clock in peripheral tissues, but not the master clock in the suprachiasmatic nucleus (*26*, *27*), and there is increasing evidence of links between diet, metabolism, and the clock (*28*, *29*). Similarly, our data show that in plants a photosynthesis-related signal, possibly sucrose or a derivative, can affect setting of the clock in roots but not in shoots. In summary, the plant clock is organ-specific but not organ-autonomous.

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30. We thank A. Millar (Edinburgh), S. A. Kay (San Diego), and C. R. McClung (Dartmouth) for *Arabidopsis* lines; I. Carré (Warwick) and E. M. Tobin (Los Angeles) for the LHY- and CCA1-specific antisera, respectively; K. Edwards (Advanced Technologies, Cambridge) for advice on COSOPT; and J. Galbraith (Faculty of Biomedical and Life Sciences, University of Glasgow) for microarray experiments. The work was supported by the Leverhulme Trust. J.A.M. holds a fellowship from the Spanish Ministry of Education and Science.

Supporting Online Material

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5 June 2008; accepted 29 September 2008
10.1126/science.1161403

A Conserved Molecular Framework for Compound Leaf Development

Thomas Blein,[1] Amada Pulido,[1] Aurélie Vialette-Guiraud,[1]* Krisztina Nikovics,[1]† Halima Morin,[1,2] Angela Hay,[3] Ida Elisabeth Johansen,[4] Miltos Tsiantis,[3] Patrick Laufs[1]‡

Diversity in leaf shape is produced by alterations of the margin: for example, deep dissection leads to leaflet formation and less-pronounced incision results in serrations or lobes. By combining gene silencing and mutant analyses in four distantly related eudicot species, we show that reducing the function of *NAM*/*CUC* boundary genes (*NO APICAL MERISTEM* and *CUP-SHAPED COTYLEDON*) leads to a suppression of all marginal outgrowths and to fewer and fused leaflets. We propose that *NAM/CUC* genes promote formation of a boundary domain that delimits leaflets. This domain has a dual role promoting leaflet separation locally and leaflet formation at distance. In this manner, boundaries of compound leaves resemble boundaries functioning during animal development.

Leaves of seed plants can be simple, with a single leaf blade, or compound when divided into distinct leaflets (*1*, *2*). Additionally, margins of both simple and compound leaves can elaborate less-pronounced incisions such as serrations or lobes. Regardless of the final shape, leaves are initiated as simple primordia from the shoot apical meristem. Primordia of 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*/*CUC3* genes, which are members of a large evolutionarily conserved family of plant transcription factors that are subdivided into *NAM* (*NO APICAL MERISTEM*) 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 leaflet margins, and leaflet specialization (Fig. 1, A, E, I, M, and R, and fig. S1) (*18*). We cloned 11 *NAM/CUC3* genes from these species, and phylogenetic analysis showed that they group either into *NAM* (*AcNAM*, *SlNAM*, *StNAM*, *PsNAM1*, *PsNAM2*, *ChCUC1*, and *ChCUC2*) or *CUC3* (*AcCUC3*, *StCUC3*, *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

[1]Laboratoire de Biologie Cellulaire, Institut Jean Pierre Bourgin, Institut National de la Recherche Agronomique (INRA), 78026 Versailles Cedex, France. [2]Plateforme de Cytologie et d'Imagerie Végétale, Institut Jean Pierre Bourgin, INRA, 78026 Versailles Cedex, France. [3]Department of Plant Sciences, University of Oxford, South Parks Road, Oxford OX1 3RB, UK. [4]Department of Genetics and Biotechnology, University of Aarhus, Thorvaldsensvej 40, DK-1871 Frederiksberg C, Denmark.

*Present address: Laboratoire de Reproduction et Développement des Plantes, UMR INRA-CNRS-Ecole Normale Supérieure de Lyon, Université de Lyon I, 46 allée d'Italie, 69364 Lyon Cedex 07, France.

†Present address: Unité INSERM U552, Institut Universitaire d'Hématologie, Batiment HAYEM, Hôpital Saint Louis, 1, avenue Claude Vellefaux, 75475 Paris Cedex 10, France.

‡To whom correspondence should be addressed. E-mail: laufs@versailles.inra.fr

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. *NAM* genes were also expressed later in association with *A. caerulea* and *S. lycopersicum* leaflet margin dissection (Fig. 1, D and H), as shown for the simple *Arabidopsis thaliana* leaf (*17*).

Next, we undertook a series of functional analyses in *A. caerulea*, *S. lycopersicum*, *P.*

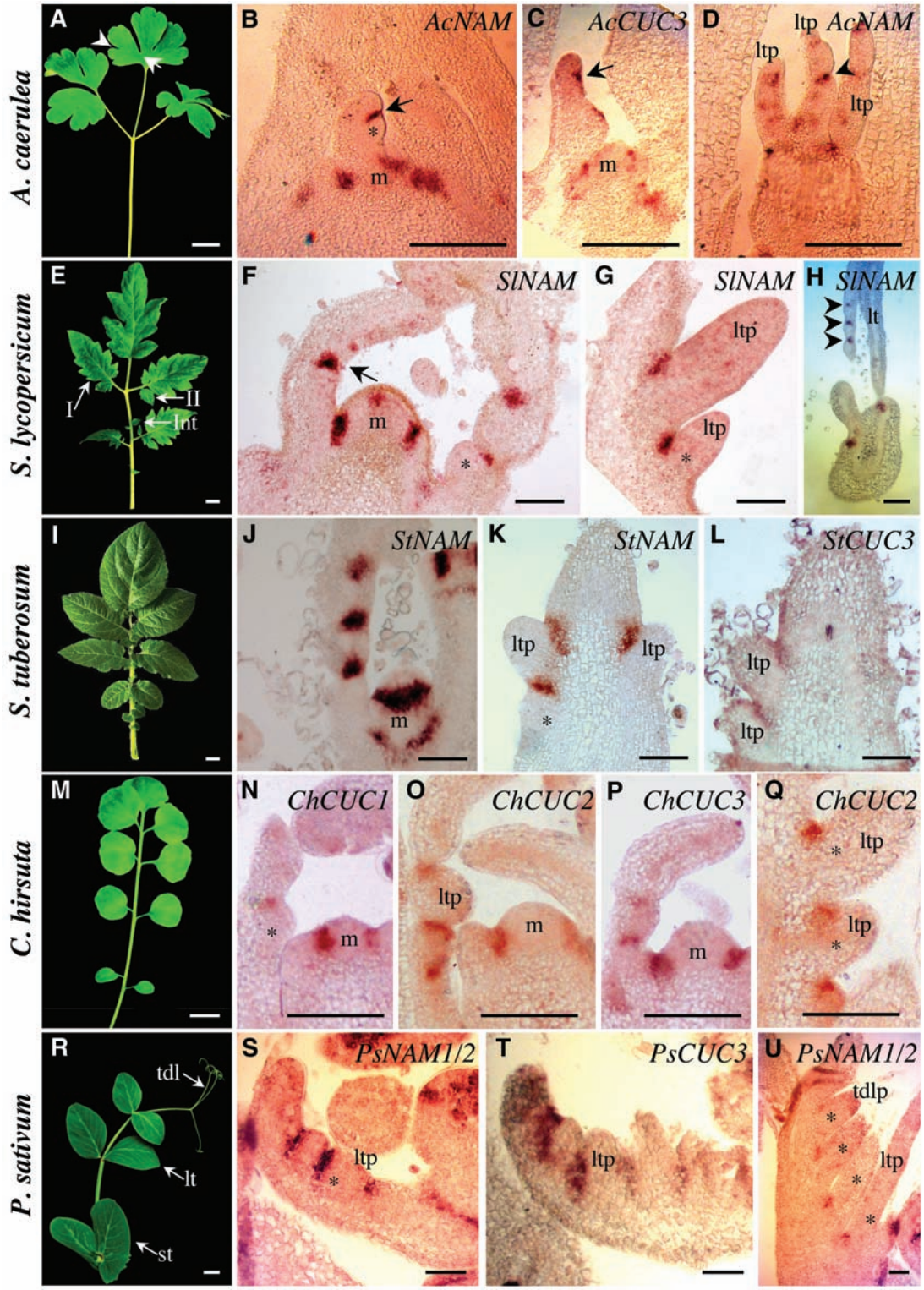

Fig. 1. Leaf shape and expression of the *NAM*/*CUC3* genes during leaf development in eudicots: *A. caerulea* [(A) to (D)], *S. lycopersicum* [(E) to (H)], *S. tuberosum* [(I) to (L)], *C. hirsuta* [(M) to (Q)], and *P. sativum* [(R) to (U)]. (**A**) *A. caerulea* leaf formed by three leaflets subdivided into three majors lobes (arrow), each of which is dissected (arrowhead). (**B** and **C**) In a young leaf primordium, *AcNAM* and *AcCUC3* are expressed in relation to the formation of the leaflet primordia (arrows). *AcNAM* expression is restricted to the distal side of the primordium marked in (B) by an asterisk. (**D**) *AcNAM* expression is coincident with further leaflet primordia (ltp) dissection (arrowhead). (**E**) *S. lycopersicum* leaf formed by primary (I), secondary (II), and intercalary (Int) leaflets that have dissected margins. (**F** and **G**) *SlNAM* expression precedes leaflet outgrowth [arrow in (F)] and marks the distal boundary of young or older leaflet primordia (asterisks). (**H**) *SlNAM* is expressed in relation with the serration of older leaflet (lt) margins (arrowheads). (**I**) *S. tuberosum* leaf formed by primary leaflets with entire leaf margins. (**J** to **L**) *StNAM* and *StCUC3* are expressed during early stages of leaflet initiation (J) and are still detected at later stages [(K) and (L)]. Asterisk in (K) indicates a young leaflet primordium showing *StNAM* expression only on its distal part. (**M**) A rosette leaf of *C. hirsuta* formed by several leaflets that show mild incision of their margins. (**N** to **Q**) *ChCUC1*, *ChCUC2*, and *ChCUC3* are expressed during leaflet initiation and at later stages. *ChCUC* expression is limited to the distal part of young [asterisk in (N)] and older [asterisks in (Q)] primordia. (**R**) *P. sativum* leaf formed by several pairs of proximal leaflets (lt) and distal tendrils (tdl). Leafletlike stipules (st) subtend the leaf. (**S** to **U**) The *PsNAM1/2* and *PsCUC3* genes are expressed during leaflet and tendril primordia development. *PsNAM1/2* is expressed 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)].

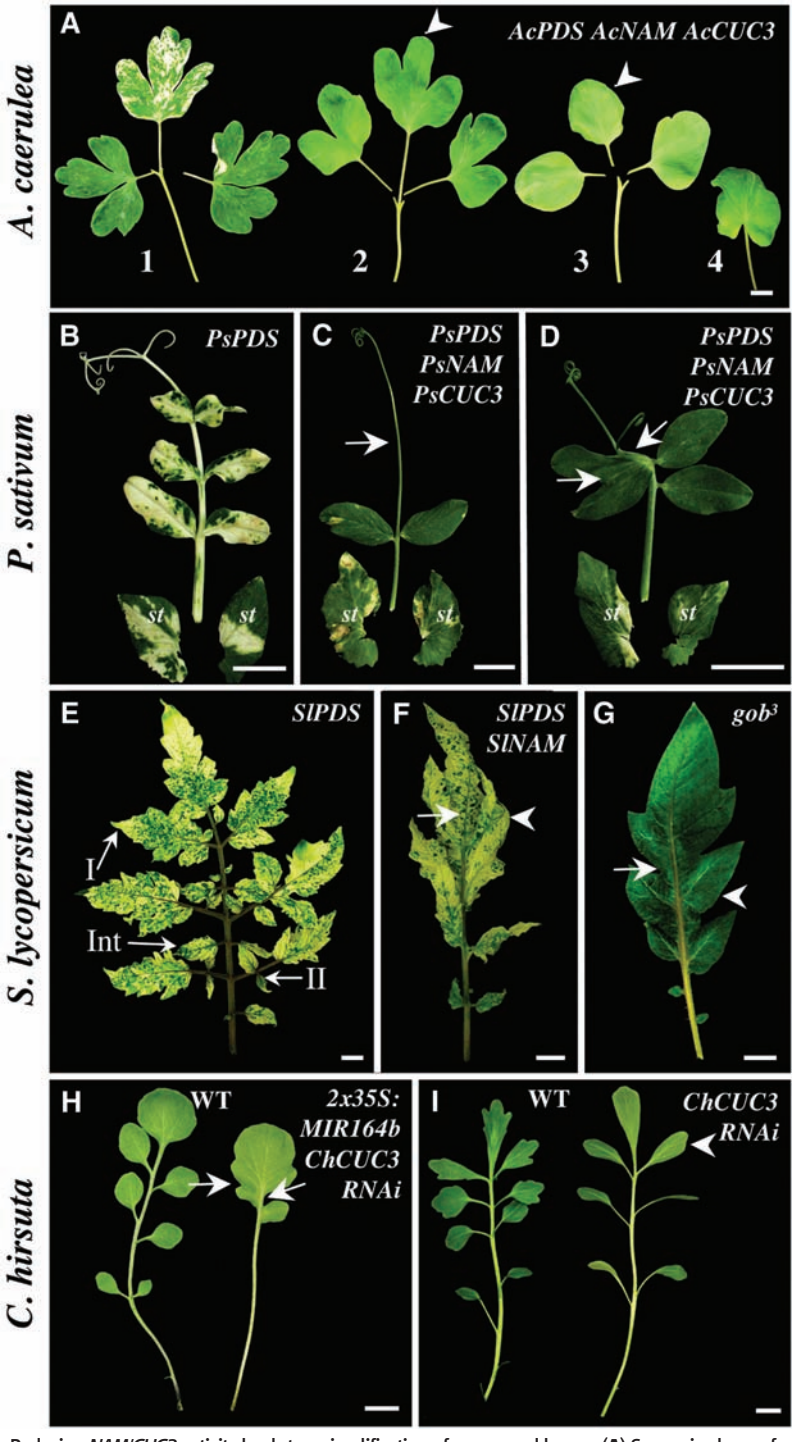

Fig. 2. Reducing *NAM/CUC3* activity leads to a simplification of compound leaves. (**A**) Successive leaves formed on an *A. caerulea* plant silenced for the *AcPDS*, *AcNAM*, and *AcCUC3* genes. Note the progressive smoothening of the leaflet margins from leaf 1 to 3 (arrowheads). At the final stage (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* formed by one pair of leaflets and one pair of tendrils separated by a long, organless rachis (arrow). (**D**) *P. sativum* leaf silenced for *PsPDS*, *PsNAM1/2*, and *PsCUC3* showing fusions between leaflets (left arrow) and between a leaflet and the rachis (right arrow). (**E**) Control leaf of *S. lycopersicum* silenced for *SlPDS* showing primary (I), secondary (II), and intercalary (Int) leaflets. (**F**) *S. lycopersicum* leaf silenced for *SlPDS* and *SlNAM* showing smoothed leaf margins (arrowhead), fusions between leaflets (arrow), and fewer leaflets. (**G**) Leaf of *gob*^{3} that harbors a mutation in the *SlNAM* gene and is similar to a *SlNAM*-silenced leaf. (**H**) Rosette leaf number 8 of wild-type (WT) *C. hirsuta* and of a line with reduced *CUC* expression (*2x35S:MIR164b ChCUC3 RNAi*). A reduced number of leaflets leads to a long, leafletless petiole and the fusion of the leaflets (arrows). (**I**) First cauline leaf of WT *C. hirsuta* and of a plant silenced for *ChCUC3* showing fewer and smoothed leaflets (arrowhead). Scale bars, 1 cm.

sativum, and *C. hirsuta* combining three different methods. Down-regulation of *NAM* and/or *CUC3* expression in *A. caerulea*, *P. sativum*, and *S. lycopersicum* by transient virus-induced gene silencing (VIGS) (fig. S5, A, C, and D) led to three specific leaf developmental defects that were never observed in control VIGS experiments (Fig. 2, fig. S6, and tables S1 to S3). First, the extent of serration or lobing at the leaf margin was reduced. Silencing of *SlNAM* was sufficient to produce smooth leaflet margins in *S. lycopersicum* (Fig. 2F and fig. S6), whereas full smoothing of the *A. caerulea* leaf margin required the simultaneous silencing of *AcNAM* and *AcCUC3* (Fig. 2A and fig. S6). Second, all lines silenced for *NAM*/*CUC3* except *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* silenced for *AcNAM* or *AcNAM*/*AcCUC3* formed 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 *SlNAM*-silenced plants, whereas secondary and intercalary leaflet numbers were highly reduced (Fig. 2F and fig. S6).

The *S. lycopersicum goblet* mutant confirmed the role of the *SlNAM* 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 *SlNAM* gene (fig. S7). Leaves regenerated from ectopic meristems of these mutants showed the same morphological defects as *SlNAM*-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. S5B). 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. 2, H 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 regulate leaflet formation.

Conversely, we tested whether *NAM/CUC3* genes had an effect on the expression of *KNOXI* and *LFY*-like genes. Accumulation of *KNOXI* (*Tkn1* and *Tkn2* in *S. lycopersicum*) and *LFY*-like (*SlLFY* 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 (*20*–*22*) may limit growth and prevent leaflet formation.

In contrast to the *KNOX* and *LFY*-like pathways, whose contributions vary between the compound-leafed 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.

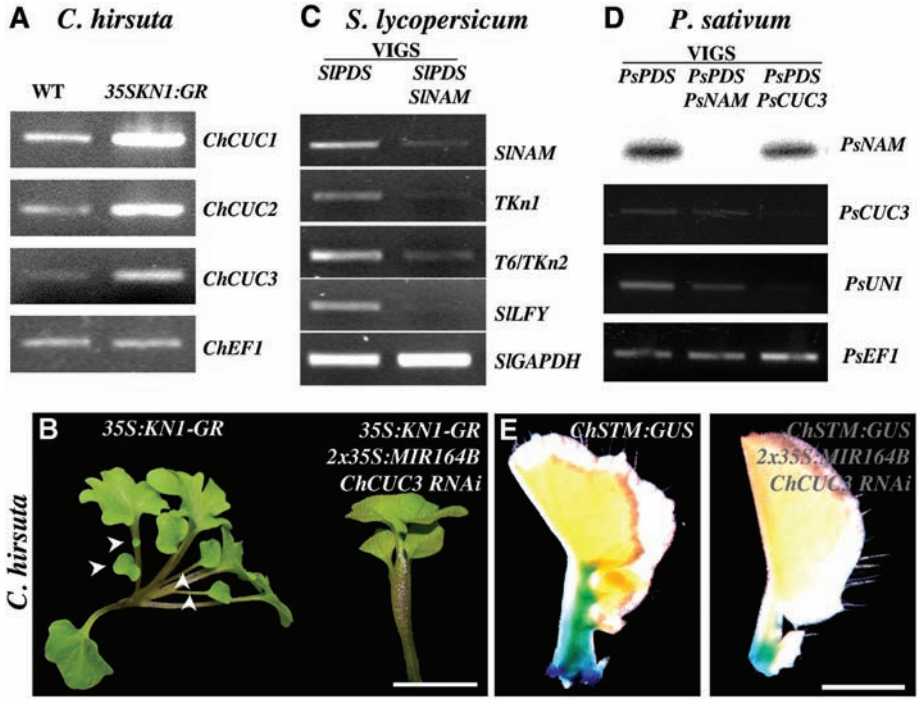

Fig. 3. Interplay between the *NAM/CUC3* genes and other regulators of compound leaf development. (**A**) After 2 days of induction of the *KN1:GR* fusion, the expression of *ChCUC1*, *ChCUC2*, and *ChCUC3* was increased compared with that of WT *C. hirsuta* control plants. *ChEF1* was used as an internal control. (**B**) Upon dexamethasone induction, ectopic leaflets were formed on the primary leaflets in *C. hirsuta* expressing a *KN1-GR* fusion (arrowheads). No ectopic leaflets were formed when the same construct is induced in a background with reduced *ChCUC* activity (*2x35S:MIR164B ChCUC3 RNAi*). (**C**) A reduction of *SlNAM* expression after *SlPDS SlNAM* VIGS was correlated with reduced expression of *SlLFY* and of the two *KNOXI* genes *TKn1* and *T6/TKn2*. *SlGAPDH* was used as an internal control. (**D**) *PsNAM1/2* and *PsCUC3* expression was reduced in plants silenced for *PsPDS PsNAM1/2* or *PsPDS PsCUC3*, respectively, and correlated with lower *UNI* expression. *PsEF1* was used as an internal control. (**E**) The *ChSTM:GUS* reporter was expressed in the petiole and rachis of a WT *C. hirsuta* leaf, and its expression was strongly reduced in a background with reduced *ChCUC* activity (*2x35S:MIR164B ChCUC3 RNAi*). Scale bars, 1 mm in (D) and 1 cm in (E).

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26. This work was partly supported by an Action Concertée Incitative "Jeunes Chercheuses et Jeunes Chercheurs" and the trilateral Génoplante GENOSOME program TRIL-046. T.B. was supported by a Ph.D. fellowship from the Ministère de l'Enseignement Supérieur et de la Recherche, and A.P. was supported by the Fundacíon Española para la Ciencia y la Tecnología (Ministerio de Ciencia e Innovacíon) and the Région Ile-de-France. M.T. is supported by the UK Biotechnology and Biological Sciences Research Council and the Gatsby Charitable Foundation and is the recipient of a European Molecular Biology Organization young investigator award and a Royal Society Wolfson Merit award. A.H. is a recipient of a Royal Society Research Fellowship. The authors thank S. Biemelt, D. Zamir, C. Rameau, J. Hofer, B. Letarnec, C. Navarro, E. Kramer, and the Tomato Genetics Resource Centre for providing material; the Cell Biology Laboratory gardener team for their plant care; J. D. Faure, H. Höfte, J. Hofer, A. Mallory, G. Mouille, J. C. Palauqui, F. Parcy, and C. Rameau for their comments on the manuscript; and N. Ori for discussions and sharing results before publication. Sequences have been deposited in GenBank, accessions FJ435156 to FJ435166.

Supporting Online Material
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19 September 2008; accepted 12 November 2008
10.1126/science.1166168

Human Fetal Hemoglobin Expression Is Regulated by the Developmental Stage-Specific Repressor *BCL11A*

Vijay G. Sankaran,[1,2] Tobias F. Menne,[1] Jian Xu,[1] Thomas E. Akie,[1] Guillaume Lettre,[3,4] Ben Van Handel,[5] Hanna K. A. Mikkola,[5] Joel N. Hirschhorn,[3,4] Alan B. Cantor,[1] Stuart H. Orkin[1,2,6]*

Differences 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 assessed.

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 erythroid cells, 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 "high-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 erythroleukemia 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.

[1]Division of Hematology/Oncology, Children's Hospital Boston, Harvard Stem Cell Institute, Harvard Medical School, Boston, MA 02115, USA. [2]Department of Pediatric Oncology, Dana-Farber Cancer Institute, Boston, MA 02115, USA. [3]Program in Medical and Population Genetics, Broad Institute of MIT and Harvard, Cambridge, MA 02142, USA. [4]Divisions of Genetics and Endocrinology and Program in Genomics, Children's Hospital Boston, Boston, MA 02115, USA. [5]Department of Molecular, Cell, and Developmental Biology, University of California at Los Angeles, Los Angeles, CA 90095, USA. [6]Howard Hughes Medical Institute, Boston, MA 02115, USA.

*To whom correspondence should be addressed. E-mail: stuart_orkin@dfci.harvard.edu

A Conserved Molecular Framework for Compound Leaf Development

Thomas Blein, Amada Pulido, Aurélie Vialette-Guiraud, Krisztina Nikovics, Halima Morin, Angela Hay, Ida Elisabeth Johansen, Miltos Tsiantis and Patrick Laufs

Science **322** (5909), 1835-1839.
DOI: 10.1126/science.1166168