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Current Opinion in Genetics & Development

Cardamine hirsuta: a comparative view

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Current advances in developmental genetics are increasingly underpinned by comparative approaches as more powerful experimental tools become available in non-model organisms. *Cardamine hirsuta* is related to the model plant *Arabidopsis thaliana* and comparisons between these two experimentally tractable species have advanced our understanding of development and diversity. The power of forward genetics to uncover new biology was evident in the isolation of *REDUCED COMPLEXITY*, a gene which is present in *C. hirsuta* but lost in *A. thaliana*, and shapes crucifer leaf diversity. Transferring two *Knotted1-like homeobox* genes between *C. hirsuta* and *A. thaliana* revealed a constraint imposed by pleiotropy on the evolutionary potential of *cis* regulatory change to modify leaf shape. *FLOWERING LOCUS C* was identified as a heterochronic gene that underlies natural leaf shape variation in *C. hirsuta*.

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Current Opinion in Genetics & Development 2016, **39**:1–7

This review comes from a themed issue on **Developmental mechanisms, patterning and evolution**

Edited by **Detlev Arendt** and **Cassandra Extavour**

http://dx.doi.org/10.1016/j.gde.2016.05.005

Introduction

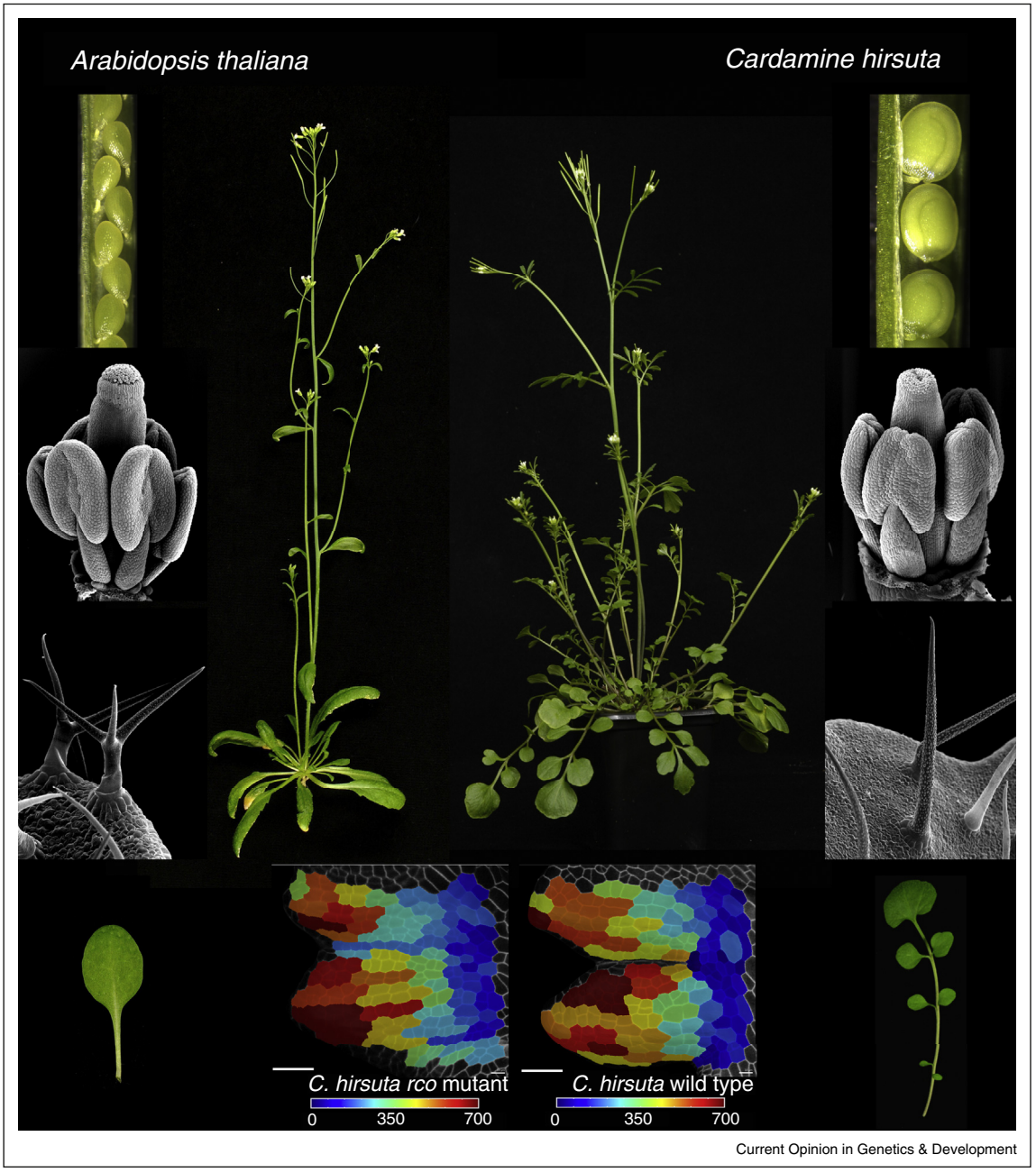
Understanding the genetic basis of phenotypic diversity is a common goal in many areas of research, from evolutionary biology to plant breeding and human genetics. The explosion of high throughput sequencing methods and advances in bioinformatics means that this research is no longer limited by sequence information, but rather by efficient ways to causally connect sequence variation to phenotypic diversity. Comparative genetic studies in the phylogenetic neighbourhood of model organisms have emerged as an important way to address this problem [1]. This approach uses genetic analyses in related taxa to identify molecular changes that underlie phenotypic differences that are of evolutionary significance [2–6]. Key to the success of this comparative approach is that it allows causal genetic differences to be identified and studied within an otherwise broadly comparable genotype to phenotype landscape. Such studies have particularly benefited from the use of interspecific gene transfers to test the evolved functions of sequence variants [7,8]. Other methods that utilize genetic recombination, such as quantitative trait locus analysis (QTL), also provide strong evidence for the genetic basis of morphological diversity and evolutionary change at the species level and between interfertile species [9,10].

Why *Cardamine hirsuta*?

In plants, such comparative studies are particularly attractive in the Brassicaceae family, which includes *Arabidopsis thaliana* — the primary model organism for plant science. This allows the technical and conceptual frameworks established in *A. thaliana* to be exploited in novel contexts to understand the origin of traits or character states not present in the model species, and to provide an evolutionary and ecological perspective [11•,12]. Recent studies have focused on mating system transitions in *Arabidopsis halleri* [13•] and *Capsella* [14,15], alternative life histories in *Arabis alpina* [16] and *Cardamine flexuosa* [17], fruit opening in *Lepidium campestre* [18,19], adaptation to extreme soil conditions in *Arabidopsis lyrata* [20] and *A. halleri* [21,22], and hybridization barriers between *Arabidopsis* species [23]. Within this small group of *A. thaliana* relatives, *Cardamine hirsuta* has emerged as a particularly powerful genetic system for comparative studies of development with *A. thaliana* [24,25•] (Figure 1).

Like *A. thaliana*, *C. hirsuta* was selected as a laboratory subject for its short generation time, small size, inbreeding habit, abundant progeny and ease of large scale cultivation. Importantly, it is a diploid species with a small genome and eight chromosomes, which follows the ancestral genome structure in the Brassicaceae [25•]. Simple, high frequency genetic transformation is routine in *C. hirsuta*, which together with a dense genetic map and chemically mutagenized populations, provide the necessary tools to investigate how genetic changes influence morphogenesis [25•]. The major motivation for studying and developing resources for the *A. thaliana* relative *C. hirsuta* is to understand the genetic basis for morphological evolution. Key to this comparative approach is the abundance of morphological diversity between these reproductively isolated species [25•] (Figure 1). In this review, we will discuss recent work over the past two years that has elucidated how differences in leaf morphology between *A. thaliana* and

Figure 1



Cardamine hirsuta: a genetic system for comparative studies with *Arabidopsis thaliana.* Whole plant and selected parts are compared between *A. thaliana* (left) and *C. hirsuta* (right). Divergent seed morphology, stamen number, trichome branching and leaf shape are shown from top to bottom. Quantitative image analysis of lateral leaflet growth in *C. hirsuta* is shown in bottom, centre panels for *rco* (left) and wild-type (right). Heat maps show relative surface area increase over 48 h of growth (color bar: percentage increase); scale bars: 30 μm.

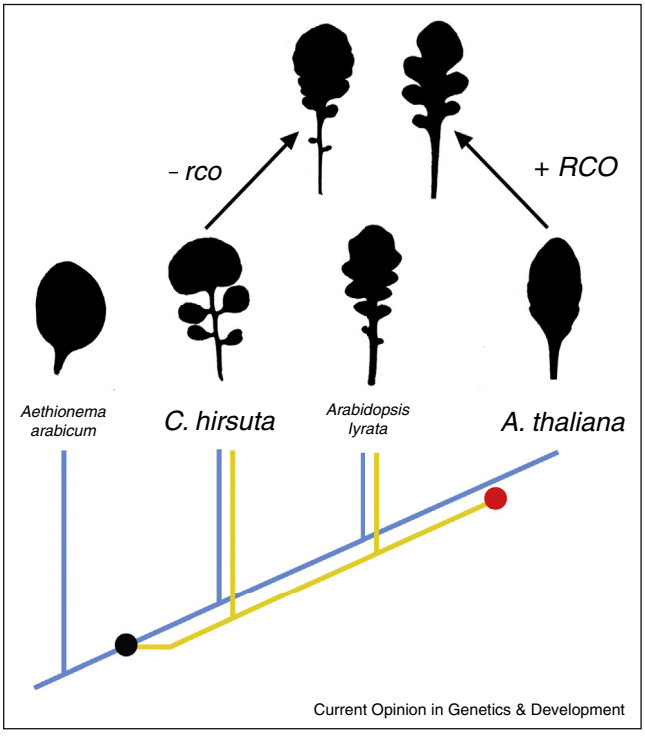
C. hirsuta are produced and how these differences evolved. We will also highlight studies of natural variation in *C. hirsuta* leaf shape and petal number that are beginning to show whether the same or different genetic pathways underlie morphological diversity within and between species.

Mutant screen uncovers homeobox gene shaping leaf diversity

Leaves show enormous variation in shape, both within and between species, and differ markedly between *A. thaliana* and *C. 43. A. thaliana* leaves are simple with an entire margin while *C. hirsuta* leaves are dissected, also

called compound, with the margin separated into individual leaflets (Figure 1). Previous work has shown that the co-option of gene networks active in the shoot apical meristem make a significant contribution to leaf shape differences between *A. thaliana* and *C. hirsuta* [24,26,27]. However, no gene had been identified that expresses specifically at developing leaflets and is sufficient to convert leaf shape from simple to more complex. To determine whether such a gene exists in *C. hirsuta*, mutagenized plants were screened for mutants that convert leaf shape from dissected to simple, resembling *A. thaliana*. This forward genetics approach led to the identification of REDUCED COMPLEXITY (RCO), a HD-ZIP class I transcription factor that promotes leaflet formation in *C. hirsuta* [28••]. *RCO* arose in the Brassicaceae through gene duplication of the floral regulator *LATE-MERISTEM IDENTITY1* (*LMI1*), which is a conserved gene in seed plants (Figure 2). The presence of *RCO* in related species with dissected or lobed leaves indicated that this gene was lost from the genome of *A. thaliana*, contributing to its simple leaf shape [28••] (Figure 2). Moreover, transformation of *RCO* from *C. hirsuta* into *A. thaliana* was sufficient to reverse this evolutionary transition and make the *A. thaliana* leaf more complex (Figure 2). This result provides a rare example where the presence or absence of a single gene has a key role in shaping diversity. Through swapping regulatory and coding sequences, the authors showed that diversification of *LMI1* and *RCO* function arose through the evolution of a novel *RCO* expression domain at the base of developing leaflets [28••]. Thus, regulatory evolution coupled with gene duplication played a major role in generating and maintaining the diversity of leaf shapes found in the Brassicaceae. Comparative genomics can provide significant resources for future studies of regulatory evolution in the Brassicaceae. For example, tens of thousands of potential regulatory sequences were recently identified as conserved non-coding sequences across nine genomes from this family [29].

Figure 2



RCO evolution and its consequences for leaf shape diversity in crucifers. An early divergent crucifer, *Aethionema arabicum*, contains a single *LMI1*-type gene (blue line). *RCO*-type genes (yellow line) arose from duplication (black dot) of an *LMI1*-type gene. Dissected-leafed *C. hirsuta* and lobed-leafed *Arabidopsis lyrata* contain both *LMI1*-type and *RCO*-type genes. *RCO* was secondarily lost in *A. thaliana* (red dot), contributing to the evolution of a simple leaf. Loss of *RCO* in the *C. hirsuta rco* mutant (– *rco*) causes leaf simplification while gain of *RCO* in an *A. thaliana* transgenic (+ *RCO*) causes leaf complexity.

How genetic differences are translated to different morphologies through the process of development is a central question in evolutionary developmental biology, and this question was specifically addressed for *RCO*. Advanced time lapse imaging methods were developed using the MorphoGraphX platform [30] that allowed tissue growth to be tracked at cellular level throughout leaf development. Results from these experiments showed that RCO acts locally at the base of leaflets to repress growth, allowing the leaf margin to separate and grow out as distinct leaflets [28••] (Figure 1). Overexpression analysis showed that *LMI1* also represses growth, demonstrating that this gene function was ancestral to the duplication event that gave rise to *RCO* in the Brassicaceae, and was likely to precede the split of eudicots from other seed plants [28••]. These findings suggest that leaf shape evolved via the targeted deployment of this growth-repressing activity to highly specific regions of the leaf. The simplicity of this model may explain why independent mutations at the *RCO* locus were responsible for repeated evolutionary changes in leaf shape in the Brassicaceae, including the sister species *Capsella rubella* and *C. grandiflora* [31], and *LMI1* orthologs outside the Brassicaceae [32]. In future studies it will be important to identify how *RCO* interacts with other genes to pattern the growth of *C. hirsuta* leaves. *simple leaf3* is another *C. hirsuta* leaf shape mutant, caused by a mutation in RNase L inhibitor 2; an ATP binding cassette-type ATPase required for ribosome recycling [33]. This suggests a possible input of translational control in the growth and development of dissected leaves.

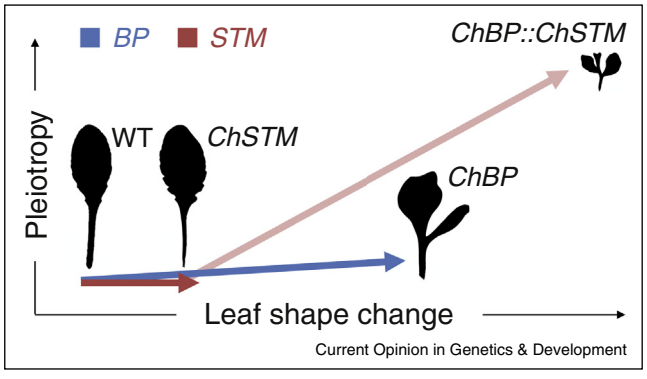
Pleiotropy shapes the evolutionary potential of two KNOX genes

Evolutionary modification of the leaf margin has occurred via two main developmental routes, one patterns the initiation of margin protrusions and the other modulates growth between these protrusions [34]. RCO acts in the

latter process, while transcription factors encoded by class I *Knotted1-like homeobox* (*KNOX*) genes are crucial for the patterning process that creates auxin activity peaks along the leaf margin [24,26]. KNOX activity is required to maintain the shoot apical meristem of most plants and KNOX presence or absence shows a broad association with dissected or simple leaf shape respectively [35,36]. Previous work in *C. hirsuta* had shown that the *KNOX* gene *SHOOT MERISTEMLESS* (*STM*) was necessary for leaflet formation, and that *cis* regulatory divergence at two *KNOX* genes, *STM* and *BREVIPEDICELLUS* (*BP*), was associated with the difference in leaf shape between *A. thaliana* and *C. hirsuta* [24]. However, it was not known whether these changes in *cis* to *STM* and *BP* were sufficient to change leaf shape, or whether other changes in *trans* were additionally required. It was also unclear how these changes in *KNOX* gene expression impacted the gene regulatory network operating in simple versus dissected leaves.

These questions were recently addressed using comparative genetics and cross-species gene transfers between *C. hirsuta* and *A. thaliana* [37••]. These loss and gain-of-function experiments provided evidence for an inverse relationship between the pleiotropy of each gene and its ability to modify leaf form. Specifically, *stm* mutants are more pleiotropic than *bp* mutants, yet the *BP* genomic locus from *C. hirsuta* is sufficient to modify *A. thaliana* leaf shape to a much greater extent than the *C. hirsuta STM* locus (Figure 3). This relationship was explored further by uncoupling the coding and regulatory regions of *STM*, and showing that the STM protein can dramatically modify *A. thaliana* leaf shape but also causes pleiotropic effects throughout plant development [37••] (Figure 3). By contrast to this, the BP protein consistently modified *A. thaliana* leaf shape with a lower pleiotropy penalty than STM [37••]. Therefore, the less pleiotropic gene, *BP*, evolved *cis* regulatory differences between *C. hirsuta* and *A. thaliana* that were sufficient to change leaf shape. Consistent with this finding, *STM* evolved in a more constrained fashion than *BP* in both coding and noncoding sequences throughout the Brassicaceae [37••]. These experiments provide empirical evidence for the general principle that regulatory evolution, constrained by pleiotropy, can drive morphological diversity [38,39].

Figure 3



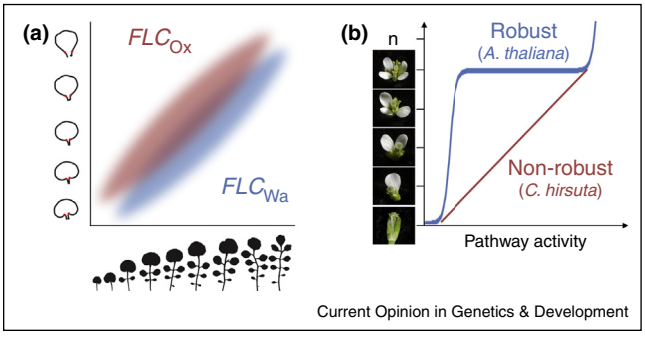
Pleiotropy influences the regulatory evolution of *KNOX* genes. Of two *KNOX* genes, *BP* (blue) and *STM* (red), the less pleiotropic *KNOX* gene, *BP*, evolved a greater capacity to contribute to leaf shape divergence between *C. hirsuta* and *A. thaliana*. Transfer of *C. hirsuta KNOX* genes into *A. thaliana* changes wild-type (WT) leaf shape either a little (*ChSTM*, red arrow) or a lot (*ChBP*, blue arrow), as shown by the arrow length along the *x*-axis. These leaf shape changes occur with only modest pleiotropic effect, shown on the *y*-axis. *STM* is able to cause increased leaf shape change when driven by the *BP* promoter (*ChBP::ChSTM*, pale red arrow) but only at the expense of increased pleiotropy.

The power of forward genetics in *C. hirsuta* was also used to identify novel regulators of *BP* and study the difference in gene regulatory network (GRN) architecture between *C. hirsuta*, where *BP* is expressed in leaves, and *A. thaliana* where it is not [37••]. These experiments showed two alternative network configurations. In *C. hirsuta* leaves, *BP* expression is negatively regulated by the myb transcription factor ASYMMETRIC LEAVES1 (AS1) [24] and the regulatory module consisting of microRNA164A and its NAC transcription factor target CUP-SHAPED COTYLEDON2 (CUC2) [37••]. This creates cross-talk between components of the leaf network, *MIR164A/CUC2* and *AS1*, that does not occur in *A. thaliana* [37••]. Thus, *cis* regulatory changes not only partition *BP* activity between the shoot apical meristem and the leaf of *A. thaliana* and *C. hirsuta*, but also result in different genetic interactions between genes that are common to the leaf GRN of both species. Therefore, network rewiring via the flexible engagement of weakly pleiotropic regulators like *BP* may provide a favourable path for morphological evolution.

Mining natural variation

Leaf shape variation that is found within and between species may or may not have a similar genetic basis. A recent QTL study addressed this question by determining the genetic architecture of leaf shape variation in *C. hirsuta* and cloning the major effect QTL in a recombinant inbred line (RIL) population [40••]. Interestingly, none of the leaf shape QTL that were identified in these RILs mapped to genes that controlled between-species variation in leaf shape, such as *RCO, MIR164A*, *CUC* or *KNOX* genes, suggesting that the causes of morphological diversity at these two evolutionary scales were divergent [40••]. Instead, the major leaf shape QTL was caused by regulatory sequence variation in the floral repressor *FLOWERING LOCUS C* (*FLC*), which encodes a MADS-box transcription factor [41]. Naturally occurring *FLC* alleles showed different levels of gene expression and affected leaf shape by modulating the pace of acquisition of adult leaf traits [40••]. *C. hirsuta*, in common with most plants, shows age-dependent or 'heterochronic'

Figure 4



Mining natural variation. **(a)** *C. hirsuta FLC* is a major QTL for leaf shape that acts via influencing age-dependent or 'heterochronic' variation. Within a shape space defined by two heterochronic traits: leaflet number (*x*-axis) and shape (*y*-axis), distinct combinations of these traits can be attributed to specific *FLC* alleles (FLC_{Ox}, red, and FLC_{Wa}, blue, are naturally occurring *C. hirsuta FLC* alleles in a RIL population derived from *C. hirsuta* Ox and Wa accessions). **(b)** *A. thaliana* petal development is buffered within the normal range of pathway activity to give a robust petal number of four. This robustness is lost in *C. hirsuta* such that petal number varies between zero and four. A polygenic architecture of many small to moderate effect QTL underlies this petal number variation.

variation in leaf shape such that leaflet number increases and leaflet shape changes as the plant ages from juvenile to adult [42] (Figure 4a). Distinct combinations of leaflet number and shape created leaf shapes that were specific to each of the natural *FLC* alleles (Figure 4a), showing how heterochronic variation can be a major source of leaf shape variation in *C. hirsuta* [40••]. Moreover, the authors propose that regulating leaf shape in this age-dependent manner allows the plant to adjust leaf growth according to the timing of reproduction, that is, flowering time. As evidence for this, early flowering plants that failed to accelerate their acquisition of adult leaf shape had reduced seed weight [40••]. Given that flowering time is variable between plant populations and highly sensitive to environmental cues, strong selection for flowering time could make this mode of leaf shape variation pervasive.

Phenotypic robustness and petal number variation

Flowers with four petals arranged in the shape of a cross characterize the Brassicaceae (Figure 4b) and gives this family its alternative name Cruciferae. This reproducibility of petal number reflects the robustness of floral organ patterning to natural genetic or environmental variation (Figure 4b). For example, diverse *A. thaliana* accessions produce the same phenotype of four petals per flower despite considerable genetic variation. By contrast to this, petal number varies between zero and four in *C. hirsuta* (Figure 4b). Two recent QTL studies that mapped the natural genetic variation influencing petal number in five *C. hirsuta* RIL populations have, therefore, advanced our knowledge of phenotypic diversity that is not accessible for study in *A. thaliana* [43•,44]. The authors identified a polygenic architecture of many small to moderate effect QTL that shift petal number in both positive and negative directions, which likely contributes to maintaining *C. hirsuta* petal number within its variable range below four [43•]. QTL were identified for both average petal number and its stochastic variation in *C. hirsuta* RILs, showing that both aspects of the phenotypic distribution are under genetic control [44]. The variation in *C. hirsuta* petal number is not entirely stochastic but age-dependent, and shares a common genetic basis with another age-dependent trait, sepal trichome number, which suggests that selection on such pleiotropic traits may contribute to maintaining petal number within its variable range [43•]. These studies suggest that evolutionary change in *C. hirsuta* likely allowed cryptic genetic variation to read out as morphological divergence, and it will be exciting to understand from future studies what these genes are and how they influence petal development.

The shape of things to come

The key point to emerge from this review is how comparative approaches not only inform us about the genetic basis for evolutionary change, but also uncover fundamental features of development that cannot be comprehended by studying a single model species in isolation. For example, *RCO* was isolated as an important gene for leaf development and diversity [28••], *KNOX* gene regulatory changes were found to rewire leaf networks [37••], natural regulatory variation at *FLC* highlighted a different genetic basis for between and within species leaf shape variation [40••], and genetic variation that is usually cryptic for petal number was mapped in *C. hirsuta* [43•]. Moreover, an interdisciplinary study that combined biological and modeling approaches, recently identified the mechanism and origin of explosive seed dispersal in *C. hirsuta* — a key life history trait associated with the invasiveness of this weed [45••]. As the ability to identify causal variants underlying morphological diversity increases, the sophisticated experimental tools in *C. hirsuta* for quantitative 4D image analysis of gene expression and growth [28••,30] will become increasingly important to understand the paths from genotype to phenotype. Placing these comparisons between *A. thaliana* and *C. hirsuta* in an ecological context [46,47] will also give an important perspective on the genetic and phenotypic diversity in plants.

Acknowledgements

We acknowledge support from Deutsche Forschungsgemeinschaft priority programme 'Adaptomics' grant HA 6316/1-3 to A.H. and M.T. and Collaborative Research Centre SFB 680 grant to M.T., a core grant of the Max Planck Society to M.T., Human Frontiers Science Programme young investigator's grant RGY0087/2011 to A.H., the Max Planck Society W2 Minerva programme to A.H., and the Cluster of Excellence on Plant Sciences to M.T.

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