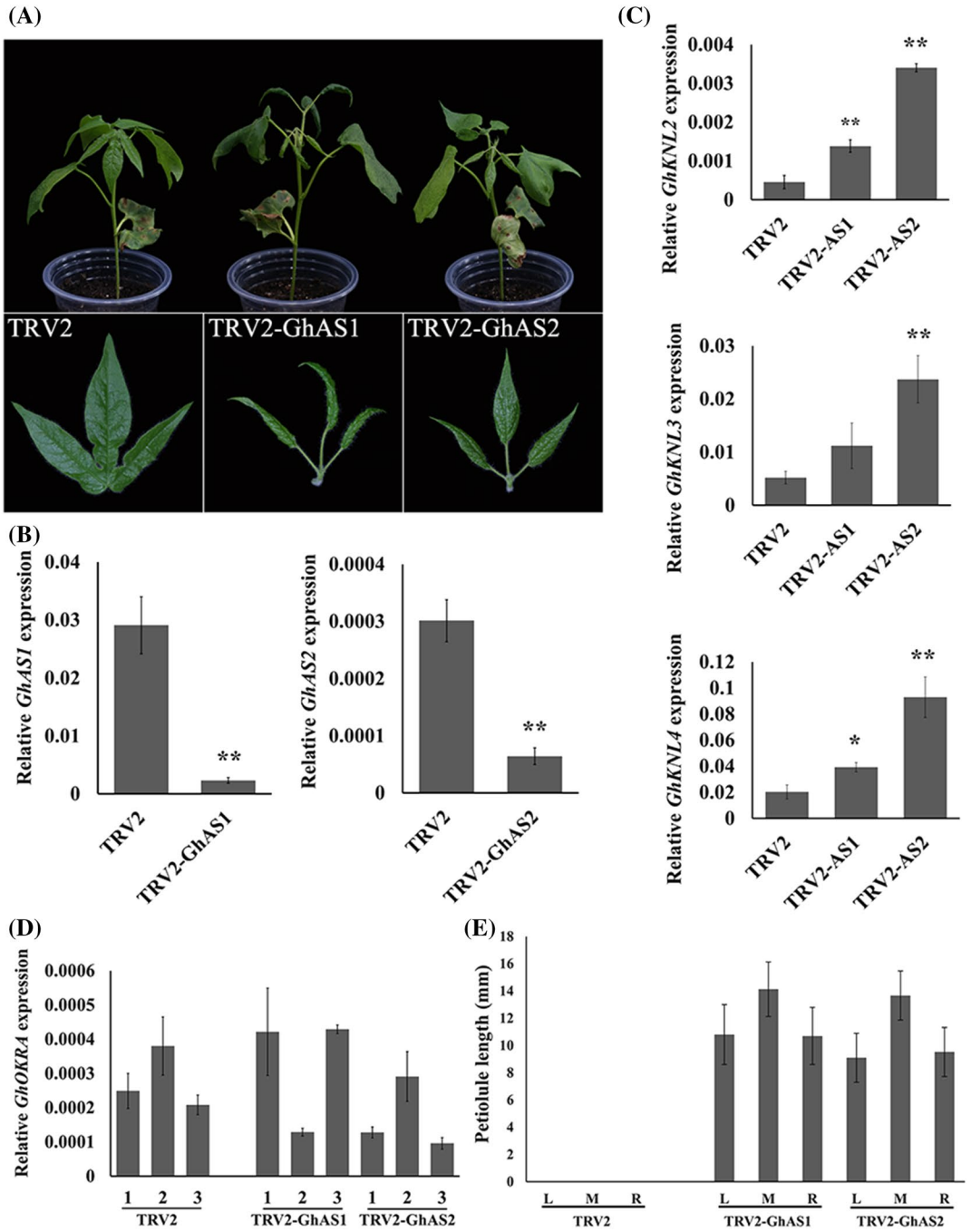


Fig. 3 Leaf phenotypes of VIGS *GhAS1* or *GhAS2*- silenced okra cotton plants. **a** Plant phenotypes from left to right: pTRV2, pTRV2-*GhAS1* and pTRV2-*GhAS2*. Leaf shape of negative control plants (left), okra cotton plants silenced by pTRV2-*GhAS1* (middle), and okra cotton plants silenced by pTRV2-*GhAS2* (right). The leaf lobes deepened and the blade at the base of the leaf vein disappeared in either *GhAS1* or *GhAS2*-silenced plants. **b** *GhAS1* transcript levels in *GhAS1*-silenced and negative control plants. *GhAS2* transcript levels in *GhAS2*-silenced and negative control plants. **c** *GhKNL2, GhKNL3*, and *GhKNL4* transcript levels in *GhAS1* or *GhAS2*-silenced and negative control plants. The expression of *GhKNL2, GhKNL3*, and *GhKNL4* was higher in silenced cotton than in controls. **d** *GhOKRA* transcript levels in three individuals silenced either *GhAS1* or *GhAS2* and negative control. There is no significant difference in comparision with the negative control. Data represent the means ± SDs of three replicated experiments. Asterisks indicate a significant difference at $P < 0.05$; Double asterisks indicate a significant difference at $P < 0.01$. **e** We measured the length of the formed left (L), middle (M) and right (R) petiolules in the fourth leaf of the VIGS plants in one of our VIGS assays by 37 days post infiltration (dpi). There is no petiolules in the control. Data represent the means ± SDs of 15 individuals

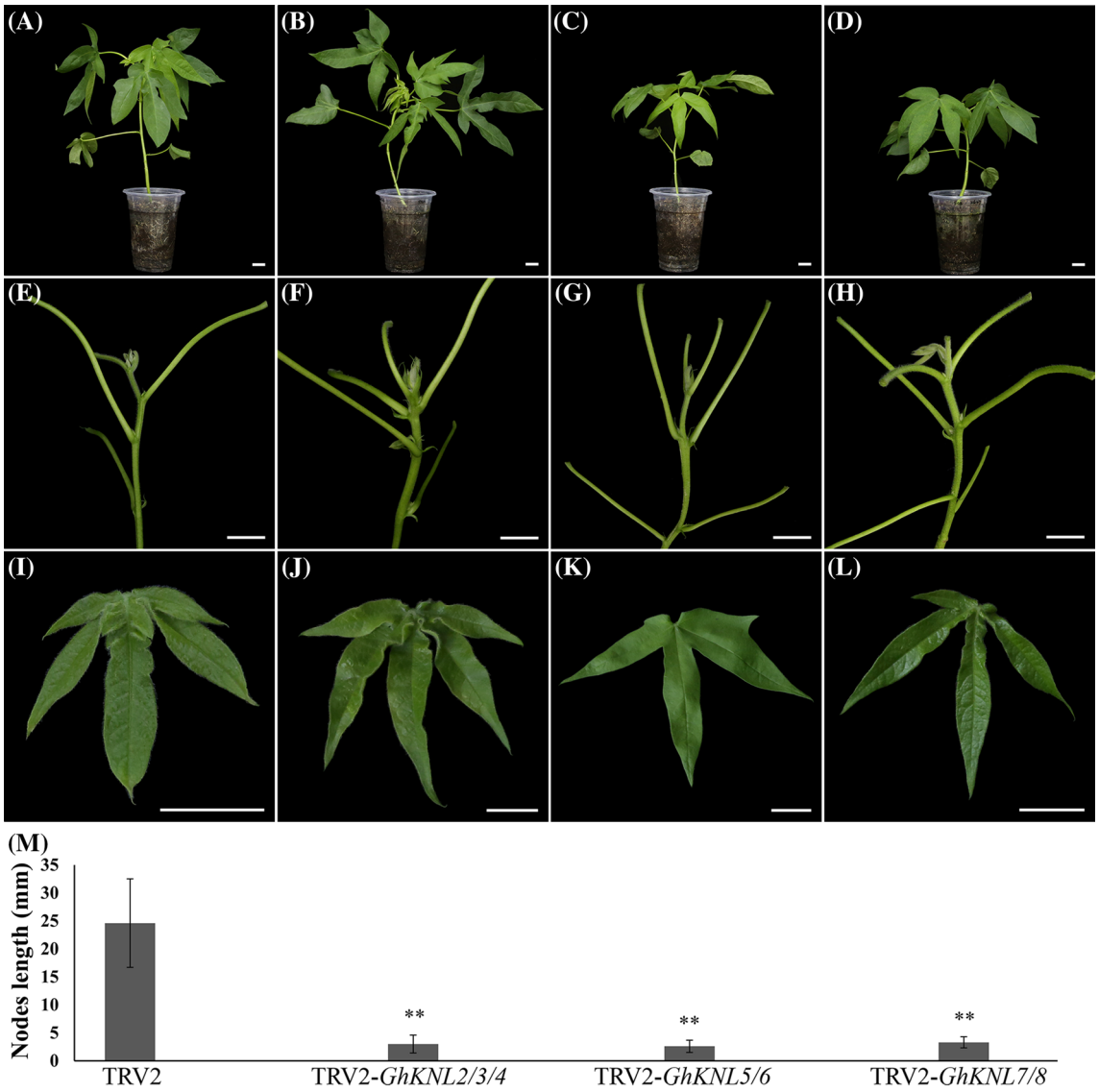
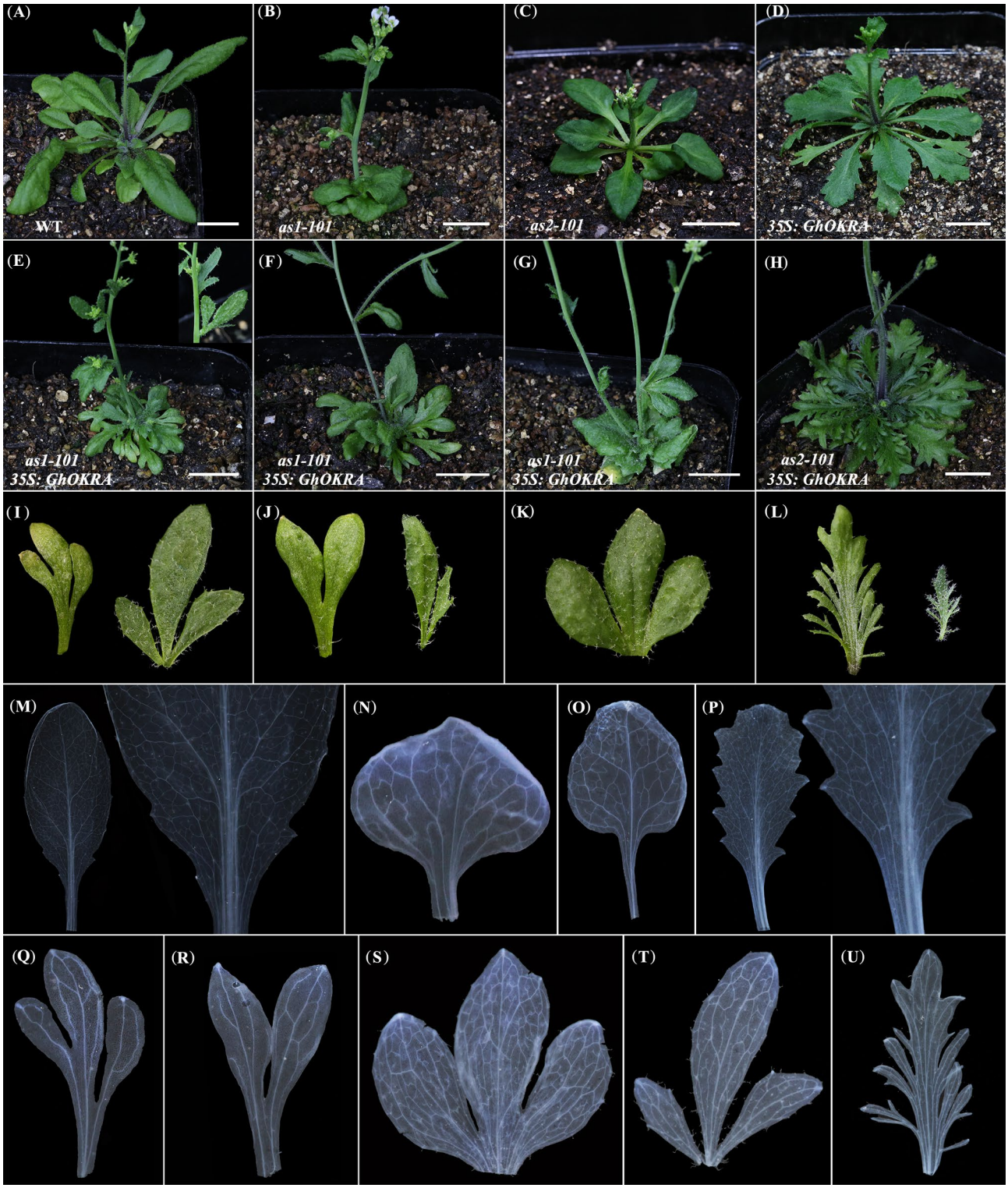


Fig. 4 The phenotype of okra cotton silenced by several paralogous genes from the *KNOX1* family. Plant phenotypes from left to right: pTRV2, pTRV2-*GhKNL2/3/4*, pTRV2-*GhKNL5/6*, and pTRV2-*GhKNL7/8*. **a–d** Whole plant scaning; **e–h** feature of the distance between the nodes; **i–l** Feature of the first leaf. **m** We measured the length of the nodes between fifth and sixth nodes of the VIGS plants in one of our VIGS assays by 61 dpi. Data represent the means ± SDs of 15 individuals. Double asterisks indicate a significant difference at $P < 0.01$. Plants with pTRV2-*GhKNLs* grew slowly and their leaves became compact in shoot apex of cotton, the distance between the nodes were decreased in comparision with the negative control, while the leaf shape showed no change. Scale bars = 2 cm

plants with a greater number of lobes than constitutively expressing *GhOKRA A. thaliana* plants were self-pollinated to generate F_2 populations. The F_2 segregated out several novel leaf types (Table S3) that were different from the WT, *as1-101* and *as2-101* mutant parents, and constitutively expressing *GhOKRA A. thaliana* plants. In Fig. 5, we show some typical individuals with novel leaf types from the two F_2 populations. Their genotypes were identified by cloning and sequencing (Figs. S2, S3). The rosette leaves of three F_2 individuals genotyped as *GhOKRA/as1-101* were divided into two or three parts along the proximal–distal axis (Fig. 5e–g). Their leaf lobes extended to leaf petioles. Some of their cauline leaves became compound-like leaves (Fig. 5i–k) and leaf-like structures were also observed at the petioles of cauline leaves (Fig. 5j). One individual from the cross between the *as2-101* and constitutively expressing *GhOKRA A. thaliana* plants showed extremely deeply lobed leaves (Fig. 5h), and their secondary lobes appeared on lobed leaves, thus increasing the leaf complexity (Fig. 5l).

To further confirm whether these novel leaves were formed from one divided leaf or the fusion of several leaves, we analyzed the leaf vein and found a difference between



rosette leaves and cauline leaves in the F_2 generated by crossing *as1-101* and constitutively expressing *GhOKRA* *A. thaliana*. The aberrant rosette leaves in F_2 plants had a reduced vascular structure. Lobes were divided between main veins, which indicates that the leaf was formed from a single complete leaf (Fig. 5q, r). The cauline leaves from F_2 plants had a complex vascular structure. Each leaflet vein was similar to those of wide-type leaves, which suggests that these cauline leaves became compound-like leaves through the fusion of simple leaves (Fig. 5s, t).

◂**Fig. 5** Phenotypes and leaf vein patterns of parents and their F_2 individuals. **a–d** Morphological observations of wild-type Col-0 (**a**), mutant *as1-101* (**b**), mutant *as2-101* (**c**) and constitutively expressing *GhOKRA A. thaliana* (**d**) plants. **e–g** The F_2 individuals of *as1-101* and constitutively expressing *GhOKRA A. thaliana* plants. **h** The F_2 individuals of *as2-101* and constitutively expressing *GhOKRA A. thaliana* plants. **i–k** The rosette leaf (left) and cauline leaf (right) of F_2 individuals of *as1-101* and constitutively expressing *GhOKRA A. thaliana* plants. **l** The rosette leaf (left) and cauline leaf (right) of F_2 individuals of *as2-101* and constitutively expressing *GhOKRA A. thaliana* plants. Rosette leaves were divided from leaf petioles, and cauline leaves became compound leaves in the F_2 individuals of *as1-101* and constitutively expressing *GhOKRA A. thaliana* plants. The F_2 individual of *as2-101* and constitutively expressing *GhOKRA A. thaliana* plants showed extremely deeply lobed leaves with secondary lobes. **m–p** Leaf vascular structure of wild-type Col-0 (**m**), mutant *as1-101* (**n**), mutant *as2-101* (**o**) and constitutively expressing *GhOKRA A. thaliana* plants (**p**). **q–t** Leaf vascular structure of F_2 individuals of *as1-101* and constitutively expressing *GhOKRA A. thaliana* plants. Two different leaf vein patterns of F_2 individuals indicated different methods of leaflet-like rosette and cauline leaf formation. **u** Leaf vascular structure of F_2 individuals of *as2-101* and constitutively expressing *GhOKRA A. thaliana* plants. Scale bars = 1 cm

The vascular structure of rosette leaves of F_2 plants from the cross between *as2-101* and constitutively expressing *GhOKRA A. thaliana* was more complex. Secondary lobes increased the complexity of the vascular structure (Fig. 5u).

In the *A. thaliana* F_2 populations generated in this study, the phenotypic segregation did not follow a Mendelian pattern. The constitutively expressing *GhOKRA A. thaliana* plants showed a deeply lobed leaf in the T1 progeny (Chang et al. 2016), but most had disappeared in the T_2 progeny. It is due to the limitations of the heterologous overexpression system which we used. Therefore, the number of progeny with dissected leaves was lower than expected (Table S3).

Discussion

Temporal regulation of *GhOKRA* and *KNOX1* genes during compound leaf development

We previously reported that *GhOKRA*-silenced plants display palmate leaves changed from deeply lobed okra leaves (Chang et al. 2016). Based on the present study, it seems like that elevated expression of *KNAT1* homologous genes in lobed leaf primordia results in the formation of dissected leaves. This is the first report of the generation of compound leaves in cotton (Fig. 3a). As we know, transient gene silencing is the limitation of the VIGS method. While the VIGS method seems like have advantages over transgenic plant methods in research into gene temporal regulation for gene function identification. We can explore the temporal expression differences between two genes by observing their phenotypic variation via VIGS. It has been reported that leaf marginal structures, such as leaflets, lobes and serrations, are formed during the phase of primary morphogenesis when leaves are primordia (Bar and Ori 2014). However, in the present study, we observed the phenotypes of leaf marginal structures after the leaves were fully expanded. In our VIGS assay, Okra cotton seedlings were infected with pTRV2-*GhAS1*, pTRV2-*GhAS2* and pTRV2-*GhOKRA* at the same time. We found that the phenotypes of either *GhAS1* or *GhAS2* gene-silenced plants were changed from the lobed to compound leaf at the fifth fully expanded leaf (L5). However, in the *GhOKRA*-silenced plants, the phenotype variations from the lobed to broad leaf were firstly observed at the seventh fully expanded leaf (L7) rather than at L5. These results suggest that silencing of *GhAS1* or *GhAS2* was effective in the primordium of L5 and those of younger leaves including L6 and L7, whereas silencing of *GhOKRA* affected leaf morphology only when silencing took place from a very early developmental stage (e.g., primordium of L7). The primordium of L7 is a younger initiating leaf than the primordium of L5. Therefore, it is likely that *GhOKRA* is involved in leaf primordia development at a very early stage, before *KNOX1* genes start to express in the *GhAS1* or *GhAS2*-silensed plants. We speculate that the expression of the *LMI1-like* gene allows lobe formation or limited lamina growth between leaflets first, and then *KNOX1* expresses to maintain the indeterminate nature of meristem cells in the developing primordia for leaflet formation (Fig. 6).

LMI1-like and *KNOX1* genes may regulate the development of different leaf shapes

The function of *LMI1-like* genes has been widely reported in different plants (Hofer et al. 2009; Vlad et al. 2014; Ni et al. 2017). Constitutively expressing homologous genes in *A. thaliana* produced lobed leaves (Chang et al. 2016; Ni et al. 2017). In the compound leaf plant, *Cardamine hirsuta*, the homologous gene, *RCO*, repressed lamina growth at the leaf margin (Vlad et al. 2014). However, the molecular context of the *LMI1-like* gene is not clear. *KNOX1* transcription factors maintain an indeterminate cell fate in SAMs and complex leaf primordia in most plants (Lincoln et al. 1994; Hay and Tsiantis 2010). Based on the present results, a hypothetical model was proposed to illustrate how the *LMI1-like* and *KNOX1* genes regulate the formation of different leaf shapes, including unlobed leaves, lobed leaves and compound leaves (Fig. 6). If primordia only expressed the *LMI1-like* gene without *KNOX1*, the leaf shape was lobed (Andres et al. 2016), as in the WT okra cotton (Fig. 1c), and as shown by constitutively expressing *LMI1-like* genes in *A. thaliana* (Fig. 5d). Removing the activity of the *LMI1-like* gene from lobed primordia led to an unlobed simple leaf type (Fig. 6), as seen in *A. thaliana* and TM-1 (Vlad et al. 2014; Chang et al. 2016). The *GhAS1* or *GhAS2*-silenced

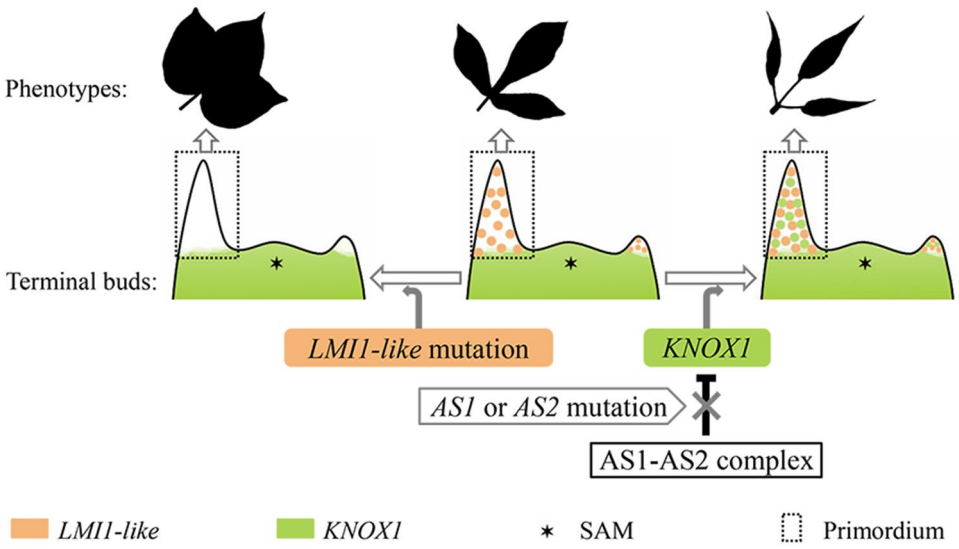


Fig. 6 Hypothesis of regulatory model for the *LMI1-like* and *KNOX1* genes. Different expression patterns of the *LMI1-like* and *KNOX1* genes regulate the formation of different leaf marginal structures. The primordia of lobed leaf is only developed when the *LMI1-like* gene express (Andres et al. 2016), but not *KNOX1*. The inactivity of the *LMI1-like* gene from lobed primordia will lead to the development of an unlobed simple leaf type (Chang et al. 2016). The AS1–AS2 protein complex repress the activity of *KNOX1* in leaf primordia, but their mutations can remove this repression. This repression might be responsible for the change from lobed simple leaves to compound leaves in the *GhAS1* or *GhAS2*-silenced plants. Orange mark *LMI1-like* gene; green mark *KNOX1* genes; asterisks mark the SAM; dashed panes mark primordia

plants with lobed leaves produce compound leaves (Fig. 3a). As the *GhAS1* and *GhAS2* repress the expression of *KNOX1* (Fig. 3c). So, the *KNOX1* expression in lobed primordia might result in compound leaves (Fig. 6). When primordia expressed *KNOX1* without the *LMI1-like* gene, the leaf shape was either unlobed or lobed, possibly depending on the expression levels of *KNOX1* genes. Neither *GhAS1* nor *GhAS2*-silenced TM-1 plants had altered leaf shapes (Fig. S1), while ectopic expression of *KNAT1* under the control of the 35S promoter in *A. thaliana* produced lobed leaves (Chuck et al. 1996). Therefore, co-expression of *LMI1-like* and *KNOX1* genes in primordia could result in compound leaves: we generated leaflet-like *A. thaliana* leaves by crossing *as1-101* and *as2-101* mutants with constitutively expressing *GhOKRA* plants (Fig. 5). The deeply lobed leaves of *C. hirsuta rco* mutant caused by the fusion of leaflets also supports this hypothetical model (Vlad et al. 2014).

Intricate and complicated factors regulate leaf development

Many factors repress the activity of *KNOX1* in leaf primordia, such as polar auxin transport and *TEOSINTEBRANCHED1/CYCLOIDEA/PROLIFERATING CELL FACTOR* (*TCP*), as well as the AS1–AS2 protein complex (Scanlon 2003; Guo et al. 2008; Li et al. 2012). *POLYCOMB REPRESSIVE COMPLEX* (*PRC*)2 interacts with the AS1–AS2 protein complex to stably silence *KNAT1* and *KNAT2* in leaf primordia (Lodha et al. 2013). In addition, in pea, the *FLORICAULA/LEAFY* ortholog, *UNIFOLIATA*, rather than *KNOX1*, was found to control the development of compound leaves (Hofer et al. 1997). It has also been reported that *CUC* genes define boundaries between leaflets in compound leaf plants (Blein and Laufs 2008; Berger et al. 2009). Leaf development is changeable and complicated and is regulated by many factors. Ectopic -expressing *GhOKRA A. thaliana* plants develop many lobes in leaves in the same direction along the medial–lateral axis (Chang et al. 2016). The *A. thaliana* F_2 progeny showed lobes in two directions; along the medial–lateral and proximal–distal axes (Fig. 5i–l). *KNOX1* might affect the position or pattern of *GhOKRA* expression. The dissected leaves of *GhAS1* and *GhAS2*-silenced okra cotton plants indicate that compound leaves are formed from deeply lobed simple leaves (Fig. 3a). There were two types of leaf vein distribution in *A. thaliana* F_2 progeny (Fig. 5q–u), and leaf vein development also affected leaflet formation (Runions et al. 2017). Whether compound leaves are formed from collections of simple leaves deserves further research (Champagne and Sinha 2004). In this study, we propose that the *LMI1-like* and *KNOX1* genes regulate the formation of several margin structures (Fig. 6).

Acknowledgements This work was financially supported in part by Supported by the earmarked fund for China Agriculture Research System, and the Distinguished Discipline Support Program of Zhejiang University. We thank Prof. Lin Xu and Prof. Hai Huang for *A. thaliana* mutant seeds of *as1-101* and *as2-101*. We thank for Medium-term

Gene Bank of Cotton in China, Institute of Cotton Research of CAAS providing seeds of Super okra used in the present study.

Author contributions TZZ designed the research; LJC, GFM performed research; TZZ, LJC, GFM analyzed all data and wrote the manuscript. LJC, GFM, JQD constructed VIGS assay. LJC, GFM, YH analyzed qPCR result. GFM constructed *A. thaliana* crosses. All authors discussed results and commented on the manuscript.

Compliance with ethical standards

Conflict of interest The authors declare that they have no conflict of interest.

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