# Hox Control of Organ Size by Regulation of Morphogen Production and Mobility

### Michael A. Crickmore<sup>1</sup> and Richard S. Mann<sup>2\*</sup>

Selector genes modify developmental pathways to sculpt animal body parts. Although body parts differ in size, the ways in which selector genes create size differences are unknown. We have studied how the *Drosophila* Hox gene *Ultrabithorax* (*Ubx*) limits the size of the haltere, which, by the end of larval development, has ~fivefold fewer cells than the wing. We find that *Ubx* controls haltere size by restricting both the transcription and the mobility of the morphogen Decapentaplegic (Dpp). *Ubx* restricts Dpp's distribution in the haltere by increasing the amounts of the Dpp receptor, *thickveins*. Because morphogens control tissue growth in many contexts, these findings provide a potentially general mechanism for how selector genes modify organ sizes.

hanges in body part sizes have been critical for diversification and specialization of animal species during evolution. The beaks of Darwin's finches provide a famous example for how adaptation can produce variations in size and shape that allowed these birds to take advantage of specialized ecological niches and food supplies (*I*). Sizes also vary between homologous structures with-

in an individual. For example, vertebrate digits and ribs vary in size, likely due to the activities of selector genes such as the Hox genes (2-4). Although the control of organ growth by selector genes is likely to be common in animal development (2, 5, 6), little is known about the mechanisms underlying this control.

The two flight appendages of *Drosophila* melanogaster, the wing and the haltere, provide

a classic example of serially homologous structures of different sizes (Fig. 1A). Halteres, appendages used for balance during flight, are thought to have been modified from full-sized hindwings during the evolution of two-winged flies from their four-winged ancestors (7, 8). All aspects of haltere development that distinguish it from a wing, including its reduced size, are under the control of the Hox gene Ultrabithorax (Ubx), which is expressed in all haltere imaginal disc cells but not in wing imaginal disc cells (8, 9) (Fig. 1B). At all stages of development, haltere and wing primordia (imaginal discs) are different sizes. In the embryo, the wing primordium has about twice as many cells as the haltere primordium (7, 10). By the end of larval development, the wing disc has ~five times more cells ( $\sim$ 50,000) than the haltere disc ( $\sim 10,000$ ) (11) [Fig. 1B and Supporting Online Material (SOM) Text]. The wing and haltere appendages will form from the pouch region of these mature discs (fig. S1). The final step that contributes to wing and haltere size differences occurs during metamorphosis, when

<sup>1</sup>Department of Biological Sciences, Columbia University, New York, NY 10027, USA. <sup>2</sup>Department of Biochemistry and Molecular Biophysics, Columbia University, HHSC 1104, 701 West 168th Street, New York, NY 10032, USA. \*To whom correspondence should be addressed. E-mail: rsm10@columbia.edu

Fig. 1. Reduced Dpp production and transduction in the haltere. (A) Wild-type adult wing and haltere (arrow). (B) Third instar wing (W), haltere (H), and T3 leg (L) imaginal discs stained for Ubx (green) and a ubiquitous nuclear protein (blue). Ubx is present in all haltere disc cells but not in wing disc cells. (C) Removing Ubx activity (lack of GFP) from more than 50% of a haltere disc during larval development using the  $M^+$  (*Minute*) technique (13) (SOM Text) increased its size [compare with discs in (B) and (F)]. (D) Isolated  $Ubx^{-}$  clones (black, -/-) were not larger than  $Ubx^+$  twin spots (bright white, +/+) in a *Ubx* heterozygous haltere (gray, -/+). (E) Ubx mutant:twin spot and neutral:twin spot clone size ratios. Error bars indicate SEM. (F to H) Wild-type wing and haltere discs stained for dpp-lacZ and P-Mad patterns. In the haltere, *dpp-lacZ* was reduced (arrowheads) and overlapped with a compacted P-Mad gradient (arrows).



wing, but not haltere, cells flatten, thus increasing the surface area of the final appendage (12).

Nonautonomous control of haltere size by **Ubx.** To confirm that Ubx has a postembryonic role in limiting the size of the haltere disc, we generated  $Ubx^{-}$  clones midway through larval development (13). Haltere discs-bearing large  $Ubx^{-}$  clones generated at this time become much larger than wild-type discs (Fig. 1C and SOM Text). Ubx could limit haltere size cellautonomously by, for example, slowing the cell cycle of haltere cells relative to wing cells. We tested this by comparing the sizes of isolated  $Ubx^{-}$  clones in the haltere with those of their simultaneously generated wild-type twin clones. Contrary to the prediction of a cell-autonomous function for Ubx in size control, Ubx mutant clones did not grow larger than their twins (Fig. 1, D and E), a result that is consistent with earlier experiments suggesting that wing and haltere cells have similar mitotic rates during development (14). Hence, Ubx limits the size of the haltere during larval development by modifying pathways that control organ growth cell-nonautonomously.

Ubx regulation of Dpp signaling. In the fly wing, Decapentaplegic (Dpp) [a long-range morphogen of the bone morphogenetic protein (BMP) family] has been shown to promote growth (15-17). In both the wing and the haltere, Dpp is produced and secreted from a specialized stripe of cells called the AP organizer, which is induced by the juxtaposition of anterior (A) and posterior (P) compartments, two groups of cells that have separate cell lineages (18). The AP organizer is a stripe of A cells that are instructed to synthesize Dpp by the shortrange morphogen Hedgehog (Hh) secreted from adjacent P compartment cells (18-22). Dpp has a positive role in appendage growth. When more Dpp is supplied to the wing disc, either ectopically or within the AP organizer, more cells are incorporated into the developing wing field (22-24). Conversely, mutations that reduce the amount of Dpp lead to smaller wings (fig. S3) (25).

A comparison of the expression patterns of Dpp pathway components in the wing and the haltere demonstrates that *Ubx* is modifying this pathway (Fig. 1, F to H, fig. S1, and SOM Text). Compared with the wing, the stripe of dpp expression in the haltere was reduced in both its width and intensity, as reported by a *lacZ* insertion into the *dpp* locus (*dpp-lacZ*). There was also a difference in the profile of Dpp pathway activation, as visualized by an antibody that detects P-Mad, the activated form of the Dpp pathway transcription factor Mothers against Dpp (Mad). In the wing, P-Mad staining was low in the cells that transcribe dpp (Fig. 1, F to H, and fig. S1) (26). Immediately anterior and posterior to this activity trough, P-Mad labeling peaked in intensity and then gradually decayed further from the Dpp source,

revealing a bimodal activity gradient. In contrast, in the haltere intense P-Mad staining was detected only in a single stripe of cells that overlaps with Dpp-producing cells of the AP organizer (Fig. 1, F to H, and fig. S1).

Because of the coincidence between dpp transcription and peak P-Mad staining in the haltere, we hypothesized that Dpp might be less able to move from haltere cells that secrete this ligand. We tested this idea by generating clones of cells in both wing and haltere discs in which the actin5c promoter drove the expression of a green fluorescent protein (GFP)-tagged version of Dpp (Dpp:GFP) (13, 27, 28). By using an extracellular staining protocol to analyze simultaneously generated clones (29), we observed Dpp:GFP and P-Mad much further from producing cells in the wing than in the haltere (Fig. 2, A to D). These observations strongly suggest that, compared with the wing, Dpp's mobility-and consequently the range of Dpp pathway activation-is reduced in the haltere.

We also tested whether the decreased production of Dpp in the haltere contributes to the different pattern of pathway activation observed in this tissue compared with the wing. This is unlikely because, even in haltere discs that overexpress Dpp in its normal expression domain, peak P-Mad staining was still observed

Fig. 2. Reduced Dpp mobility in the haltere. (A to D) Simultaneously generated actin5c promoter flp-out clones expressing UAS-dpp::gfp and UAS-GFP in the wing and haltere stained for extracellular GFP (red and white) and P-Mad (blue). The green channel shows GFP autofluorescence and marks the clone. The extracellular Dpp::GFP pattern closely correlates with the P-Mad pattern. In (C) and (D), enlarged images of the regions boxed in (A) are shown. (E and F) Overexpressing dpp with ptc-Gal4 (visualized with UAS-GFP) increased the scale and intensity of P-Mad staining in the wing and the haltere, but the patterns remained qualitatively similar to those of wild type.

close to Dpp-expressing cells (Fig. 2, E and F) (13). Despite increased dpp expression, no P-Mad activity trough was observed in these haltere discs. Further, although they become larger, these discs remained smaller than wild-type wing discs. We conclude that the decreased Dpp production in the haltere contributes to its reduced growth, but there must be mechanisms that also limit the extent of Dpp pathway activation, even in the presence of increased Dpp production.

One way in which Dpp's activation profile can be modified is by varying the production of the type I Dpp receptor, Thickveins (Tkv) (26, 30). In the wing, tkv expression is low within and around the source of Dpp, resulting in low Dpp signal transduction in these cells and robust Dpp diffusion (26, 30, 31) (Fig. 3, A and B, and fig. S1). Low the expression in the medial wing is due to repression by both Hh and Dpp (26, 30). Accordingly, tkv expression is highest in lateral regions of the wing disc, where Hh and Dpp signaling are low. In contrast to the wing, tkv transcription and protein levels were high in all cells of the haltere (Fig. 3, A and B). Thus, the more restricted Dpp mobility and P-Mad pattern in the haltere may result from a failure to repress tkv medially. To test this idea, we supplied all cells of the wing disc with uniform  $UAS-tkv^+$ expression, to mimic the haltere pattern (Fig.



3C) (13). The resulting P-Mad pattern in these wing discs was very similar to that found in the wild-type haltere: The P-Mad trough was gone, and the activity gradient was compacted into a single stripe that coincides with Dpp-producing cells. Conversely, lowering the amount of Tkv in the haltere by expressing an RNA interference (RNAi) hairpin construct directed against tkv (UAS-tkvRNAi) in Dpp-producing cells induced a bimodal pattern of P-Mad staining similar to that of the wild-type wing disc (Fig. 3, D to F) (13). Thus, different amounts of Tkv result in qualitative differences in the P-Mad profiles of the wing and the haltere.

tkv expression and appendage size. We hypothesized that the more limited pathway activation in the haltere might contribute to its smaller size. If correct, increasing tkv expression in the wing should reduce its size. Adult wings from flies expressing uniform UAS-tkv<sup>+</sup> were  $\sim 30\%$  smaller than control wings; however, wing cell size remained the same (Fig. 3, G and H, and fig. S2) (13, 30). Similar results were seen in staged imaginal discs and when UAS- $tkv^+$  expression was limited to the wing and the haltere (fig. S2). Conversely, reducing Tkv amounts by uniformly expressing UAS-tkvRNAi in wings and halteres increased haltere size by 30 to 60% (Fig. 3, I and J). In a complementary experiment, we reduced tkv transcription in the haltere by expressing a known tkv repressor, master of thickveins (mtv) (32). In this experiment, we measured haltere discs instead of the adult appendage and found, consistently, that the appendage-

Fig. 3. Tkv production influences Dpp signaling and appendage size. (A) tkv-lacZ expression was high throughout the haltere, whereas in the wing it was low medially and high laterally. (B) Tkv antibody staining showed a pattern similar to that of the *tkv-lacZ* enhancer trap, with less resolution. (C) Driving uniform UAS- $tkv^+$  expression in the wing using tubGal4 compacted the Dpp activity gradient and created halterelike P-Mad staining pattern (arrow). (D to F) Expressing UAS-tkvRNAi in the haltere using ptc-Gal4 (visualized with UAS-GFP) reduced Tkv staining [yellow arrow in (D) and (E)] and resulted in a bimodal P-Mad staining pattern [vellow arrowheads in (F), which shows a magnification of the region boxed in (D)]. (G) Adult wings uniformly expressing UAS-tkv<sup>+</sup> using *tubGal4* were  $\sim$  30% smaller than control wings. (H) Quantification of wing size reduction caused by uniform UAS-tkv<sup>+</sup> expression (orange) compared to controls (green). Error bars indicate SEM. (I) Adult halteres uniformly expressing UAS-tkvRNAi using vg-tubGal4 were up to 60% larger than control halteres. (J) Quantification of haltere size increase caused by uniform tkvRNAi expression (orange) compared to controls (green). The average increase seen is 46%.

generating region of these discs increased in size by ~40% (fig. S2). Thus, different amounts of Tkv not only affect Dpp pathway activation but also affect organ size. The fact that manipulating only Tkv production does not fully transform the sizes of these appendages suggests that additional mechanisms, such as the reduced amounts of *dpp* transcription and the modulation of other morphogen pathways by *Ubx*, also contribute to size regulation. Consistently, when Dpp production is decreased in wing discs that uniformly express *UAS-tkv*<sup>+</sup>, wing size was reduced more than it was by either single manipulation (fig. S3).

Ubx regulation of tkv. We next address how Ubx up-regulates tkv in the haltere. tkvlacZ expression and amounts of Tkv protein were cell-autonomously reduced in medial  $Ubx^{-}$  clones, whereas lateral Ubx mutant tissue retained high amounts of Tkv (Fig. 4, A to D, and fig. S4). Because tkv is repressed by Dpp and Hh signaling in the wing (26, 30), these results suggest that, in the haltere, these signals are not able to repress tkv. Consistently, activation of the Dpp pathway by expressing a constitutively active form of Tkv (TkvQD) resulted in cell-autonomous tkv-lacZ repression in the wing pouch (Fig. 4, E and F), whereas repression is not observed in the corresponding region of the haltere disc (Fig. 4, G and H).

In *Ubx* mosaic haltere discs, we also found that medial  $Ubx^+$  tissue showed stronger P-Mad staining than  $Ubx^-$  tissue at the same distance from the Dpp source (Fig. 4, A to D). We interpret this observation as evidence that  $Ubx^+$ 

tissue is more effective at trapping and transducing Dpp than  $Ubx^-$  tissue because of higher Tkv production in  $Ubx^+$  cells.

To further understand the control of tkv by Ubx, we examined the known tkv repressor, mtv (32). In medial wing disc cells, mtv expression is approximately complementary to tkv expression (Fig. 4, I and J, and fig. S1), and  $mtv^{-}$  clones in this region of the wing disc cell autonomously derepressed tkv (fig. S4) (32). In the haltere, very low *mtv-lacZ* expression was detected in the cells that stained strongly for P-Mad, suggesting that *mtv* is repressed by Dpp in this appendage (Fig. 4, I and J). Accordingly, strong repression of mtv-lacZ was seen in UAS*tkv<sup>QD</sup>*-expressing haltere pouch clones, whereas weak or no repression was seen in analogous wing clones (Fig. 4, K and L). We also found that, as expected,  $Ubx^{-}$  clones in the medial haltere cell autonomously derepressed mtv-lacZ (fig. S4).

In the wing, Dpp and mtv are mandatory repressors of tkv: In the absence of either, tkvexpression is high. In the haltere in the presence of Ubx, Dpp is a repressor of mtv. Consequently, high levels of these obligate tkvrepressors (Dpp signaling and mtv) do not coexist in the haltere, resulting in tkv derepression. Consistent with this model, when we forced mtv expression in the medial haltere, where it coexists with Dpp signaling, it repressed tkv-lacZ (fig. S4). We note, however, that Ubx is likely to control tkv through additional means, because mtv mutant wing clones did not derepress tkv-lacZ expression to



## RESEARCH ARTICLES

Fig. 4. Dpp and Ubx collaborate to repress a tkv repressor in the haltere. (A to D) Ubx mutant tissue in the medial haltere (absence of GFP) shows a cell-autonomous reduction in tkv-lacZ and reduced P-Mad staining. High P-Mad and tkv-lacZ staining can be detected in a *Ubx*<sup>+</sup> island (yellow arrow) that is separated from Dpp-producing cells by Ubx<sup>-</sup> tissue (\*). The approximate position of the AP boundary is indicated by a white arrow in (D). (E to H) Clones expressing UAS-tkv QD (marked with GFP) repress tkv-lacZ in the wing pouch (cyan arrow) but not in the analogous domain of the haltere (yellow arrowheads). (I and ]) Wild-type wing and haltere discs stained for *mtv-lacZ* and P-Mad. *mtv-lacZ* is strongly expressed in Dppproducing cells of the wing (cyan arrow) but is



repressed in Dpp-producing cells of the haltere (yellow arrow). (K and L) Clones expressing UAS-tkv<sup>QD</sup> (marked with GFP) strongly repress mtv-lacZ in the haltere (yellow arrows). Similar clones in the wing repress mtv-lacZ moderately in the P compartment (cyan arrow) and not at all in the A compartment (cyan arrowheads).



Fig. 5. Contributions of dpp transcription and mobility to growth. (A and B) dpp-lacZ is up-regulated in Ubx mutant haltere tissue (marked by loss of GFP) within the AP organizer. (**C** and **D**) UAS-tkv<sup>+</sup> expression in dorsal cells using ap-Gal4 results in dpp-lacZ down-regulation (thin arrow) compared with ventral cells (thick arrow). (E to G) Examples of  $+/Ubx^{-}$  (E),  $pbx/Ubx^{-}$  (F), and  $pbx/Ubx^{-}$ ; actGal4>tkv<sup>+</sup> (G) haltere discs stained for Nubbin (Nub), a marker of the appendage, and a marker of the AP compartment boundary (yellow line). (H) P:A ratios of the Nub domains of +/Ubx<sup>-</sup>, pbx/Ubx<sup>-</sup>, and *pbx/Ubx<sup>-</sup>; actGal4>tkv*<sup>+</sup> haltere discs. Error bars indicate SEM. (I) Summary of the consequences of different spatial relationships of Dpp and Hh signaling for *dpp* transcription in the wing and haltere. (**J**) Summary of how Dpp represses *mtv* in the presence of Ubx to control tkv expression, Dpp mobility, and growth in the haltere compared with the wing.

haltere levels (fig. S4), and ectopic mtv in the haltere did not repress tkv-lacZ expression to the extent seen in the medial wing (fig. S4).

**Control of the relative position of Dpp and Hh signaling by** *tkv* **regulation.** Because of high Tkv production in the wild-type haltere disc, peak Dpp signal transduction occurs in the AP organizer, the same cells that transduce the Hh signal. Thus, in the haltere, the activity profiles for these two signal transduction pathways coincide with each other (Fig. 1, F to H, and 5I). In contrast, low tkv expression in the wing AP organizer results in two peaks of Dpp signaling that are on either side of Hhtransducing cells. This difference will have important consequences for the expression of genes that are targets of both pathways. For example, dpp is activated by Hh and repressed by Dpp signaling (19-22, 33). In the haltere, these two conflicting inputs occur in the same cells, possibly contributing to reduced dpp expression compared with the wing.  $Ubx^{-}$  clones cellautonomously up-regulated dpp-lacZ in the haltere (Fig. 5, A and B). To test whether Ubx lowers dpp transcription in part by aligning Dpp and Hh signaling, we expressed uniform UAS $tkv^+$  in the dorsal half of the wing disc. As a result, in this region of the wing disc both signals peaked in the same cells, and dpp-lacZexpression was reduced compared with the ventral half of these wing discs (Fig. 5, C and D, and fig. S5). Conversely, expressing tkvRNAi in dorsal haltere cells increased dpp*lacZ* expression (fig. S5). Thus, *Ubx* reduces dpp transcription in part by changing where peak Dpp signaling occurs in the disc (Fig. 5I). We note that *Ubx* is likely to reduce *dpp* expression in additional ways, because increasing tkv expression does not lower dpp*lacZ* expression to that observed in wild-type haltere. Nevertheless, varying the relative spatial relationships between signal transduction pathways is a potentially powerful mechanism for modifying the outputs from commonly used pathways. We suggest that selector genes may work through molecules that control ligand distribution to vary the spatial relationships between these and other signal transduction pathways in diverse contexts during development.

Dpp mobility versus dpp transcription. The finding that increased tkv expression results in decreased dpp transcription reveals an unexpected link between Dpp mobility and Dpp production. Because of this link, the above experiments do not discriminate between growth effects due to differences in Dpp mobility per se as opposed to secondary consequences on Dpp production. To distinguish between these scenarios, we made use of a compartmentspecific Ubx regulatory allele, posterior bithorax (pbx), that lacks detectable Ubx in the P compartment when paired with a Ubx null allele but still has normal Ubx expression in the A compartment (fig. S6) (8). Consequently, in  $pbx/Ubx^{-}$  haltere discs, the P compartment increased in size such that the P:A size ratio was 1.45 (Fig. 5, E and F); the P:A ratio of  $+/Ubx^{-}$  haltere discs was ~0.35 (13). We suggest that Dpp more readily diffuses into and through the P compartments of  $pbx/Ubx^{-}$  discs because of the wing-like expression pattern of tkv and that this wing-like diffusion results in its robust growth.

To test whether differences in Tkv-regulated Dpp diffusion affect tissue growth independently of an effect on Dpp production, we examined the consequences of expressing UAS $tkv^+$  uniformly in  $pbx/Ubx^-$  haltere discs. If Tkv's effect on growth is mediated only by lowering Dpp production, both compartments should be reduced in size and thus maintain the same size ratio. However, if reducing Dpp mobility directly affects growth, the P compartment should be reduced in size more than the A compartment, which, in  $pbx/Ubx^{-}$  discs, already has high tkv expression. We found that expressing uniform  $tkv^+$  in  $pbx/Ubx^$ discs decreased the size of the P compartment more than the A compartment, resulting in a P:A ratio of 0.83 (Fig. 5, E to H). Because uniform  $tkv^+$  returned the P:A ratio back to the wild-type ratio by  $\sim 56\%$  (from 1.45 to 0.83, whereas  $+/Ubx^{-}$  discs have a P:A ratio of  $\sim 0.35$ ), these results suggest that this single variable is sufficient to provide  $\sim 50\%$  rescue of the size of an otherwise Ubx mutant P compartment.

Discussion. We have investigated the mechanism underlying a classic yet poorly understood phenomenon in biology: how size variations are genetically programmed in animal development. Many experiments show that organ size is not governed by counting cell divisions but instead depends on disc-intrinsic yet cellnonautonomous mechanisms, possibly relying on morphogen signaling (34). Our results support this idea by showing that alterations in a morphogen gradient contribute to size differences between appendages. In the example investigated here, Ubx limits the size of the haltere by reducing both Dpp production and Dpp mobility. Moreover, both of these effects are due, in part, to higher tkv expression in the medial haltere (Fig. 5, I and J). In many morphogen systems, the receptors themselves have been shown to control the distribution of the ligand and, consequently, pathway activation (30, 35-37). We show that a selector gene exploits this phenomenon to modify organ size.

Although the mechanism by which Dpp controls proliferation is not fully understood, recent results argue that, in the medial wing disc, cells may compare the amount of Dpp transduction with their neighbors, whereas lateral cells proliferate in response to absolute Dpp levels (17). Our results suggest several ways in which the altered Dpp gradient in the haltere could limit its growth. First, proliferation of lateral haltere cells may be limited because they perceive less Dpp. Second, the narrower Dpp gradient results in fewer cells exposed to the gradient in the medial haltere. Another notable difference is that, because there are two peaks of Dpp signaling in the wing but only one in the haltere, the wing has four distinct slopes whereas the haltere has only two. The less complex Dpp activity landscape of the haltere may also contribute to its reduced growth.

On the basis of these results, we suggest that altering the shape and intensity of morphogen gradients may be a general mechanism by which selector genes affect tissue sizes in animal development. Consistent with this view, *wingless* (*wg*), another long-range morphogen in the wing, is partially repressed in the haltere (*38*). Intriguingly, some of the size and shape differences in the beaks of Darwin's finches are controlled by alterations in the production of the Dpp ortholog BMP4 (*39*). Our results suggest that differences in the diffusion of this ligand may also contribute to the range of beak morphologies that have evolved in these species.

#### References and Notes

- 1. P. R. Grant, B. R. Grant, Curr. Biol. 15, R614 (2005).
- J. Zakany, D. Duboule, Cell Tissue Res. 296, 19 (1999).
- D. M. Wellik, M. R. Capecchi, Science 301, 363 (2003).
- 4. R. Krumlauf, Cell 78, 191 (1994).
- A. C. Burke, C. E. Nelson, B. A. Morgan, C. Tabin, Development 121, 333 (1995).
- 6. M. Kessel, P. Gruss, Cell 67, 89 (1991).
- S. B. Carroll, S. D. Weatherbee, J. A. Langeland, *Nature* 375, 58 (1995).
- 8. E. B. Lewis, Nature 276, 565 (1978).
- P. A. Beachy, S. L. Helfand, D. S. Hogness, *Nature* 313, 545 (1985).
- S. M. Cohen, in *The Development of* Drosophila melanogaster, M. Bate, A. Martinez Arias, Eds. (Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY, 1993), vol. II, pp. 747–842.
- 11. P. Martin, J. Exp. Zool. 222, 97 (1982).
- 12. F. Roch, M. Akam, Development 127, 97 (2000).
- 13. See Materials and Methods on Science Online.
- G. Morata, A. Garcia-Bellido, W. Roux, Arch. Dev. Biol. 179, 125 (1976).
- 15. T. Lecuit et al., Nature 381, 387 (1996).
- D. Nellen, R. Burke, G. Struhl, K. Basler, Cell 85, 357 (1996).
- 17. D. Rogulja, K. D. Irvine, Cell 123, 449 (2005).
- 18. P. A. Lawrence, G. Struhl, Cell 85, 951 (1996).
- 19. K. Basler, G. Struhl, Nature 368, 208 (1994).
- 20. I. Guillen et al., Development 121, 3447 (1995).
- T. Tabata, C. Schwartz, E. Gustavson, Z. Ali, T. B. Kornberg, Development 121, 3359 (1995).
- M. Zecca, K. Basler, G. Struhl, *Development* **121**, 2265 (1995)
- 23. R. Burke, K. Basler, *Development* **122**, 2261 (1996).
- 24. ]. Capdevila, I. Guerrero, *EMBO J.* **13**, 4459 (1994).
- J. Sekelsky, S. J. Newfeld, L. A. Raftery, E. H. Chartoff, W. M. Gelbart, *Genetics* 139, 1347 (1995).
- 26. H. Tanimoto, S. Itoh, P. ten Dijke, T. Tabata, *Mol. Cell* **5**, 59 (2000).
- E. V. Entchev, A. Schwabedissen, M. Gonzalez-Gaitan, Cell 103, 981 (2000).
- 28. A. A. Teleman, S. M. Cohen, Cell 103, 971 (2000).
- 29. T. Y. Belenkaya *et al.*, *Cell* **119**, 231 (2004).
- 30. T. Lecuit, S. M. Cohen, Development 125, 4901 (1998).
- 31. T. J. Brummel et al., Cell 78, 251 (1994).
- 32. Y. Funakoshi, M. Minami, T. Tabata, *Development* **128**, 67 (2001).
- T. E. Haerry, O. Khalsa, M. B. O'Connor, K. A. Wharton, Development 125, 3977 (1998).
- S. J. Day, P. A. Lawrence, *Development* **127**, 2977 (2000).
- K. M. Cadigan, M. P. Fish, E. J. Rulifson, R. Nusse, *Cell* 93, 767 (1998).
- 36. J. Casanova, G. Struhl, Nature 362, 152 (1993).
- 37. Y. Chen, G. Struhl, Cell 87, 553 (1996).
- S. D. Weatherbee, G. Halder, J. Kim, A. Hudson, S. Carroll, Genes Dev. 12, 1474 (1998).

## RESEARCH ARTICLES

- A. Abzhanov, M. Protas, B. R. Grant, P. R. Grant, C. J. Tabin, *Science* **305**, 1462 (2004).
- 40. Y. S. Lee, R. W. Carthew, *Methods* **30**, 322 (2003).
- We thank S. Cohen, M. Gonzalez-Gaitan, T. Jessell, L. Johnston, E. Laufer, B. McCabe, M. O'Connor, G. Struhl, T. Tabata, the Bloomington Stock Center, and the Developmental Studies Hybridoma Bank for antibodies and fly stocks and R. Axel, O. Hobert, T. Jessell,

L. Johnston, D. Rogulja, and G. Struhl for comments on the manuscript. This work was supported by a grant from the NIH to R.S.M. and an NIH training grant (M.A.C.).

Supporting Online Material

www.sciencemag.org/cgi/content/full/1128650/DC1 Materials and Methods SOM Text Figs. S1 to S6

12 April 2006; accepted 25 May 2006 Published online 1 June 2006; 10.1126/science.1128650 Include this information when citing this paper.

## Hierarchical Action and Inhibition of Plant Dicer-Like Proteins in Antiviral Defense

Angélique Deleris,<sup>1</sup> Javier Gallego-Bartolome,<sup>1</sup> Jinsong Bao,<sup>2</sup> Kristin D. Kasschau,<sup>2</sup> James C. Carrington,<sup>2</sup> Olivier Voinnet<sup>1\*</sup>

The mechanisms underlying induction and suppression of RNA silencing in the ongoing plant-virus arms race are poorly understood. We show here that virus-derived small RNAs produced by *Arabidopsis* Dicer-like 4 (DCL4) program an effector complex conferring antiviral immunity. Inhibition of DCL4 by a viral-encoded suppressor revealed the subordinate antiviral activity of DCL2. Accordingly, inactivating both DCL2 and DCL4 was necessary and sufficient to restore systemic infection of a suppressor-deficient virus. The effects of DCL2 were overcome by increasing viral dosage in inoculated leaves, but this could not surmount additional, non-cell autonomous effects of DCL4 specifically preventing viral unloading from the vasculature. These findings define a molecular framework for studying antiviral silencing and defense in plants.

n RNA silencing, ribonuclease (RNase) III-like enzymes in the Dicer family produce short interfering (si)RNA and micro (mi)RNA from RNA with doublestranded (ds) features (1). These molecules guide RNA-induced silencing complexes (RISCs) to suppress gene expression at the transcriptional, RNA-stability, and translational levels (2). Arabidopsis thaliana has four specialized Dicer-like (DCL) proteins. DCL1 processes fold-back precursors to release miRNAs (3). DCL3 produces 24-nucleotide (nt)-long, DNA repeat-associated siRNAs guiding heterochromatin formation (4). DCL4 generates 21-nt-long siRNAs that mediate posttranscriptional silencing of some endogenous genes [trans-acting (ta)-siRNAs; (5, 6)] and of transgenes mediating RNA interference (7). DCL2 synthesizes stress-related natural-antisensetranscript (nat)-siRNAs (8), siRNAs derived from at least one virus (4), and, in dcl4 mutant plants, it alternately processed ~22-nt siRNAs from ta-siRNA precursors (5, 6).

The observations that virus-derived siRNAs accumulate in plant and insect infected tissues and that many viruses encode suppressor proteins targeting DCL, RISC, or small RNA activities strongly suggest that RNA silencing has antiviral roles (9-11). In plants, one or more of the six RNA-dependent RNA-polymerase (RDR) paralogs, including *Arabidopsis* RDR6 and RDR1, may strengthen primary silencing responses by producing dsRNA from viral templates (12) and by amplifying mobile silencing signals conditioning antiviral immunity in noninfected tissues (7, 13). Nevertheless, the genetic bases of silencing induction and suppression by plant viruses remain unclear. Even the existence of an antiviral RISC ("slicer") is arguable because DCL-mediated processing of virusderived dsRNA could be, in principle, sufficient to dampen infections. It remains also uncertain how, when, and where antiviral silencing and its suppression impact susceptibility and defense in whole plants. This study addresses these issues using *Arabidopsis* silencing mutants and three distinct RNA viruses.

**DCL4-** and DCL2-dependent siRNAs recruit an antiviral RISC. Arabidopsis plants were inoculated with modified Tobacco rattle virus (TRV-PDS) (Fig. 1A) containing a fragment of the Arabidopsis phytoene desaturase (PDS) gene in place of the RNA2-encoded 2b and 2c sequences. Like TRV-infected tissues (Fig. 1B), TRV-PDS–infected tissues are free of disease symptoms, because of a strong silencing response that dramatically reduces viral titers (14), and exhibit extensive photobleaching due to virus-induced gene silencing (VIGS) of PDS (Fig. 1C) (7).

TRV-PDS-specific siRNAs accumulated as discrete 21-nt and 24-nt species in wildtype (WT) *Arabidopsis* (Fig. 1D), a pattern unchanged in *rdr1*, *rdr2*, *rdr6* [supporting online material (SOM), fig. S1], and *dcl2* mutants (Fig. 1D). However, the 24-nt and 21-nt siRNAs were undetectable in *dcl3* and *dcl4* mutants, respectively. Loss of 21-nt siRNAs in *dcl4* mutants (Fig. 1D). Identical siRNAs in *dcl4* mutants (Fig. 1D). Identical siRNA patterns were detected with an RNA2(TRV)specific probe, whereas probes specific for cellular *PDS* sequences absent in TRV-PDS





Fig. 1. (A) Genome organization of TRV and its TRV-PDS derivative. (B and C) Asymptomatic infection (B) and extensive photobleaching (C) caused by TRV and TRV-PDS, respectively. (D and E) Analysis of (top) low- and (bottom) highmolecular-weight RNAs from TRV-PDS-infected

plants carrying single (D) and double (E) *dcl* mutations [14 days post inoculation (dpi)]. The probe was specific for viral PDS. The numbers of infected plants showing photobleaching are from four independent experiments involving four plants

each. (**F** to **L**) Disease symptoms and VIGS in *dcl* mutants (14 dpi). (**M**) TRV-PDS siRNA analysis in *dcl2-dcl3-dcl4* triple mutants (14 dpi). rRNA shown by ethidium bromide staining.

<sup>&</sup>lt;sup>1</sup>Institut de Biologie Moléculaire des Plantes, CNRS Unité Propre de Recherche (UPR) 2357, 12, rue du Général Zimmer, 67084 Strasbourg Cedex, France. <sup>2</sup>Center for Genome Research and Biocomputing, Oregon State University, Corvallis, OR 97331, USA.

<sup>\*</sup>To whom correspondence should be addressed. E-mail: olivier.voinnet@ibmp-ulp.u-strasbg.fr