# Pathway toward gene silencing described in plants

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Biologists at Washington University in St. Louis have made an important breakthrough in understanding a pathway plant cells take to silence unwanted or extra genes using short bits of RNA. Basically, they have made it possible to see where, and how, the events in the pathway unfold within the cell, and seeing is believing, as the old saying goes.

Craig Pikaard, Ph.D., Washington University professor of biology in Arts & Sciences and his collaborators have described the roles that eight proteins in Arabidopsis plants play in a pathway that brings about DNA methylation, an epigenetic function that involves a chemical modification of cytosine, one of the four chemical subunits of DNA. Without proper DNA methylation, higher organisms from plants to humans have a host of developmental problems, from dwarfing in plants to certain tumors in humans, and death in mice.

One role of DNA methylation is to turn off repetitive genes, such as transposable elements that can move or spread throughout a genome and disrupt other gene functions if left unchecked. There is also interest in DNA methylation because understanding how some genes are selectively silenced and how silenced alleles can be turned on again may someday have practical benefits. For instance, tumor suppressor genes that normally help keep cells from dividing uncontrollably are often silenced by DNA methylation and histone (proteins that wrap DNA) modifications in cancer cells, contributing to tumor growth. And certain blood disorders resulting from defective genes expressed in adults might be alleviated if versions of those same genes that are only expressed very

early in development, but are then silenced in adults, could only be turned on again.

"The pathway we are studying is part of an interesting phenomenon that occurs in plants, and reportedly in humans, too, called RNA-directed DNA methylation," Pikaard explained. "This pathway takes place in the nucleus, and it involves short RNAs, called small interfering RNAs -- siRNAs."

Those little tykes, just 24 nucleotides long, are somehow responsible for methylation of DNA sequences that match the sequence of the siRNAs, but not without a lot of help from their friends. The friends in this case are the team of eight known proteins of the RNA-directed DNA methylation pathway.

Using an impressive toolkit of sophisticated techniques, Pikaard and his collaborators not only have described the locations of the eight proteins in the pathway but also have provided the sequence of events that leads to methylation. It is a twisted, and ultimately circular path, but Pikaard and his colleagues are the first researchers to literally see the pathway and thereby provide a clearer understanding of the steps leading to methylation and gene silencing.

The results were published in the July 14, 2006 issue of *Cell*. The study was funded by the National Institutes of Health, Howard Hughes Medical Institute (HHMI) and Monsanto Company. Pikaard's collaborators include Olga Pontes, the first author of the study, other group members from his Washington University laboratory and the group of Steven E. Jacobsen, Ph.D., an HHMI investigator and professor of biology at the University of California, Los Angeles.

Using mutants, antibodies, and fluorescence microscopy techniques known as RNA fluorescence in situ hybridization (RNA-FISH) and

DNA-FISH, Washington University postdoctoral researcher Olga Pontes, Ph.D., was able to unravel where the eight team players are located and in what order events in the RNA-directed DNA methylation pathway transpire. Using antibodies to detect the proteins, together with DNA-FISH to detect the DNA sites that give rise to the siRNAs, Pontes found that half of the team is located with the genes that match the siRNAs.

"The combination of DNA FISH and protein localization allowed us to say which proteins are sitting on the DNA that give rise to the siRNAs and also the loci that are modified by the siRNAs," Pikaard said.

Pontes found the other half of the team located within a special nuclear compartment known as the nucleolus, long known to be the production center for ribosomes. "She got a brilliant signal in the nucleolus, a brilliant dot in the same place for each of the proteins," said Pikaard. Using RNA-FISH, Pontes also found that the siRNAs were in that same dot within the nucleolus.

Pontes and Pikaard were able to deduce the order of events by studying mutations of all eight genes that give rise to the proteins, finding out what happens to the different proteins as the different genes are mutated, one by one. For instance, the researchers found the importance of RNA Polymerase IVa (Pol IVa) by looking at a Pol IVa mutant and noting that the rest of the proteins didn't localize properly. In the RNA-dependent RNA polymerase 2 (RDR2) mutant, Pol IVa is unaffected, but the function of all the other proteins downstream is lost, inferring that it came into the act second. The picture that emerged from this logical approach is that Pol IVa gets things started, churning out RNA that then goes to the nucleolus where it is acted on by RDR2, which turns the single-stranded RNA into double-stranded RNA. The Dicer-like 3 protein, DCL3 then chops the RNA into small interfering RNAs (siRNAs). Along comes ARGONAUTE4 (AGO4), which grabs hold of

the siRNAs while also binding to NRPD1b, the largest subunit of an alternative form of RNA Polymerase IV, Pol IVb. The AGO4-siRNA-NRPD1b complex is then thought to leave the nucleolus, acquire the second-largest Pol IV subunit, NRPD2, which serves both Pol IVa and Pol IVb, and then seek out the DNA sequences that match the siRNAs. At these sites, the chromatin remodeler DRD1 presumably bulldozes histones and other proteins out of the way to make the DNA accessible for methylation by the de novo cytosine methyltransferase, DRM2.

A paradoxical aspect of the pathway is that siRNAs direct DNA methylation but DNA methylation is also required for the production siRNAs. "It's a circular pathway. You have to produce the siRNA in order to have them come back and methylate the loci, which somehow induces more siRNA production involving Pol IVa". Pikaard said.

A combination of genetic mutants, transgenes, antibodies, RNA-FISH and DNA-FISH were key to the study. "This toolkit is really powerful," Pikaard said.

"It enabled us to look at a complex pathway and figure out not only the order of events but also the spatial organization of the pathway in the nucleus. Our hope for the future is to develop tools that will enable us to watch the pathway function in live cells using fluorescent proteins and time-lapsed microscopy, to learn even more."

Source: Washington University in St. Louis