A Conserved Molecular Framework for Compound Leaf Development

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Diversity in leaf shape is produced by alterations of the margin: for example, deep dissection leads to leaflet formation and less-pronounced incisions result in serrations or lobes. By combining data from the shoot apical meristem, we propose that compound leaves maintain an organogenic region at their margin from which leaflet primordia emerge (1, 2). Two different pathways have been recruited to promote this organogenic activity during the multiple independent origins of compound leaves in seed plants. One pathway involves expression in the primordia of compound leaves of class 1 homeodomain KNOTTED1-like (KNOXI) transcription factors that were initially identified for their role in maintenance of meristem identity (3–5). This pathway is active in a wide range of flowering seed plants, including Solanum lycopersicum and Cardamine hirsuta. A second pathway involving the UNIFOLIATA (UNI) gene is found in Pisum sativum, which does not express KNOXI genes in the leaf primordium. UNI encodes a member of the LEAFY (LFY) family of transcription factors, initially identified for its role in floral meristem identity (6, 7). Despite progress in understanding what promotes the organogenic potential of compound leaves, the mechanistic basis of leaflet formation and delimitation is less clear. The generation of activity maxima of auxin, a small indolic hormone, is one such mechanism that facilitates initiation and separation of both leaves at the shoot apical meristem and leaflets from the rachis (8–10). Other key regulators of organ initiation and delimitation are the NAM/CUC genes, which are members of a large evolutionarily conserved family of plant transcription factors that are subdivided into NAM/NO APICAL MERISTEM (NOM) and CUC3 (CUP-SHAPED COTYLEDON3) clades (11–14). They are expressed in the boundary of organ primordia, where they repress growth to allow organ separation (15). In addition, they are involved in meristem establishment via their activation of KNOXI expression (16).

Because previous work showed that AtCUC2 is required for Arabidopsis leaf serration (17), we hypothesized that NAM/CUC3 genes could have a broader role in leaf dissection. To test this hypothesis, we analyzed the function of NAM/CUC3 genes in a selection of five eudicots with compound leaves (Aquilegia caerulea, S. lycopersicum, S. tuberosum, C. hirsuta, and P. sativum) that show contrasting phylogenetic positions, genetic controls and patterning of leaflet development, dissection of leaflets, and leaflet specialization (Fig. 1, A, E, I, M, and fig. S1) (18). We cloned 11 NAM/CUC3 genes from these species, and phylogenetic analysis showed that they group either into NAM (AcNAM, SinNAM, SinNAM, PsNAM1, PsNAM2, ChCUC1, and ChCUC2) or CUC3 (AcCUC3, SiCUC3, PsCUC3, and ChCUC3) clades (fig. S2). The NAM/CUC3 genes had a typical expression pattern in the boundary domain at the base of organ primordia, a pattern that is complementary to the cell proliferation marker HISTONE H4 (fig. S3). This suggested conserved roles in defining boundary and organ separation by local repression of cell proliferation.

To determine whether the NAM/CUC3 genes have a role in defining compound leaf morphology, we examined their expression during leaf development. A similar expression pattern was observed in the initiation and separation of leaflets. This suggests that the conserved expression pattern of the NAM/CUC3 genes in the boundary domain defines the leafletMargin.
observed in all species examined (Fig. 1 and fig. S4). NAM/CUC3 genes were expressed in a narrow strip of cells at the distal boundary of leaflet primordia, whereas no expression was observed in the proximal region (e.g., Fig. 1, B, G, K, N, and U, and fig. S3). NAM/CUC3 expression preceded the actual outgrowth of leaflet primordia (e.g., Fig. 1, F and J, and fig. S3). Given the diversity of the species analyzed here, this conserved NAM/CUC3 gene expression pattern is likely to reflect a fundamental mechanism of leaflet formation.

Next, we undertook a series of functional analyses in A. caerulea, S. lycopersicum, P. sativum, and S. tuberosum. NAM/CUC3 expression was observed in leaflet primordia, leaflet margins, and stipule primordia. NAM/CUC3 expression was also observed in the distal boundary of young [asterisk in (S)] and older [asterisks in (T)] leaflet primordia. Scale bars indicate 1 cm [(A), (E), (I), (M), and (R)] or 0.1 mm [(B) to (D), (F) to (H), (J) to (L), (N) to (Q), and (S) to (U)].
Reducing NAM/CUC3 activity leads to a simplification of compound leaves. (A) Successive leaves formed on an A. caerulea plant silenced for the AcPDS, AcNAM, and AcCUC3 genes. Note the progressive smoothing of the leaflet margins from leaf 1 to 3 (arrowheads). At the final stage (leaf 4), corresponding to early silencing, a simple leaf with an entire margin is formed. (B) Control leaf of P. sativum silenced for PsPDS with three pairs of leaflets and three pairs of tendrils subtended by a pair of stipules (st). (C) P. sativum leaf silenced for PsPDS, PsNAM1/2, and PsCUC3 showed a single leaf blade, and fewer leaflets and tendrils were formed in P. sativum after PsNAM1/2 and PsNAM1/2/PsCUC3 silencing (Fig. 2, A, C, and D, and fig. S6). The number of primary leaflets was slightly reduced in S. lycopersicum silenced plants, whereas secondary and intercalary leaflet numbers were highly reduced (Fig. 2F and fig. S6). This indicated that NAM/CUC3 expression at the base of outgrowing leaflets is required for their separation, a function resembling that of organ primordia separation in the meristem. Third, the number of leaflets was reduced. A. caerulea showed fusions of the leaflets with the rachis or between leaflets, and P. sativum showed tendril fusions (Fig. 2, D and F, and fig. S6). This indicated that NAM/CUC3 expression at the base of outgrowing leaflets is required for their separation, a function resembling that of organ primordia separation in the meristem. Third, the number of leaflets was reduced. A. caerulea showed fusions of the leaflets with the rachis or between leaflets, and P. sativum showed tendril fusions (Fig. 2, D and F, and fig. S6). This indicated that NAM/CUC3 expression at the base of outgrowing leaflets is required for their separation, a function resembling that of organ primordia separation in the meristem.

The S. lycopersicum goblet mutant confirmed the role of the S. lycopersicum gene during compound leaf ontogeny. The goblet mutant displays developmental defects reminiscent of nam/cuc3 mutants in other plant species (19), and sequencing of three goblet mutants revealed that each harbored a mutation in the S. lycopersicum gene (fig. S7). Leaves regenerated from ectopic meristems of these mutants showed the same morphological defects as S. lycopersicum silenced plants (Fig. 2G).

In C. hirsuta, we stably reduced the expression of all three NAM/CUC3 genes by overexpressing MIR164b, which gives rise to mature microRNA164 that directs ChCUC1 and ChCUC2 repression, and silencing ChCUC3 through hairpin-mediated RNA interference (RNAi) (fig. S8). Individually, MIR164b overexpression and ChCUC3 RNAi led to leaflet fusions and reduced leaflet lobing and number. The severity of these defects was increased in double transgenics (Fig. 2H and I, and fig. S8).

Altogether, our data revealed a conserved requirement for NAM/CUC3 genes during leaflet formation, leaflet separation, and margin dissection. In addition, we showed the existence of a morphological continuity from leaf margin to leaflet dissection, which is facilitated at the molecular level by a common underlying mechanism involving NAM/CUC3 genes.

Next, we investigated NAM/CUC3 gene expression in response to modifications of other regulators of compound leaf development. First, we observed no NAM/CUC3 expression in the simple leaves of S. lycopersicum Lanceolate (La)
mutants containing a gain-of-function TEOSINTE BRANCHED1/CYCLOIDEA/PCF (TCP) TCP gene (20) and in P. sativum uni mutants (fig. S9), consistent with NAM/CUC3 expression being required for leaflet formation. Second, we analyzed C. hirsuta transgenics bearing a KNOTTED1-GR fusion (3). A 2-day-long activation of the KNOTTED1-GR fusion led to increased ChCUC1-3 expression (Fig. 3A). ChCUC activity was required for the induction of ectopic leaflets by KNOTTED1-GR (3) because they did not form when the ChCUC genes were silenced (Fig. 3B). Altogether, these analyses showed that LA, UNI, and KNOXI genes influence NAM/CUC3 expression, which in turn regulates leaflet formation.

Conversely, we tested whether NAM/CUC3 genes had an effect on the expression of KNOXI and LFY-like genes. Accumulation of KNOXI (Tk1 and Tk2 in S. lycopersicum) and LFY-like (SLFY in S. lycopersicum and UNI in P. sativum) transcripts was reduced in lines silenced for the NAM/CUC3 genes (Fig. 3, C and D). In line with these results, KNOXI reporter (ChSTM:GUS) expression was reduced in the developing leaf of a C. hirsuta line with reduced ChCUC activity (Fig. 3E). This indicated that NAM/CUC3 genes are required for proper expression of KNOXI/LFY-like genes during compound leaf development. Together, these findings advocate the existence of a feed-forward regulatory loop between NAM/CUC3 and KNOXI/LFY-like genes and indicate that this coordinately regulated expression controls leaflet formation.

We reveal a dual evolutionarily conserved role for NAM/CUC3 genes during eudicot leaf development (fig. S10). First, NAM/CUC3 are required to dissect compound leaves into leaflets and leaflet margins into serrations or lobes. This is a local, probably cell-autonomous function of the NAM/CUC3 genes because they are expressed in the boundary domain. Second, NAM/CUC3 genes are required for leaflet formation. This is likely to be a non-cell-autonomous effect of NAM/CUC3 genes. Differences between formation of a leaflet, a lobe, or a serration could depend on different capacities of cells to respond to NAM/CUC3 expression. For example, factors such as TCP proteins (22–22) may limit growth and prevent leaflet formation.

In contrast to the KNOX and LFY-like pathways, whose contributions vary between the compound-leaved eudicots, the requirement for NAM/CUC3 activity during leaflet formation is conserved in all species tested here and is likely to be extensively conserved within eudicots. Our results suggest that species-specific activity of either the KNOXI or the LFY pathway induces expression of NAM/CUC3 genes, which are responsible for leaflet formation and maintenance of KNOXI/LFY expression through a positive feedback loop.

The dual role of the NAM/CUC genes revealed here during leaf development could also exist in the plant apex, where the topology of NAM/CUC3 expression is similar to that observed during leaflet formation [i.e., they are expressed at the boundary between the meristem and the primordium (23)]. It will therefore be interesting to determine whether NAM/CUC3 proteins, in addition to their well-established role in organ separation at the apex, also contribute to the outgrowth of the leaf primordium and whether NAM/CUC3 action in leaves is mediated by auxin maxima (8–10). This evolutionarily conserved deployment of both NAM/CUC3 genes and auxin in both leaf and leaflet formation may reflect the common evolutionary origin of leaves from branched shoots (10).

Our results highlight an unexpected role for the interleaflet boundary domain patterned by NAM/CUC3 genes in directing novel axes of growth that give rise to leaflets. This role is conceptually similar to that of boundary domains acting during animal development (24, 25) and hence provides an example of a common developmental logic operating to sculpt organ form in evolutionary lineages where multicellularity evolved independently.

References and Notes
18. Materials and methods are available as supporting material on Science Online.
Human Fetal Hemoglobin Expression Is Regulated by the Developmental Stage-Specific Repressor BCL11A

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Diffences in the amount of fetal hemoglobin (Hbf) that persists into adulthood affect the severity of sickle cell disease and the β-thalassemia syndromes. Genetic association studies have identified sequence variants in the gene BCL11A that influence Hbf levels. Here, we examine BCL11A as a potential regulator of Hbf expression. The high-Hbf BCL11A genotype is associated with reduced BCL11A expression. Moreover, abundant expression of full-length forms of BCL11A is developmentally restricted to adult erythroid cells. Down-regulation of BCL11A expression in primary adult erythroid cells leads to robust Hbf expression. Consistent with a direct role of BCL11A in globin gene regulation, we find that BCL11A occupies several discrete sites in the β-globin gene cluster. BCL11A emerges as a therapeutic target for reactivation of Hbf in β-hemoglobin disorders.

Genome-wide association studies have yielded insights into the genetics of complex diseases and traits (1, 2). In the majority of instances, the functional link between a genetic association and the underlying pathophysiology remains obscure. The level of fetal hemoglobin (Hbf) is inherited as a quantitative trait and is of enormous clinical relevance, given its role in ameliorating the severity of the principal hemoglobin disorders, sickle cell disease and β-thalassemia (3, 4). Two recent genome-wide association studies have identified three major loci containing a set of five common single-nucleotide polymorphisms (SNPs) that account for ~20% of the variation in Hbf levels (5–7). Moreover, several of these variants predict the clinical severity of sickle cell disease (5), and at least one of these SNPs may also affect clinical outcome in β-thalassemia (6). The SNP with the largest effect size is located in the second intron of a gene on chromosome 2, BCL11A. Although BCL11A has been investigated in the context of lymphocyte development (8, 9), its role in the red blood cell lineage has not been previously appreciated.

Hbf is a tetramer of two adult α-globin polypeptides and two fetal β-like γ-globin polypeptides. During gestation, the duplicated γ-globin genes constitute the predominant genes transcribed in the β-globin cluster. After birth, γ-globin is replaced by adult β-globin (4), a process referred to as the “fetal switch.” The molecular mechanisms responsible for this switch have remained largely undefined. Moreover, the extent to which γ-globin gene expression is silenced in adulthood varies among individuals (5, 6). In nonanemic individuals, Hbf makes up <1% of total hemoglobin. However, in those with sickle cell disease and β-thalassemia, higher levels of γ-globin expression partially compensate for defective or impaired β-globin gene production, which ameliorates the clinical severity in these diseases. The results of recent genetic association studies provide candidate genes to test for involvement in control of the γ-globin genes. In light of the strong association of SNPs within the BCL11A locus with Hbf levels in disparate populations (5–7, 10), we explore here the hypothesis that the product of the BCL11A locus, a multi– zinc-finger transcription factor, encodes a stage-specific regulator of Hbf expression.

As a first step in seeking how variation at the BCL11A locus might relate to γ-globin expression, we examined expression of BCL11A in erythroid cells (11). In primary adult human erythrocytes, BCL11A is expressed as two major isoforms at the protein and RNA levels (Fig. 1A). These isoforms (designated XL and L) differ only in usage of the 3′ terminal exon and function similarly in other settings (9). We have recently fine-mapped the BCL11A-Hbf association signal to a variant in close linkage disequilibrium (LD) with the SNP rs4671393 (5). Because this association has been confirmed in multiple independent European and African diasporic populations, we examined expression of the XL and L isoforms of BCL11A as a function of the genotype at rs4671393 in lymphoblastoid cell lines from the HapMap European (CEU) and African (YRI) groups. The utility of this strategy has been shown in prior studies examining the consequences of common genetic variation on gene expression (12–14). We observed a striking difference in expression for both isoforms between individuals of different SNP genotypes (Fig. 1B). Cells homozygous for the “low-Hbf” allele expressed a lower level of BCL11A transcripts than those homozygous for the “low-Hbf” allele or heterozygous for both alleles. The difference in expression between the “high” and “low” Hbf–associated BCL11A alleles is 3.5-fold. Hence, relatively modest differences in BCL11A expression appear to be associated with changes in Hbf expression.

To our surprise, we observed that the embryonic erythroblast cell line K562 expressed very little, if any, of the XL and L isoforms but, instead, expressed shorter variant proteins (Fig. 1C). To assess whether the difference between adult erythroblasts and K562 cells reflected developmental stage-specific control of BCL11A or the malignant nature of these cells, we examined stage-matched, CD71+/CD235+ erythroblasts isolated from adult bone marrow, second-trimester fetal liver (FL), and circulating first-trimester primitive cells. FL and primitive erythroblasts, which both robustly express γ-globin (15), expressed predominantly shorter BCL11A variants (Fig. 1C). Although we continue to investigate the structure of these variant proteins, our findings indicate that the BCL11A locus is developmentally regulated, such that full-length XL and L isoforms are expressed almost exclusively in adult-stage erythroblasts. Independently, the genetic data strongly argue that the level of XL and L isoforms is influenced by sequence variants in the BCL11A gene.