

FOCUS

Over the course of evolution, plants have developed a wide array of leaf shapes. Every shape is the result of adaptation to the environment, which is why the way that leaves look is no real indication of whether or not plants are related. Even closely related species may have different leaves, in some cases as a way of adapting to different environmental conditions.



DIVERSITY IN THE LEAFY FOREST

TEXT: TIM SCHRÖDER

Lanceolate, ovate, elliptical, entire, serrated, and uni- or multi-pinnate – there are numerous names to describe the variety of leaf morphology. But how does this diversity come about? Miltos Tsiantis from the Max Planck Institute for Plant Breeding Research in Cologne and his team are looking for genes that control leaf growth. They have already found one central regulatory element.



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- In botanical guides, the lists of leaf morphology descriptions can fill several pages. These classifications distinguish between simple leaves with continuous leaf surfaces and outlines, and so-called compound leaves, which feature leaf surfaces consisting of separate units called leaflets. The vast range of morphology is well researched, because the shape of a leaf is an important characteristic for the identification of plants and because leaves are the sites of fixing atmospheric carbon into sugars in a process known as photosynthesis. But the origin of different leaf forms and why even leaves of closely related species can look very different remained a mystery until quite recently.
- Together with his team at the Max Planck Institute in Cologne, Miltos Tsiantis is analyzing when and where genes that are important for shape development are switched on and off as a leaf bud slowly takes shape. He's utilizing modern technologies to do so, including advanced microscopy methodologies that allow scientists to zoom deep into leaf tissue and computer models to grow virtual leaves. "We want to find out which genes make leaves of different species look the way they do. Since flowering plants typically have over twenty thousand genes, it's a bit like searching for a needle in a haystack," says Tsiantis. For many years, he has been working with *Arabidopsis thaliana*, which is essentially

the fruit fly for botanists. Thousands of researchers have analyzed the plant's genetic material, metabolism, and development. Many of its genes are therefore relatively well studied.

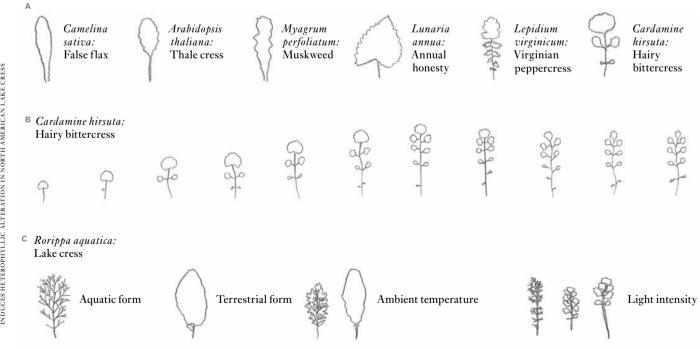
But studying Arabidopsis thaliana alone cannot tell Tsiantis how various leaf forms evolve. For this, he is researching its close relative, Cardamine hirsuta, a delicate plant with white flowers that also belongs to the Brassicaceae family and that the Cologne research group has used to develop a model system for comparative studies in plant genes. Although the two plants are closely related, their leaves have different shapes. Those of Arabidopsis thaliana resemble round spinach leaves, which have a few delicate serrations at the edge. Cardamine hirsuta, on the other hand, has pinnate leaves (i.e., one leaf is divided into several pinnae or leaflets). A larger, roundish leaflet grows at the tip of the petiole with small pinnate leaves on delicate stalks on the left and right below it. Tsiantis cultivates Cardamine hirsuta in the greenhouses at the MPI in Cologne, because he needs genetically different individuals for his investigations. To achieve this, he and his team treat the seeds of the plants with the chemical ethyl methanesulfonate. This substance induces point mutations (i.e. damage to the genetic material). "This helps us modify individual genes. However, the muta-



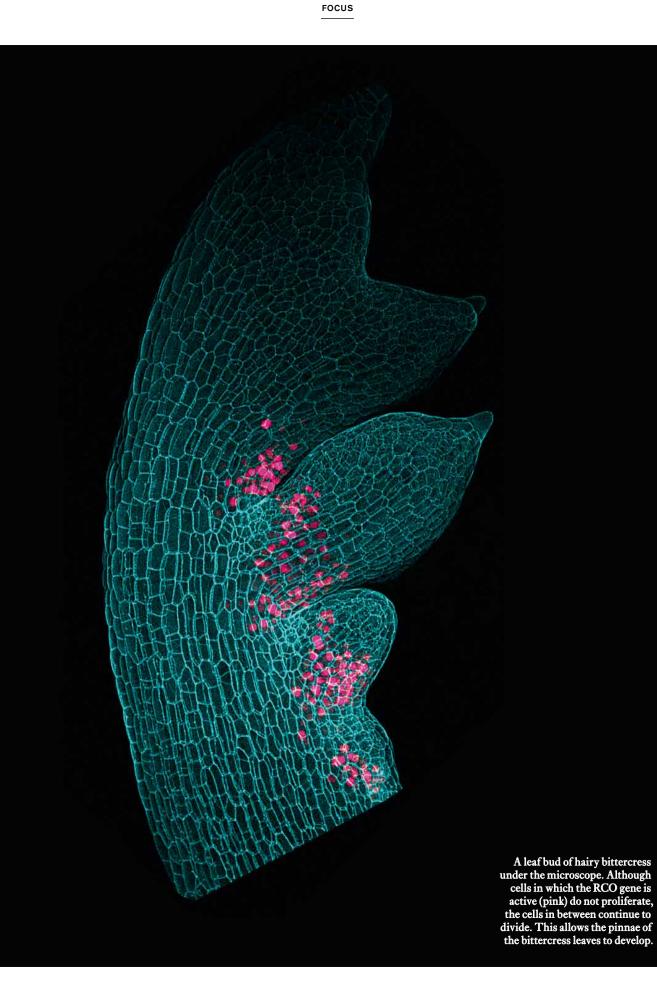
Exchange of ideas during the COVID pandemic: Miltos Tsiantis, Angela Hay, Neha Bhatia, Ziliang Hu, David Wilson-Sanchez und Shanda Liu (from left to right).

tions occur randomly. We can't influence where they occur," says Tsiantis. But if one of the treated plants develops leaves of a different shape, he and his research team can analytically reverse engineer the process to pinpoint which genes have mutated and are thus responsible for the change in shape.

This is how Tsiantis's former student, Huw Jenkins, has cultivated thousands of Cardamine hirsuta plants with varying leaf shapes - from curly, parsley-like leaves to elongated ones resembling lavender. Among all the plants, the group discovered one in which the leaves looked familiar - round, with fine indentations at the edges and completely without pinnate leaves. "This Cardamine hirsuta plant looked a bit like an Arabidopsis thaliana plant," says Angela Hay, who also uses Cardamine hirsuta in her studies. A genetic analysis showed that the chemical treatment had altered a DNA segment of Cardamine hirsuta that does not occur at all in Arabidopsis thaliana. The researchers had thus discovered a gene that was missing in the simple leaves of Arabidopsis thaliana but that, when it is damaged, causes Cardamine hirsuta to develop simplified leaves as well. These results were further confirmed by using the recent breakthrough technology of genome editing, which allowed the Cologne group to induce targeted mutations in the RCO gene: and again they observed the formation of that same, simple leaf phenotype. That made it clear that this gene controls the shape of the leaf. Because the leaves of



Leaf diversity among crucifers: differently shaped leaves occur not only in different plant species (A) but also in the same plant depending on its age (B). Environmental conditions also influence leaf shape (C).



Young leaf bud of a genetically modified thale cress (left). The microscopic image shows the contours of the cells that have been made visible with a fluorescent protein. With the help of a computer, the cells of the same bud can be color-coded (right), and the multiplication of individual cells can be investigated.

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Cardamine hirsuta have a less complex structure without the gene, the researchers named it "RCO" (Reduced Complexity).

Tsiantis's team then conducted a more detailed analysis of how leaves of *Cardamine hirsuta* develop. Using fluorescent protein labeling, the researchers were able to observe under a microscope that the RCO gene is active at certain places along the leaf edge – specifically, in distinct bands at the base of emerging leaflets. The RCO gene inhibits cell growth at these sites, while the cells in between the RCO bands continue to grow. The

"The RCO gene is one of our most important discoveries – because it gives us a clear glimpse of how evolution works."

MILTOS TSIANTIS

bulges created in this way gradually grow into the distinct leaflets or pinnae. "RCO thus helps the individual leaflets to emerge and grow into separate pinnae, instead of producing a continuous leaf surface," explains Tsiantis.

Thus, if the RCO gene is a pivotal gene in the evolution of leaf shapes, then an Arabidopsis thaliana with an artificially transferred RCO gene should also develop pinnate leaves. And indeed: an Arabidopsis thaliana plant modified to contain the RCO gene has roundish leaves with clearly visible protrusions. Although the plants do not grow pinnate leaves on stems, the resemblance to Cardamine hirsuta is truly astonishing. "The results of these experiments caused some excitement in the scientific community, because it is very rare for a single gene to influence the differences between plant species to such an extent," says Tsiantis. "Actually, we expected that the minor, respective effects of a large number of genes would work together to cause evolutionary changes in such fundamental traits." So, together with Donovan Bailey from the New Mexico State University, Tsiantis then began studying the phylogeny of Brassicaceae in order to find the origin of the gene. Apparently, the Brassicaceae family did not originally possess an RCO gene. Consequently, species of Brassicaceae that are developmentally similar to

ancient members of the family have simple leaves. When RCO emerged from a gene duplication event during the course of evolution, species such as *Cardamine hirsuta* or *Arabidopsis lyrata* (which is even more closely related to *Arabidopsis thaliana*) were able to form deeply indented or compound leaves. The gene was lost later in the phylogenetic history, so that *Arabidopsis thaliana* was able to form simple leaves. Since the discovery of the RCO gene, Miltos Tsiantis, and his team have been studying the regulation of leaf

growth even more closely. Although Tsiantis's work is basic research, his findings could certainly have practical relevance, for example in agriculture. "In light of climate change, knowledge about the development of different leaf forms could become increasingly important and contribute to the environmentally sustainable cultivation of higher-yielding plants," says Tsiantis. When Miltos Tsiantis moved from Oxford to Cologne in 2013, he was able to readily acquire state-of-the-art microscopes. They make individual cells visible to Neha Bhatia, a post-doctoral scientist in the group. With great dexterity, she first removes the mature leaves of the seedlings, and then places the tiny plant under the microscope. This allows her to observe the newly formed leaves which can be as small as a few micrometers long. Then she switches on the screen, focuses the microscope and suddenly the cells marked with fluorescent proteins begin to glow. "We can now mark several genes simultaneously with different colors and see where in the leaf they are currently active," explains Bhatia.

In the meantime, the researchers have also discovered that a previously discovered gene called the STM gene acts together with RCO to initiate leaflet formation. While RCO inhibits leaf growth, STM causes cells to continue growing. Nor-

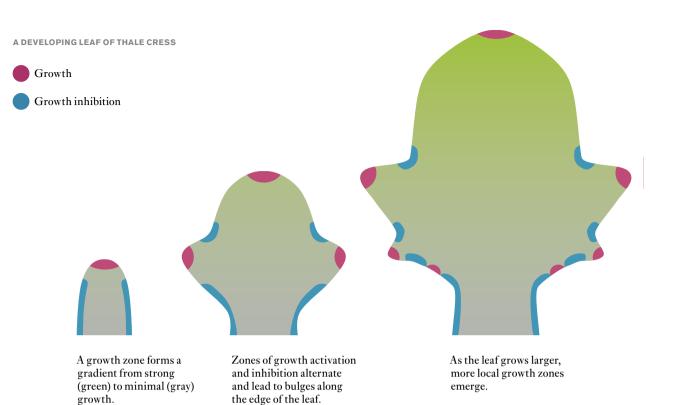
mally, cells no longer divide when they specialize. However, when STM is active, the cells can continue to multiply so that the leaf grows wider at this point. If RCO and STM are active at the same time, broad bulges form at the edge of the leaf. "When we activate both genes in the *Arabidopsis thaliana* leaves, the leaves formed by these genetically modified plants look like those of *Cardamine hirsuta*," says Tsiantis. This experiment shows that, by having an in-depth understanding of how plants differ from one another in their specific characteristics, we might then also use that understanding to modify the traits of crop plants as well. However, the shape of a leaf is not determined

SUMMARY

Developing *Cardamine hirsuta* as a model system for studying plant biology helped the scientists make new discoveries about how plants develop and diversify. For example, the 'RCO gene' is active in some species and not others, and it plays a significant role in determining the shape of a leaf.

RCO is active in the cells located at regular intervals along the edge of the leaf. It prevents those cells from growing.

STM is another important gene that is active in leaves of some species but not others. STM is active more broadly in the leaf than RCO and it allows prolonged proliferation of leaf cells. This feature, together with the more local growth repressive action of RCO, allows small bulges at the leaf margin to eventually grow into separate leaflets.



by its genes alone. The physical properties of leaf tissue also influence the development of the leaf, by means of the mechanical resistance it exerts on growing cells. Adam Runions, a computer scientist in Tsiantis's team, developed a mathematical model that takes into account physical interactions within the leaf tissue. This allows him to "program" leaves that grow virtually. "If we suspect, for example, that a gene functions in a certain way, we can use our model to test this hypothesis," says Tsiantis. "In this way, we can identify the basic rules according to which the leaves develop."

But why do plants have such dissimilar leaves in the first place? To answer this question, the Tsiantis team studies different Cardamine hirsuta plants from Europe and other parts of the globe to learn how the local environment may affect the shape of a plant's leaves. "For instance, we have observed that plants that grow in regions where they can produce flowers quickly and reproduce rapidly also produce more leaflets more quickly. This strategy may support a more efficient assimilation of carbon and consequently a better supply of nutrients for the next generation. Conversely, in areas like central Europe or Scandinavia, where plants bloom late because they have to survive a cold winter, they tend to 'wait' to attain their maximum leaflet numbers until just before they flower in spring. Perhaps this strategy also ensures the leaves' ability to supply an optimum level of nutrients for the seeds, which will form soon after flowering," the scientist explains. The various methods, the wealth of ideas, and the unusual perspectives implemented by Tsiantis and his team have made important insights into leaf growth possible. Tsiantis himself considers the RCO gene to be one of his most important discoveries, "...because it very clearly illustrates how innovations can arise during evolution." 45

Most genes have several functions. For this reason, the mutations of one gene can trigger a wide variety of changes in a plant's form and function. A mutation can improve one function and impair another. Experts refer to this as "pleiotropy". "Just imagine: a bird develops wings that can help it fly better. But if the genetic modifications responsible for this improvement also change the bird's color and thus make it less attractive to potential mates, that change in wing shape will most likely not be successful," explains Tsiantis. RCO on the other hand is a gene that is hardly pleiotropic at all. It largely controls the shape of a plant's leaves, but otherwise has few other functions. "The example of RCO lets us not only reveal the secret of leaf diversity, but also learn how evolution works." For example, the role of RCO in leaflet separation can be considered analogous to processes that lead to the separation of digits on developing limbs. In this case, however, the separation is primarily driven by programmed cell death, and not growth inhibition.

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