Compound Leaf Development and Evolution in the Legumes

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INTRODUCTION

Leaves produced by angiosperms can be either simple or compound. Simple leaves have a single blade unit supported by a petiole, whereas compound leaves have multiple blade units, called leaflets, attached to a rachis. Class 1 KNOTTED1-like (KNOX1) genes have been implicated in regulating leaf complexity across vascular plants. In all model species examined to date, KNOX1 genes are expressed in the shoot apical meristem (SAM) (Smith et al., 1992; Lincoln et al., 1994; Nishimura et al., 1998, 1999; Waites et al., 1998) and appear necessary for meristem formation and maintenance (Long et al., 1996; Vollbrecht et al., 2000). In simple-leaved model species, such as maize (Zea mays), rice (Oryza sativa), Arabidopsis thaliana, tobacco (Nicotiana tabacum), and snapdragon (Antirrhinum majus), KNOX1 genes are expressed in the SAM and are downregulated at sites of leaf initiation (P0). Overexpression of KNOX1 genes in simple-leaved plants results in formation of leaves with ectopic outgrowths, lobes, and ectopic shoots (Vollbrecht et al., 1990; Sinha et al., 1993; Schneeberger et al., 1995; Muehlbauer et al., 1999).

By contrast, in tomato (Solanum lycopersicum), which has compound leaves, KNOX1 genes are expressed in both the apical meristem and leaf primordia, and overexpression results in ramification of the compound form, suggesting a role for KNOX1 genes in compound leaf development (Hareven et al., 1996; Chen et al., 1997; Parnis et al., 1997; Janssen et al., 1998). Across vascular plants, KNOX1 gene expression in leaves is correlated with leaf compounding that arises during primary morphogenesis of the early leaf primordium, although not in cases where compounding is due to later postprimordial morphogenesis (Bharathan et al., 2002). These results hint that KNOX1 expression in compound primordia is part of the typical pathway that results in the production of a mature compound leaf. In support of this model, reduction of SHOOTMERISTEMLESS expression in Cardamine hirsuta, a compound-leaved relative of Arabidopsis, results in reduced leaflet production (Hay and Tsiantis, 2006).

The legumes (Fabaceae), a clade of angiosperms that have a diversity of simple and compound leaves, might be an exception to this trend. Pea (Pisum sativum) does not show expression of KNOX1 genes in developing compound leaves. In pea, UNIFOLIATA (UNI), an ortholog of the floral regulators FLOREALIA (FLO) and LEAFY (LFY) from snapdragon and Arabidopsis, respectively, is necessary for compound leaf development, suggesting that it might play the role otherwise fulfilled by KNOX1 genes (Hofer et al., 1997, 2001; Gourlay et al., 2000). The only other reported flo/lfy mutant in a compound-leaved species is falsiflora (fa) in tomato. Unlike UNI, FA plays only a minor role in compound leaf development: fa mutant leaves have...
the normal number of primary leaflets but a reduced number of minor leaflets (Molinero-Rosales et al., 1999).

The snapdragon flo mutant and the Arabidopsis Ily mutant have indeterminate shoots in place of flowers and are unable to make the transition from inflorescence to floral meristem (Coen et al., 1990; Schultz and Haughn, 1991; Huala and Sussex, 1992; Weigel et al., 1992). Analyses of flo/ily mutants from other species, such as aberrant leaf and flower from petunia (Petunia hybrida), fa from tomato, flo/fly (zf1) and zf2 from maize, and un from pea, indicate that these orthologs play a universal role in the specification of flower organs and the vegetative-to-reproductive phase transition (Hofer et al., 1997; Souer et al., 1998; Molinero-Rosales et al., 1999; Bombles et al., 2003). The aforementioned observations raise several questions. First, when during Fabaceae compound leaf evolution did the KNOX1 gene cascade cease to be associated with the generation of compound leaves? Here, we show that a range, and by implication perhaps the majority, of species in Fabaceae have expression of KNOX1 proteins associated with compound leaves. However, a large sub-clade of papilionoid legumes, of which pea is a member, that happens to be marked by loss of one copy of the 25-kb inverted repeat in the chloroplast genome (Figure 1; the inverted repeat-lacking clade [IRLC]; Wojciechowski et al., 2000) appears to include compound-leafed species that lack KNOX1 expression in leaves. We suggest that the role of generating compound leaves in this clade may be associated, instead, with the FLO/LFY gene. Another question is when during evolution did FLO/LFY orthologs acquire a more prominent role in compound leaf development? We addressed this by investigating the contribution of FLO/LFY to leaf complexity in a member of the Fabaceae outside of the IRLC by reducing expression of the FLO/LFY ortholog in transgenic soybean (Glycine max). Our results demonstrate that FLO/LFY functions in a minimal capacity in compound leaf development in soybean, suggesting that this gene acquired a more significant role no earlier than the divergence of the Hologalegina clade from the other legumes. Finally, in species where KNOX1 orthologs are not correlated with compound leaf development, we explore whether the genetic networks that control leaf complexity are still sensitive to KNOX1 expression in leaf primordia. Overexpression of a KNOX1 gene in alfalfa (Medicago sativa), a member of the IRLC, increases leaf complexity, answering this question in the affirmative.

RESULTS

Phylogenetic Relationships and KNOX1 Protein Expression Patterns in the Fabaceae

The Fabaceae are traditionally subdivided into three subfamilies: the monophyletic subfamilies Mimosoideae (mimosoids) and Papilionoideae (papilionoids) nested within a paraphyletic subfamily Caesalpinioideae (caesalpinoids) (Figure 1). Leaves of Fabaceae species are usually pinnately (occasionally trifoliolate or palmate) compound. Considering leaf complexity in the legumes and its sister families Polygalaceae, Quillajaceae, and Surianaceae (relationships unresolved), the ancestral state for leaf complexity in the legumes is likely to be compound. Although a number of taxa with simple leaves are known from legumes, this character state probably has originated multiple times within this group (R. Geeta, M.F. Wojciechowski, and M. Lavin, personal communication). The caesalpinoids Cercis and Bauhinia, which comprise the earliest branching lineage in the legume family (Figure 1), have leaves that appear simple (bilobed). The IRLC is a group of predominately temperate, herbaceous papilionoids that are distinguished from the rest of the legumes by several distinctive morphological and molecular features, including lack of pulvini, accumulation of the nonprotein amino acid canavanine, and loss of one copy of the inverted repeat in the chloroplast genome (Lavin et al., 1990; Wojciechowski et al., 2000). Species representative of the major legume clades were chosen for expression studies (Figure 1).

In seed plants, the sites of future leaf initiation are marked by a downregulation of KNOX1 expression (Smith et al., 1992; Lincoln et al., 1994; Nishimura et al., 1998, 1999; Waites et al., 1998). We
confirmed the efficacy of our KNOX1 antibody by examining KNOX1 downregulation at the leaf initiation site in both early-diverging and more recently diverged clades in the Fabaceae and found that in all cases KNOX1 proteins are downregulated at P0 (Figure 2).

Consistent with previous reports, we did not detect KNOX1 expression in developing pea leaves (Figure 3E). We examined KNOX1 expression in soybean and found that KNOX1 proteins were indeed expressed in its developing leaf primordia (Figure 4F). Since these results are contrary to those for pea, we undertook analysis of KNOX1 expression in a phylogenetic context. KNOX1 expression was analyzed in seven other species of compound-leafed legumes to determine whether this lack of expression was peculiar to pea. Chinese wisteria (Wisteria sinensis), alfalfa, and fava bean (Vicia faba), all members of the IRLC, resembled pea in that they lacked KNOX1 expression in developing leaves (Figures 3F to 3H). Four non-IRLC species examined, Mimosa pudica, Acacia hindsii, bean (Phaseolus vulgaris), and Lotus japonicus, two mimosoids and two papilionoids, respectively, all showed similarity to soybean in having KNOX1 expression in developing leaf primordia (Figures 4G to 4J). Therefore, legumes with compound leaves that do not have KNOX1 expression within their primordia appear to be restricted to the IRLC. Our results suggest that there may have been a loss in the regulation of leaf complexity by KNOX1 genes before the diversification of the IRLC from the other Fabaceae.

The tribe Cercideae (Cercis, Bauhinia) has species with unifoliolate, bilobate, or bifoliolate leaves. Recent work suggests that the unifoliolate blade of the Cercis leaf is a derived trait (Owens, 2000). Two main hypotheses have been put forward to explain the derivation of the unifoliolate leaf in these taxa (reviewed in Cusset, 1966; Van Der Pijl, 1951). The fusion hypothesis suggests that the ancestral leaf type was bifoliolate compound and

The Soybean FLO/LFY Ortholog Plays a Limited Role in Compound Leaf Development

The pea gene UNI and the tomato gene FA are both orthologs of the genes FLO and LFY from snapdragon and Arabidopsis,
respectively. In addition to floral abnormalities, the uná mutant in pea causes a drastic simplification of the normally compound leaf form (Hofer et al., 1997, 2001; Gourlay et al., 2000). The leaves of fa plants have the normal number of primary leaflets but a reduced number of small leaflets (Molinero-Rosales et al., 1999). Given that FA is only a minor determinant of leaf complexity in tomato and UNI is the major contributor to the same process in pea, we wanted to investigate when FLO/LFY orthologs adopted a more significant role in regulating compound leaf development. We tested this event occurred by examining the role of two FLO/LFY orthologs from soybean. In most angiosperms, FLO/LFY is a single-copy gene. However, soybean is tetraploid, accounting for the presence of two copies of this gene in the genome (see Supplemental Figures 1A and 1B online). A single RNA interference (RNAi) vector, designed to silence both of these genes, was constructed and used to transform soybean (see Supplemental Figure 1C online). Thirteen Gm LFY RNAi transgenic lines were obtained, and 11 of the lines had altered floral development, resembling that of flo/lfy mutants in other species. Leaf-like structures replaced the floral organs, and the number of whorls of organs within the flower increased (Figure 6B). Presence of the floral phenotype confirmed that expression of the Gm LFY genes was suppressed. After prolonged growth in the greenhouse (>6 months under high-light conditions), all of the lines eventually set a small amount of seed, facilitating examination of the second generation. In the second generation, the same 11 Gm LFY RNAi lines had severely altered flowers, identical to those present in the first generation. Nine of the 11 second generation Gm LFY RNAi lines with aberrant flowers produced leaves at the second node that showed reduced complexity. In some instances, the leaves produced had a single blade, while other leaves had two, rather than three, leaflets (Figures 6D, 6E, 6G, and 6H). Leaves produced at the third and subsequent nodes were trifoliolate. Therefore, in addition to the conserved role that the soybean FLO/LFY orthologs play in floral development, they also play a role, albeit minor, in soybean compound leaf development. Based on this and the

Figure 4. Mature Leaf Form and KNOX1 Immunolocalization Patterns in Select Members of Non-IRLC Fabaceae.

Compound-leafed Fabaceae outside of the IRLC soybean ([A] and [F]), M. pudica ([B] and [G]), L. japonicus ([C] and [H]), A. hindsii ([D] and [I]), and bean ([E] and [J]) have KNOX1 expression in the SAM and in developing leaves. Insets of (F), (I), and (J) show details of leaves and meristems from Acacia, soybean, and bean, respectively. Dashed boxes depict regions magnified in insets. Arrows point to KNOX1 expression in leaves. Bars = 30 \( \mu \)m in all cases, except in insets of (F) and (J) and red boxed inset of (I), where bars = 10 \( \mu \)m.

Figure 5. KNOX1 Expression Patterns Support the Fusion Hypothesis for the Unifoliolate Cercis Leaf.

(A) Morphology of the unifoliolate Cercis leaf.
(B) KNOX1 proteins are localized within the SAM and developing leaves of Cercis. Bar = 30 \( \mu \)m.
phylogenetic position of soybean within the Millettioids, we can hypothesize that the acquisition of a prominent role for FLO/LFY orthologs in compound leaf development occurred no earlier than the time of divergence of the Hologalegina and Millettioids from their common ancestor (Figure 1).

After germination and expansion of the cotyledons, wild-type soybean produces two simple leaves in opposite phyllotaxy at the first node (Figure 6C). Production of adult trifoliolate leaves in spiral phyllotaxy commences at the second node. Interestingly, in the second generation, all 11 Gm LFYRNAi lines with altered flower development produced leaves in opposite phyllotaxy at the second node (Figures 6D to 6H). Usually after the second node, and occasionally after the third node, all lines transitioned to leaf production in a spiral pattern. Thus, the phase change from the juvenile stage to the adult stage may have been delayed by at least one node in the Gm LFYRNAi plants.

**Overexpression of a KNOX1 Gene in Alfalfa, a Member of the IRLC**

We placed the tomato gene LeT6 under control of the 35S promoter and introduced it into alfalfa to determine if expression of a KNOX1 gene outside of the meristem is capable of increasing leaf complexity in a member of the IRLC. Wild-type alfalfa invariably has trifoliolate pinnate leaves (Figure 7A, Table 1). Twelve of 16 independent 35S:LeT6 transgenic lines appeared vegetatively and reproductively normal. However, four 35S:LeT6 lines had severe phenotypes, including small leaflets, increased serration of leaflets, and reduced petioles (Figures 7B and 7D). In addition, these four lines produced extra leaflets on the rachis of the leaves, indicating an increase in leaf complexity (Figure 7C). Quantitative RT-PCR was used to investigate transgene expression levels in young leaves from two lines (35S:LeT6-59-067 and 35S:LeT6-59-030) that produce leaves with extra leaflets and five lines without phenotypic alterations. The two lines with increased leaflet numbers had high levels of LeT6 transcript, while the other five lines had negligible to low levels of LeT6 transcript (Figure 8).

The two lines with strong leaf phenotypes included in the quantitative RT-PCR analysis were further characterized. Stems were randomly selected, and leaflet number per leaf for consecutive leaves was evaluated. In transgenic line 35S:LeT6-59-067, 16 of 30 leaves examined had more than three leaflets. The mean number of leaflets present on these 16 leaves was 4.63 (SD = 0.62) (Table 1). In the transgenic line 35S:LeT6-59-030, 11 of 30 leaves examined had more than three leaflets. The mean number of leaflets present in these 11 leaves was 4.81 (SD = 0.40) (Table 1). The distribution of leaves with extra leaflets along the stem appeared to be random. In addition, these lines seemed to be reproductively compromised, as none of them flowered after 10 months of growth under standard conditions, during which time wild-type plants flowered and set seed. A similar phenomenon was also observed in transgenic tobacco overexpressing the KNOX1 gene NTH15 where plants with severe phenotypes did not produce flowers (Tamaoki et al., 1997).

**Figure 6. Phenotypes of Soybean Gm LFYRNAi Lines.**

(A) Wild-type soybean flower.
(B) Gm LFYRNAi line ST40-141 with altered floral development.
(C) Wild-type soybean shoot with two simple, opposite leaves at the first node and one trifoliolate leaf at the second node.
(D) Line ST40-99 with two simplified opposite leaves at the second node.
(E) Line ST40-189 with two opposite leaves, one simple and the other trifoliolate, at the second node.
(F) Line ST40-189 with two opposite leaves at the second node. Note that one leaf appears to have two fused leaflets.
(G) Another individual from line ST40-189 with one simple leaf at the second node.
(H) Line ST40-114 with two simple opposite leaves at the first node (the second leaf is highly reduced) and a simple leaf at the second node.

**Figure 7. Overexpression of a KNOX1 Gene in Alfalfa.**

(A) Wild-type alfalfa. Bar = 2 cm.
(B) 35S:LeT6 line with reduced internodes, reduced petioles, and small leaves. Bar = 2 cm.
(C) 35S:LeT6 line 59-015 produces leaves with extra leaflets (arrow).
(D) 35S:LeT6 line 59-067 produces leaflets that are deeply lobed (asterisk) and serrated (arrowheads).
than leaves at subsequent nodes. 

These lines, the transition from juvenile to adult leaf production in the first node of wild-type soybean. One interpretation is that in phyllotaxy at the second node, reminiscent of juvenile leaves at tomato, there is context with altered phyllotaxy and bifurcated vegetative meristem development. Besides the data from UNI, a limited number of reports have indicated that FLO/LFY genes regulate, or have the potential to regulate, vegetative growth. For example, transgenic poplar overexpressing LFY have small deformed leaves that are cup-like (Rottmann et al., 2000). Furthermore, Ahearn et al. (2001) showed that transgenic tobacco plants with reduced expression of NFL1 germinated with fused cotyledons and usually died. Those that survived had a bush-like appearance with altered phyllotaxy and bifurcated vegetative meristems. The authors attributed the phenotype of these plants to NFL1 playing a role in allocation and placement of primordia at the vegetative shoot apex. Moreover, 35S:NFL1 Arabidopsis plants were noted to have abnormal leaf shape and rosette development under short-day conditions (Ahearn et al., 2001). Finally, in addition to floral abnormalities, the tomato fa mutant has a reduced number of small leaflets present on its compound leaf (Molinero-Rosales et al., 1999).

The phenotype of the Gm LFYRNAi lines is consistent with FLO/LFY playing a role in vegetative development in addition to its well-known role in flowering. With simplified leaves restricted to the second node, the phenotype of the Gm LFY RNAi plants is less overt than that of the tomato fa mutant. Absence of a more dramatic phenotype in the Gm LFY RNAi lines may be due to the fact that soybean possesses trifoliolate leaves, which have a lower order of complexity than tomato leaves. Reduction of FLO/LFY expression manifests a somewhat more palatable phenotype in tomato, perhaps because it has a higher order of complexity than soybean does. Alternatively, soybean leaves produced at the second node may be more sensitive to the loss of FLO/LFY than leaves at subsequent nodes.

Most Gm LFY RNAi plants had simplified leaves in opposite phyllotaxy at the second node, reminiscent of juvenile leaves at the first node of wild-type soybean. One interpretation is that in these lines, the transition from juvenile to adult leaf production has been delayed. Alternatively, instead of regulating a vegetative phase transition, the Gm LFY genes play a role in establishing phyllotaxy early in soybean growth. Combined with their contribution to leaf complexity, this could mimic a phase change role. In support of the latter model, transgenic tobacco plants with cosuppressed levels of the tobacco FLO/LFY gene, NFL1, produce leaves in an irregular phyllotactic pattern (Ahearn et al., 2001). Snapdragon flo mutants also fail to shift from spiral to whorled phyllotaxy during flower development (Coen et al., 1990; Carpenter et al., 1995). Additionally, effects on inflorescence phyllotaxy have been associated with the FLO/LFY genes ZFL1 and ZFL2 in maize (Bombles et al., 2003).

**Table 1. Overexpression of a KNOX1 Gene Increases Leaflet Number in Alfalfa**

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Mean No. of Leaves</th>
<th>No. of Leaves with More Than Three Leaflets</th>
<th>Mean No. of Leaflets on Leaves with Increased Compounding</th>
</tr>
</thead>
<tbody>
<tr>
<td>Wild type</td>
<td>3.00 (SD = 0.00)</td>
<td>0 (SD = NA)</td>
<td>NA (SD = NA)</td>
</tr>
<tr>
<td>35S:LeT6-59-067 (n = 30)</td>
<td>3.87 (SD = 0.94)</td>
<td>16 (SD = 4.63)</td>
<td>0.62 (SD = 0.40)</td>
</tr>
<tr>
<td>35S:LeT6-59-030 (n = 30)</td>
<td>3.67 (SD = 0.92)</td>
<td>11 (SD = 4.82)</td>
<td>0.40 (SD = 0.40)</td>
</tr>
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NA, not applicable.

**DISCUSSION**

**The Role of FLO/LFY Orthologs in Soybean Vegetative Development**

Despite intensive studies of FLO/LFY genes in flowering, relatively little is known about the functions of these genes in vegetative development. Besides the data from UNI, a limited number of reports have indicated that FLO/LFY genes regulate, or have the potential to regulate, vegetative growth. For example, transgenic poplar overexpressing LFY have small deformed leaves that are cup-like (Rottmann et al., 2000). Furthermore, Ahearn et al. (2001) showed that transgenic tobacco plants with reduced expression of NFL1 germinated with fused cotyledons and usually died. Those that survived had a bush-like appearance with altered phyllotaxy and bifurcated vegetative meristems. The authors attributed the phenotype of these plants to NFL1 playing a role in allocation and placement of primordia at the vegetative shoot apex. Moreover, 35S:NFL1 Arabidopsis plants were noted to have abnormal leaf shape and rosette development under short-day conditions (Ahearn et al., 2001). Finally, in addition to floral abnormalities, the tomato fa mutant has a reduced number of small leaflets present on its compound leaf (Molinero-Rosales et al., 1999).

The phenotype of the Gm LFY RNAi lines is consistent with FLO/LFY playing a role in vegetative development in addition to its well-known role in flowering. With simplified leaves restricted to the second node, the phenotype of the Gm LFY RNAi plants is less overt than that of the tomato fa mutant. Absence of a more dramatic phenotype in the Gm LFY RNAi lines may be due to the fact that soybean possesses trifoliolate leaves, which have a lower order of complexity than tomato leaves. Reduction of FLO/LFY expression manifests a somewhat more palatable phenotype in tomato, perhaps because it has a higher order of complexity than soybean does. Alternatively, soybean leaves produced at the second node may be more sensitive to the loss of FLO/LFY than leaves at subsequent nodes.

Most Gm LFY RNAi plants had simplified leaves in opposite phyllotaxy at the second node, reminiscent of juvenile leaves at the first node of wild-type soybean. One interpretation is that in these lines, the transition from juvenile to adult leaf production has been delayed. Alternatively, instead of regulating a vegetative phase transition, the Gm LFY genes play a role in establishing phyllotaxy early in soybean growth. Combined with their contribution to leaf complexity, this could mimic a phase change role. In support of the latter model, transgenic tobacco plants with cosuppressed levels of the tobacco FLO/LFY gene, NFL1, produce leaves in an irregular phyllotactic pattern (Ahearn et al., 2001). Snapdragon flo mutants also fail to shift from spiral to whorled phyllotaxy during flower development (Coen et al., 1990; Carpenter et al., 1995). Additionally, effects on inflorescence phyllotaxy have been associated with the FLO/LFY genes ZFL1 and ZFL2 in maize (Bombles et al., 2003).

**Activation of the KNOX1 Network in Alfalfa Leaves**

The compound leaf developmental program in the IRLC may be caused by a transient phase of indeterminacy conferred by FLO/LFY. Overexpression of the KNOX1 gene LeT6 in alfalfa may have prolonged this transient indeterminate state in developing leaves and increased leaf compounding even though KNOX1 genes are not part of the developmental cascade leading to compound leaves in the IRLC. It is possible that some of the genes regulated by FLO/LFY in the IRLC that promote leaf compounding may be the same targets normally regulated by KNOX1 genes in other compound-leaved species. Accordingly, expression of KNOX1 genes in developing leaves of alfalfa may have resulted in increased leaflet number due to a dosage effect: increased transcription of the same gene network. Alternatively, KNOX1 and FLO/LFY genes may regulate parallel pathways, both of which promote indeterminacy. At this point, it is unknown how much overlap exists between the genetic networks of LFY and KNOX1 genes in compound leaf development.

It appears that a compound leaf developmental program may need to be established before overexpression of KNOX1 genes can promote leaflet formation. Overexpression of KNOX1 genes from the 35S promoter in the simple-leaved species Arabidopsis did not result in the formation of compound leaves. Rather, the development of its simple leaves was altered such that lobed leaves were formed (Lincoln et al., 1994). This argues that perhaps the compound leaves of members of the IRLC are

![Figure 8. Quantitative RT-PCR Analysis of LeT6 RNA Levels in Several Alfalfa Transgenic Lines and the Wild Type.](image-url)
fundamentally different from leaves of simple-leafed species, despite the fact that they both lack \textit{KNOX1} expression in primordia.

\textbf{Evolution of KNOX1 and FLO/LFY Pathways in Legume Compound Leaf Development}

Our results, combined with other studies, show that \textit{KNOX1} genes are involved in establishing complexity in early leaf development in the caesalpinioioid, mimosoid, and many papilionoid lineages of the Fabaceae but that this role was lost when the IRLC, which includes pea, fava bean, alfalfa, and wisteria, diverged from the other legumes. Compound leaf development in pea is substantially controlled by the \textit{FLO/LFY} ortholog \textit{UNI} (Hofer et al., 1997). Analysis of the tomato \textit{fa} mutant (Molinero-Rosales et al., 1999) and reduction of \textit{Gm LFY} expression in transgenic soybean suggest that \textit{FLO/LFY} orthologs play a less significant role in controlling leaf complexity in these two species. Considering the subordinate role of \textit{FLO/LFY} orthologs in compound leaf development in soybean and tomato and the major role \textit{UNI} plays in pea leaf complexity, it is likely that \textit{FLO/LFY} orthologs acquired a more significant role in compound leaf development no earlier than the divergence of the Hologalegina clade from the other legumes. Coupled with the \textit{KNOX1} expression survey results presented here, it is tempting to speculate that this prominent role of \textit{FLO/LFY} orthologs in compound leaf development was established on the stem lineage of the IRLC (\~{}39 million years ago; Lavin et al., 2005). Further sampling of the role of \textit{FLO/LFY} in taxa within the Hologalegina, particularly within the Robinoids (Figure 1), would be helpful to define more precisely when this change in the role of \textit{FLO/LFY} in compound leaf development occurred.

Delineating the timing of these two events, loss of \textit{KNOX1} expression from developing leaves and \textit{FLO/LFY} adopting a critical role in compound leaf development, is important to understanding how this evolutionary phenomenon occurred. It could be argued that the lineage leading to the IRLC could not go from depending on \textit{KNOX1} expression in compound leaves to relying on \textit{FLO/LFY} for compound leaf development without passing through a simple-leafed intermediate. However, our data suggest that this transition was possible in a compound-leafed state as there was likely some or complete genetic redundancy between \textit{KNOX1} and \textit{FLO/LFY} in the ancestral lineage. This could have allowed for \textit{KNOX1} expression to be lost from leaf primordia without moving off a compound leaf adaptive peak.

\textbf{Regulatory versus Coding Sequence Evolution in KNOX1 and FLO/LFY Genes}

Variation in morphological traits in evolution can be achieved by different routes, which include, but are not limited to, changes in a transcription factor’s expression pattern, biochemical properties (DNA binding properties or the ability to interact with other protein partners), and/or array of targets, either by recruitment of new targets, or loss of old targets. Currently, there is some debate surrounding the relative significance of \textit{cis}-regulatory mutations versus mutations in coding regions of genes to drive morphological evolution (Hoekstra and Coyne, 2007; Wray, 2007). The data presented here hint that both mechanisms likely have been employed in the evolution of the \textit{KNOX1} and \textit{FLO/LFY} pathways governing compound leaf development in the legumes.

We compared \textit{KNOX1} downregulation at the leaf initiation site in both early-diverging and more recently diverged clades in the Fabaceae and found that in all cases, including members of the IRLC, \textit{KNOX1} proteins are downregulated at P0. This suggests a conserved function of the \textit{KNOX1} genes in leaf initiation throughout the Fabaceae that parallels the role of \textit{KNOX1} genes in leaf initiation across seed plants. Furthermore, comparative analysis of the promoters from two \textit{KNOX1} genes, \textit{BREVIPEDICELLUS} from \textit{Arabidopsis} and its ortholog from \textit{C. hirsuta}, which has compound leaves, shows that expression pattern differences between these genes can be attributed to their \textit{cis}-regulatory regions (Hay and Tsiantis, 2006). Interestingly, overexpression of a \textit{KNOX1} gene in alfalfa, a member of the IRLC, resulted in increased leaflet numbers. This suggests that the \textit{KNOX1} targets regulated in compound leaf development in angiosperms outside of the IRLC are present in alfalfa and are still sensitive to \textit{KNOX1} regulation. Thus, the loss of a role for \textit{KNOX1} genes in compound leaf development in the IRLC likely happened by a loss of expression, rather than an inability of \textit{KNOX1} proteins to activate targets promoting leaflet development. While this loss of expression is best explained by evolution of the \textit{cis}-regulatory sequences of the \textit{KNOX1} genes themselves, at this point it is not possible to rule out that \textit{trans}-changes to a protein upstream of the \textit{KNOX1} genes may be responsible.

The role of \textit{FLO/LFY} genes appears to have been pliable in the evolution of plant morphology, and changes in both its expression pattern and biochemical properties have been implicated in driving morphological variation (Weigel et al., 1992; Shu et al., 2000; Busch and Gleissberg, 2003; Yoon and Baum, 2004; Baum et al., 2005; Maizel et al., 2005). Notably, Busch and Gleissberg (2003) reported great variation in \textit{FLO/LFY} expression in the vegetative SAM across eudicots. Despite the fact that \textit{FLO/LFY} is expressed in developing leaves of both simple- and compound-leafed species, these authors suggested that there is a correlation between prolonged expression of the \textit{FLO/LFY} gene in leaf primordia and development of compound leaves. Compound leaves can be viewed as either equivalent to simple leaves that have been subdivided into leaflets or as transiently indeterminate structures that share properties with both shoots and leaves (Champagne and Sinha, 2004). \textit{FLO/LFY} plays a universal role in the transition from vegetative to reproductive development in angiosperms by inducing several floral identity genes. Production of a flower marks a shift in meristem fate from indeterminate to determinate, suggesting that \textit{FLO/LFY} may act to initiate a genetic cascade leading to determinacy (Coen et al., 1990). If compound leaves are transiently indeterminate, then an acquired role of \textit{FLO/LFY} in compound leaf development is to promote a transient indeterminate condition on the developing leaf. In this event, it is unlikely that only a shift in the expression domain of \textit{FLO/LFY} could account for its contribution to leaf complexity. It is more likely that the genetic cascade downstream of \textit{FLO/LFY} orthologs has been altered to affect leaflet development. \textit{FLO/LFY} may have acquired new targets for the regulation of compound leaf development throughout
angiosperm evolution. This could have occurred by two equally parsimonious events: either changes in the biochemical properties of FLO/LFY, which would permit interaction with genes that regulate compound leaf development, or by changes in the regulatory sequences of a gene that is high in the cascade-promoting compound leaf development, making it sensitive to FLO/LFY regulation.

Progress toward understanding the role of LFY in flowering has been made in the model plant Arabidopsis using microarray-based identification of targets directly upregulated by LFY (William et al., 2004). Similarly, direct FLO/LFY downstream targets that promote compound leaf development could be determined in transgenic tomato and alfalfa using microarray analyses in concert with inducible FLO/LFY overexpression. Comparison of FLO/LFY interactions with their targets between select simple- and compound-leafed species may help provide insight into how FLO/LFY has evolved to acquire a role in compound leaf development.

METHODS

Plants Sampled for Molecular Study and Sources of Material

Representative plants with simple and compound leaves were sampled across the Fabaceae. Plant material for immunolocalization experiments was obtained as follows: Phaseolus vulgaris, Glycine max, Vicia faba, and Pisum sativum were grown from seed purchased at Ace Hardware. All other plant material was generously provided by Tim Metcalf and Ernesto Sandoval at the Botanical Conservatory of the University of California at Davis.

Immunolocalization Protocol and Antibodies

KNOX1 immunolocalizations were performed as described previously (Bharathan et al., 2002) using a polyclonal antibody against KN1 (a generous gift from Sarah Hake, prepared as described in Smith et al., 1992). Sections were photographed on an Olympus BX50 microscope equipped with an Olympus PM-20 exposure control unit.

Genomic DNA Extractions, RNA Extractions, and cDNA Synthesis

Genomic DNA was extracted from mature leaf tissue using the CTAB extraction protocol (Doyle and Doyle, 1990) with minor modifications. RNA was extracted from apical meristems using the RNAwiz reagent (Ambion) according to the manufacturer’s instructions. cDNA was synthesized from ~5 μg of total RNA using random primers and the Superscript II cDNA synthesis kit (Invitrogen) following the manufacturer’s suggested protocol.

Quantitative RT-PCR Analysis

Glycerinaldehyde-3-phosphate dehydrogenase C subunit (Ms GAPDH) was used as the constitutively expressed control gene and the primers were qMsGAPDHf/qMsGAPDHR (5'-CTGGAGAGGTGGAAAGAC-3' / 5'-GCTCAAAACTGACACATCC-3'). The primers used for quantitative RT-PCR for LeT6 were qLeT6f/qLeT6r (5'-GGCTCATCCTCTACTA CCATCGTCTC-3' / 5'-ATTCCACCACTACTGCTAC-3'). The sequence for alfalfa GAPDH was found by searching Medicago sativa ESTs using the BLAST utility (National Center for Biotechnology Information) with a protein query. The resulting sequence (accession number ABA07956) was used for primer design. All primers were designed using the BeKton Designer software (PREMIER Biosoft International). The quantitative RT-PCR experiment was validated by an independent quantitative RT-PCR replicate. The data shown are of a representative experiment. The quantitative RT-PCR was performed according to published protocols (Yamagishi et al., 2005) except that IQ SYBR Green Supermix was used. The annealing temperature for the experiment was 50°C.

Cloning FLO/LFY Orthologs

FLO/LFY orthologs were cloned from either genomic DNA or cDNA by PCR amplification of sequences using degenerate primers whose sequence was based on previously published data from other species. The PCR products were cloned using the TOPO-TA cloning kit (with the PCR 2.1-TOPO cloning vector: Invitrogen). Plasmids containing amplified fragments were sequenced commercially by Davis Sequencing.

Construction of Gm LFYRNAi Vector

The second exon of Gm LFY1 was combined with the third exon of Gm LFY2 (total length of ~700 bp) and was used to create the Gm LFYRNAi vector. The combined fragment was cloned into pRNA69 (Kwong et al., 2003) in two locations in opposite orientation. The RNAi cassette was subcloned into pTF101.1 (Paz et al., 2004) and used for soybean transformation.

Plant Transformations

Soybean transformation was conducted as described by Paz et al. (2004). 3SS:FLAG-LeT6 was introduced into M. sativa by Agrobacterium tumefaciens EHA105. Medicago transformations were performed according to Shahin et al. (1986) by the Ralph M. Parsons Foundation Plant Transformation Facility at the University of California at Davis (College of Agricultural and Environmental Sciences).

Accession Numbers

Sequence data from this article can be found in the GenBank/EMBL data libraries under accession numbers EF198177 and EF198178 for Gm LFY1 and Gm LFY2, respectively.

Supplemental Data

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

Supplemental Figure 1. Gm LFY RNAi Cassette Present in the Gm LFYRNAi Vector.

Supplemental Data Set 1. Alignment of Pea UNIFOLIATA and Deduced Partial Gm LFY1 and Complete Gm LFY2 Proteins.

Supplemental Data Set 2. Alignment of Partial Genomic DNA and Complete Genomic DNA Sequences of Gm LFY1 and Gm LFY2.

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