

the sequence data from the second generation sequencers. By integrating paired end sequences generated from 454 and Solexa sequencers, scaffolding and gap filling of current contigs are on going. Status of this integration approach will also be presented.

#### Structure–function studies in the wheat genome

**Keeble, G<sup>1</sup>**, Black, M<sup>1</sup>, Moolhuijzen, P<sup>1</sup>, Barerro, R<sup>1</sup>, Bellgard, M<sup>1</sup>, Appels, R<sup>1</sup>

<sup>1</sup>Centre for Comparative Genomics, Murdoch University, Perth, Australia

Two separate 1Mb regions of the wheat genome comprising extensive stretches of repetitive sequences were assembled based on sequence data from overlapping BAC clones located in a section of the chromosome 3B of particular interest with regards to disease resistance. The sequencing of the 24 BAC clones combined standard Sanger sequencing (5-8 x coverage) and Solexa/Illumina short read sequencing (>300 x coverage). In addition, available BAC end and 454-based sequences were utilized to provide a good quality reference sequence that was annotated with a particular focus on the repetitive elements. Alignment to the rice and *Brachypodium* genome sequences identified a repetitive locus that is conserved between these genomes and the wheat genome. The conserved repetitive protein kinase like genes at this conserved locus showed a good alignment to the bacterial effector protein AvrPto (Weiman et al. 2007).

### Sym162: Molecular and genetic analysis of features important for economically significant plants – 29 July

#### A systems approach to defining C<sub>4</sub> networks in the grasses

**Brutnell, TP<sup>1</sup>**

<sup>1</sup>Boyce Thompson Institute, USA

C<sub>4</sub> photosynthesis has evolved at least 60 times in the angiosperms and at least 17 times in the grasses, leading to some of the most productive food, feed, and bioenergy crops on the planet. Yet, we know very little of the regulatory networks that drive this process in any major crop plant. I will describe our efforts to use maize and *Setaria viridis* as models for a systems biology approach to understanding regulatory networks driving C<sub>4</sub> differentiation. We have recently analyzed the maize leaf transcriptome using Illumina sequencing (RNAseq) to define gene structure and to quantify transcript abundance along a calibrated leaf developmental gradient and in mature bundle sheath and mesophyll cells. Complementing this analysis, detailed histological, proteomic and metabolite datasets have been collected along the same gradient and analyzed by several collaborators. We are now developing methods to integrate these diverse datasets and I will discuss our current strategies and findings. To provide community access to this data, we implemented Gbrowse and an electronic fluorescent pictograph browser to display and

query the datasets. Finally, I will describe our recent efforts to develop *Setaria viridis* as a model system for dissecting C<sub>4</sub> gene expression.

#### Plant epigenetics : stories from beyond the double helix

**Rival, A<sup>1</sup>**, Jaligot, E<sup>1</sup>, Beule, T<sup>1</sup>, Richaud, F<sup>1</sup>, Aberlenc, F<sup>1</sup>, Adler, S<sup>1</sup>, Debladis, E<sup>1</sup>, Ilbert, P<sup>1</sup>, Finnegan, J<sup>2</sup>

<sup>1</sup>UMR DIADE. IRD, Cirad BioS, Montpellier, France; <sup>2</sup>CSIRO Plant Industry, Canberra, Australia

Epigenetics is the study of heritable changes in gene function that occur without a change in the DNA sequence. In recent years, this field has attracted much attention as more epigenetic controls of gene activities are being discovered. Such controls involve a complex interplay of DNA methylation, histone modifications, and RNA-mediated pathways from non-coding RNAs, notably silencing RNA (siRNA) and microRNA (miRNA). In plants, although epigenetic mechanisms help to protect cells from parasitic elements, this defence can complicate the genetic engineering process through transcriptional gene silencing. Furthermore, these phenomena have economic relevance, for example, in somaclonal variation: a genetic and phenotypic variation among clonally propagated plants from a single donor genotype. The loss of phenotypic fidelity is now a major impediment to the development of large scale propagation of plants through in vitro processes such as somatic embryogenesis. Examples of aberrant phenotypes in regenerated plants include abnormal leaf structures and variant floral morphology, both organs being of paramount importance for applications in horticulture and/or agriculture. Changes in DNA methylation have been hypothesised playing a key role in the mechanism underlying tissue-culture induced changes. Indeed, studies of both global methylation levels and the methylation of specific sites show that variation in DNA methylation occurs frequently during growth in tissue culture. In vitro plant regeneration, like somatic embryogenesis, bypasses the normal developmental process of fertilisation and plant development, thus potentially resulting in the instability of epigenetic patterns. The large-scale clonal propagation of oil palm (*Elaeis guineensis* Jacq.) is being hampered by the occurrence of the mantled somaclonal variation. Indeed, this abnormality which presents a homeotic-like conversion of male floral organs into carpelloid structures, hampers oil production since the supernumerary female organs are either sterile or produce fruits with poor oil yields. Beyond its primary interest in the search for discriminating markers against an economically crippling phenotype, the study of the mantled abnormality also provides a unique opportunity to investigate the regulation of reproductive development in a perennial tropical plant. The present interest on food and energy security as well as the concerns raised by the possibility of climate change further stress the need for a global comprehension of how crop plants react to their fluctuating environment and how their productivity can be affected.

## **Plant epigenetics: stories from beyond the double helix**

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# Plant epigenetics

## Stories from beyond the double helix ...

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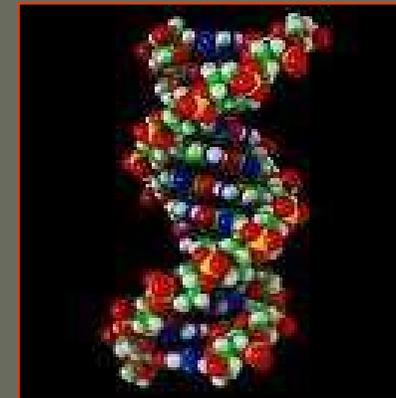
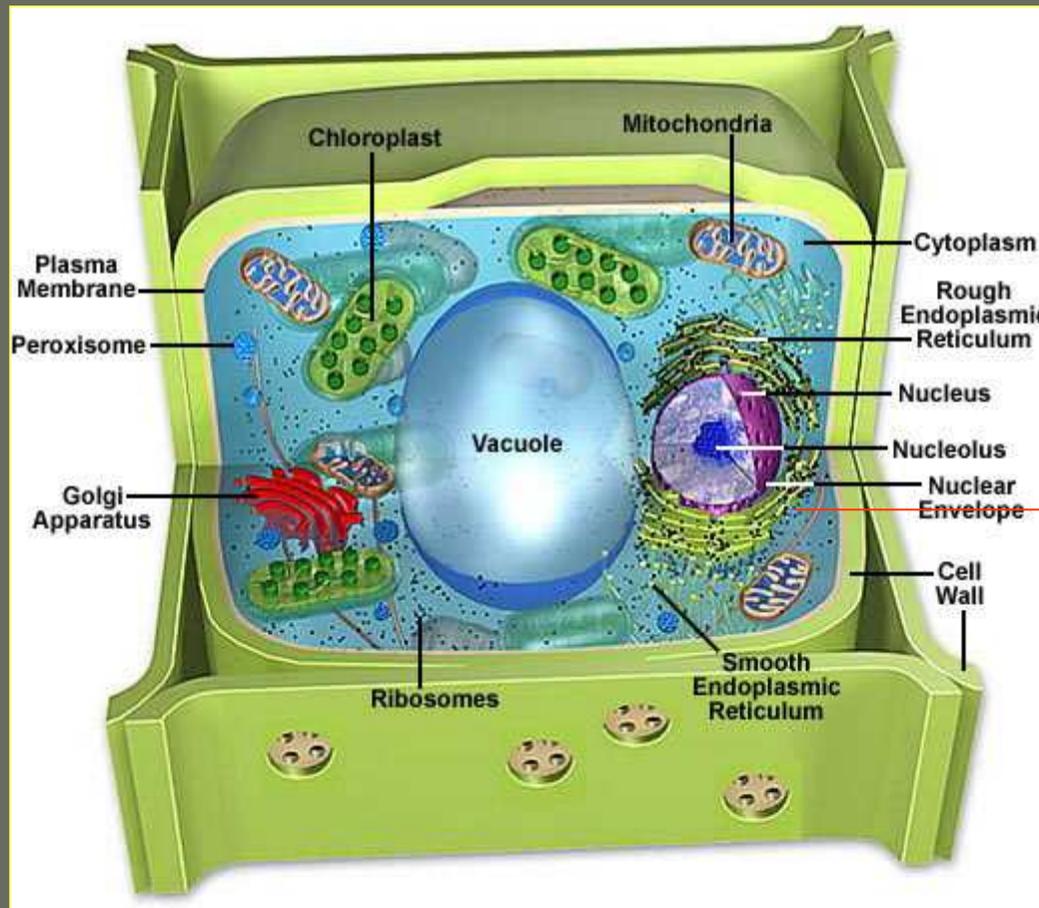
# What is Epigenetics?

« The interactions of genes with their environment, which bring the phenotype into being ».

Waddington, C. (1942) The Epigenotype. Endeavour, 1, 18–20

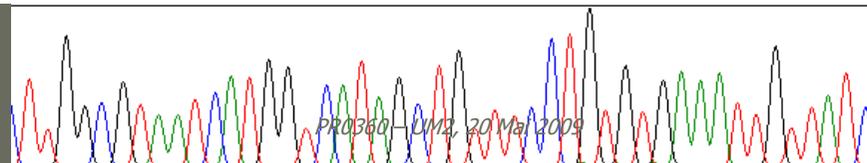
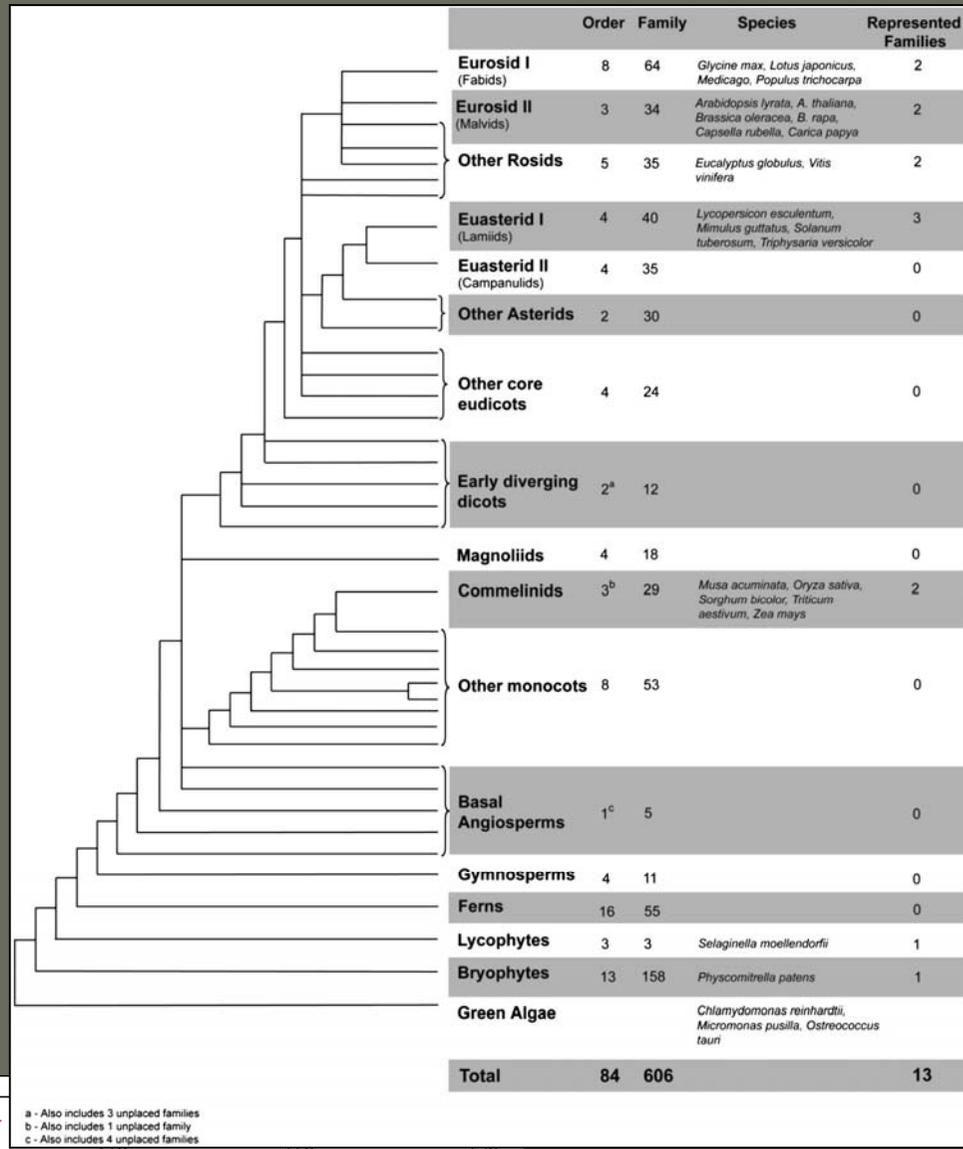
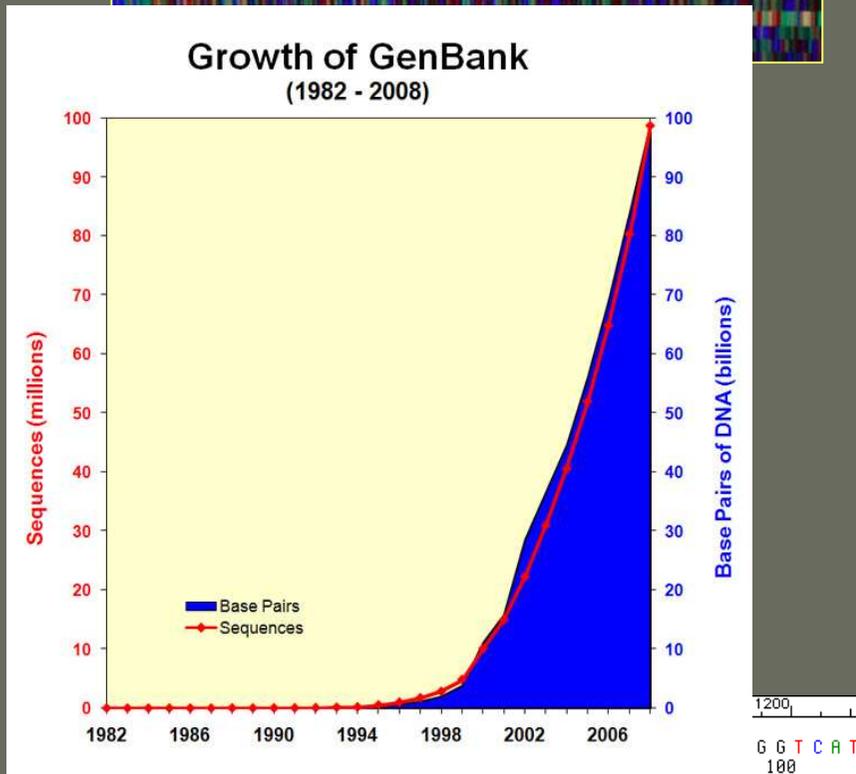
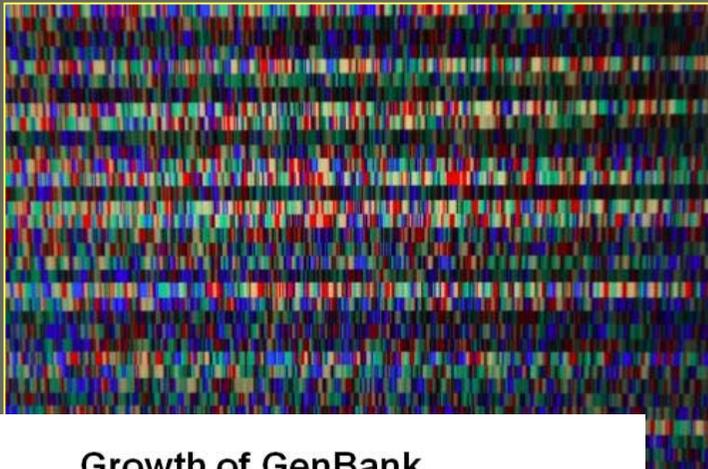
“Changes in gene expression not attributable to nucleotide sequence variation”.

# The Cell and the Genome



The cell nucleus contains the majority of the plant cell's genetic material

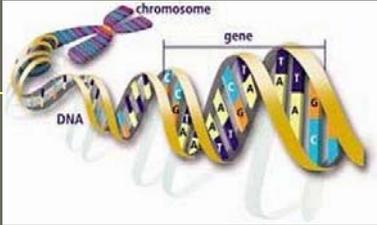
# Genomics: sequencing plant genomes



# Beyond the sequencing of plant genomes

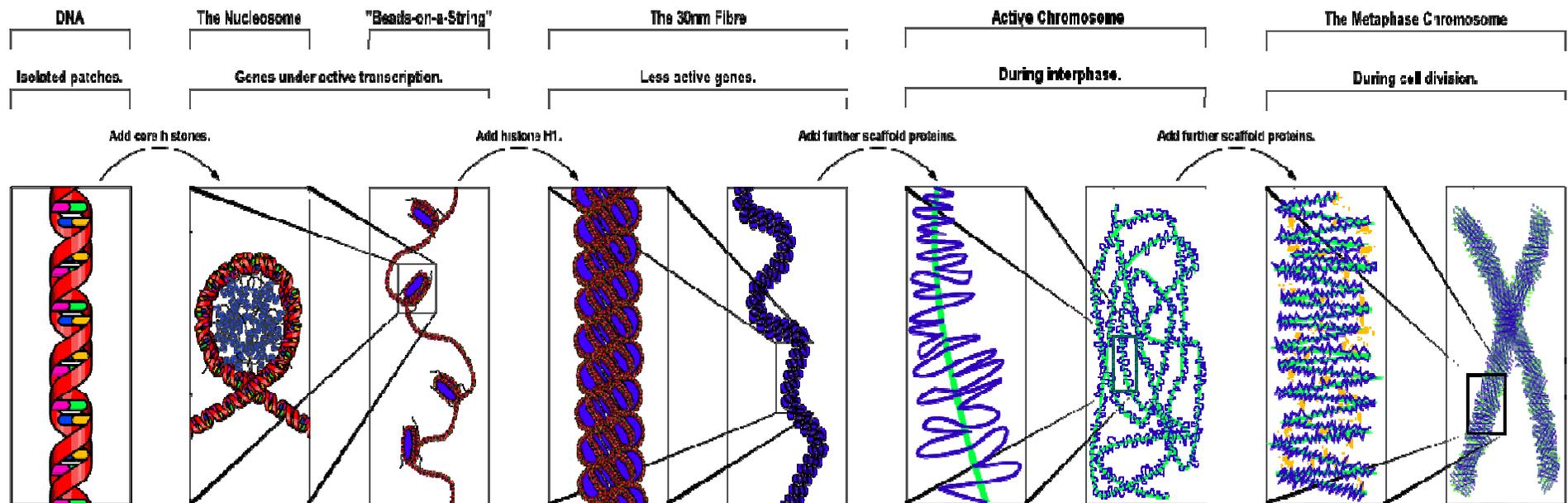
- Several new sequencing technologies are emerging that have the potential to provide **increases in throughput and reductions in cost**
- Companies such as 454 Life Sciences, Solexa, and Helicos Biosciences all have **competing technologies**, vying to be widely adopted for the next generation of sequencing machines
- The success of genome sequencing project has created wide-spread interest **in exploring epigenomes** in order to elucidate **how the genome executes the information it holds.**

# An organised DNA packaging



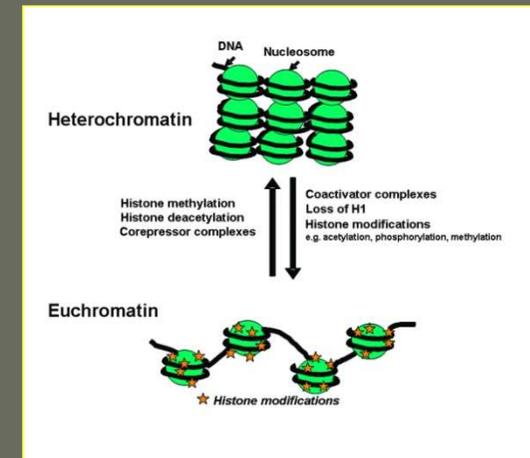
- A mammal diploid cell contains in average **6000 Mb of DNA**, corresponding to **1.8 meters**, which is packed within a nucleus of **6 microns** diameter, following a strongly organized packaging
- Each chromosome yields **one single molecule of linear DNA** which is folded several times and distributed over the centromere
- Within the nucleus, DNA is never encountered under a free form but **associated to other molecules**, principally histones. Histones are small basic proteins which are present with the same proportion as DNA.
- Other proteins associated to DNA are **non histones acidic proteins**, which account for 10 to 30% of the whole structure.
- This **DNA-Protein complex** is called chromatin (as it is easily stained) and it constitutes the chromosomes

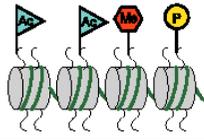
# DNA: increasing orders of packaging



# Chromatin structure and gene activity

- **Heterochromatin** refers to regions of the genome that have **low gene density**, contain **satellite repeat elements** and are late replicating.
- **Euchromatin** is a **lightly packed form of chromatin** that is rich in **gene** concentration, and is often under active transcription. Euchromatin comprises the most active portion of the genome within the cell nucleus.
- Heterochromatin and euchromatin are associated with **distinct DNA methylation and histone modification patterns** that correlate with particular states of gene activity

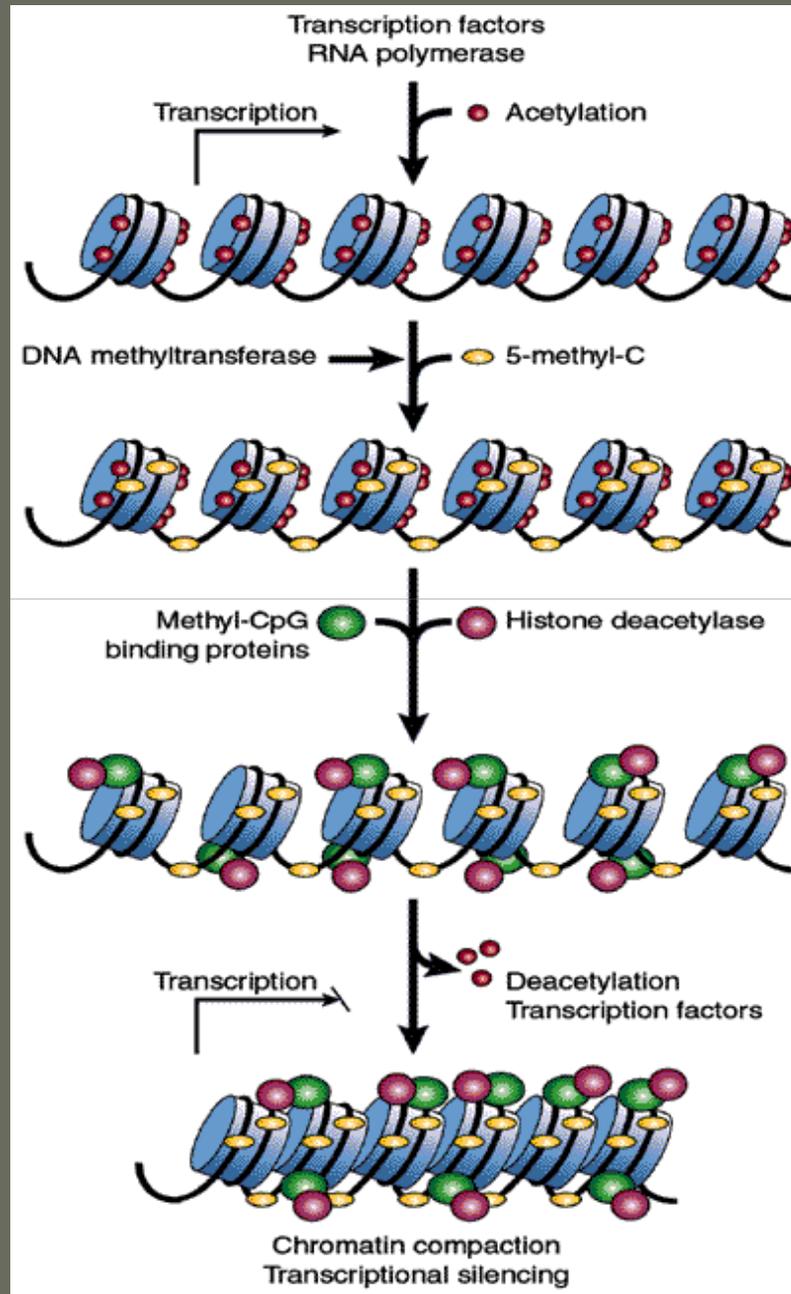




# Chromatine structure and gene activity

- **Acetylation of histones loosens the chromatin**, facilitating replication and transcription.
- **Methylated histones hold the DNA more tightly**, thus restricting access and impeding transcription.
- Methylation of Lysine 4 and Lysine-27 on Histone3 may be involved in development.
- Specifically, there is fewer methylated Lysine 27 in the chromatin of differentiated cells.
- Lysine-4 methylation acts to promote transcription by recruiting nucleosome remodeling enzymes and histone acetylase.

# Epigenetic prints on chromatin



Transcriptionally active chromatin regions tend to be **hyperacetylated** and **hypomethylated**.

If a region of DNA or a gene is destined for silencing, **chromatin remodeling enzymes** such as histone deacetylases and ATP-dependent chromatin remodelers likely begin the gene silencing process.

One or more of these activities may recruit **DNA methyltransferase** resulting in DNA methylation, followed finally by recruitment of the methyl-CpG binding proteins.

The region of DNA will then be **heritably maintained in an inactive state**.

# The Da-Histone Code ...



Covalent histone modifications, including acetylation, methylation, phosphorylation and ubiquitylation, represent a 'histone code' that controls the transcriptional status of genes.

# From genome to epigenome ...

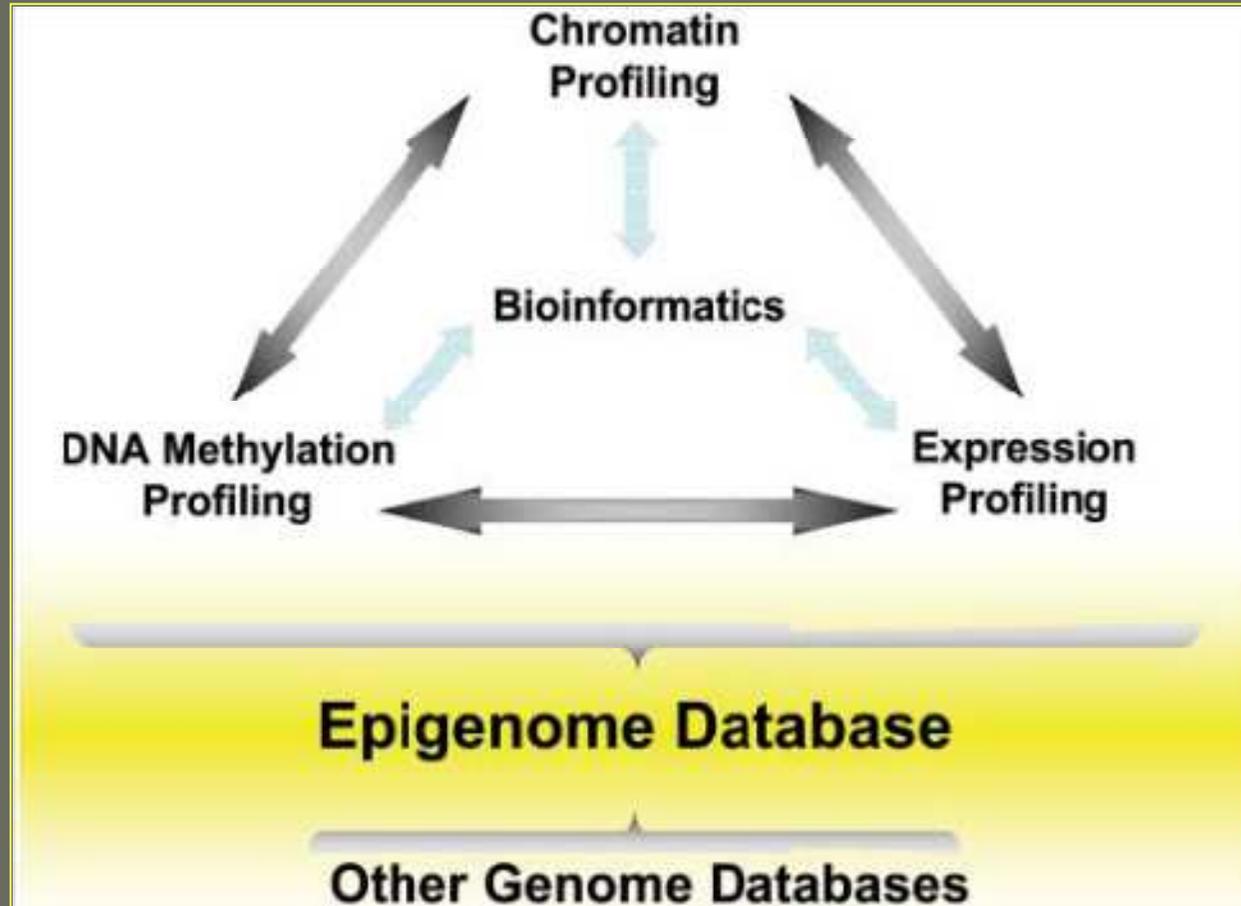
**Epigenomics** is the large scale study of epigenetic marks on the genome including:

- Covalent modifications of histone tails  
(acetylation, methylation, phosphorylation, ubiquitination)
- DNA methylation.
- Small RNAs

Epigenetic components are all amenable to genome-wide studies

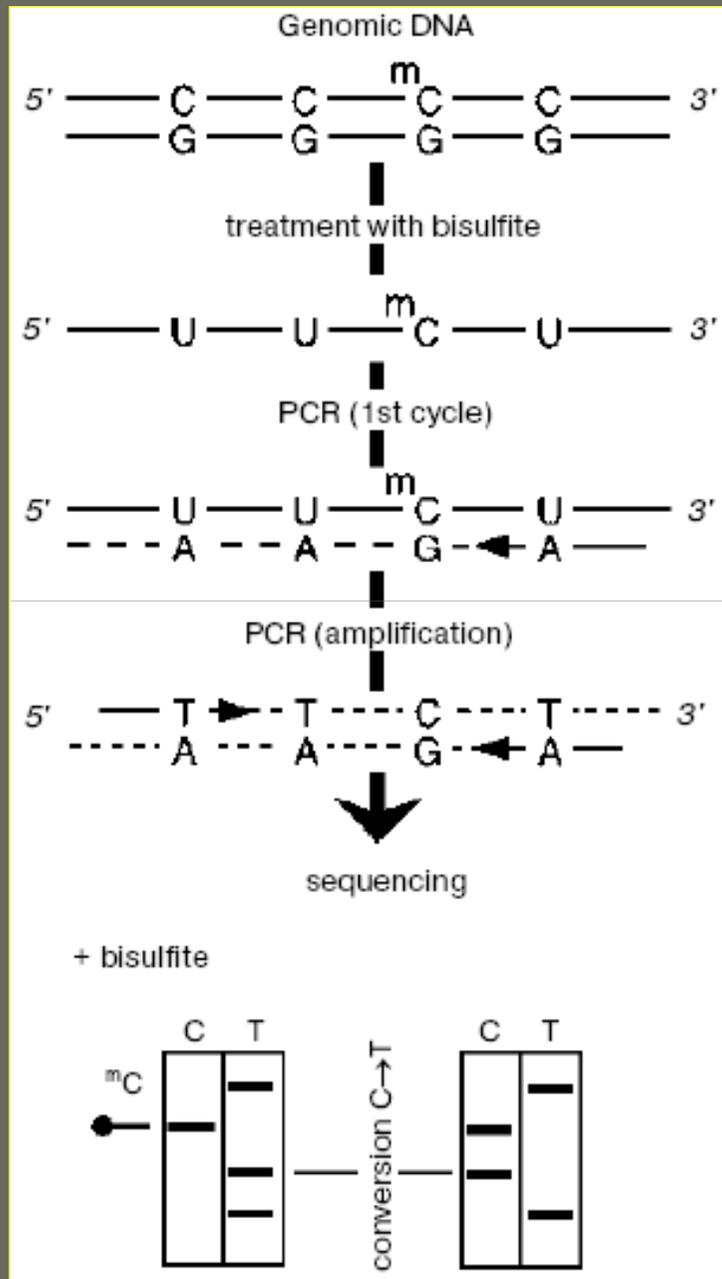
Integrated approaches that correlate gene expression with DNA methylation and chromatin profiles are being designed.

# From genome to epigenome



Murell et al, Human Molecular Genetics 2005 14(Review Issue 1):

# Analysing DNA Methylation: the bisulfite reaction



➤ Cytosine is converted stoichiometrically to uracil, but **5-methylcytosine remains nonreactive**.

➤ PCR amplification of the region of interest in the bisulfite-reacted DNA yield a fragment in which all uracil, formerly cytosine, and thymine residues have been amplified as thymine and only 5-methylcytosines have been amplified as cytosine

➤ Sequences will provide **methylation maps of single DNA strands** from individual DNA molecules in the original genomic DNA sample.

➤ **The position of each 5-methylcytosine** will be given by a positive band on a sequencing gel

## ➤ Array-based methylation profiling

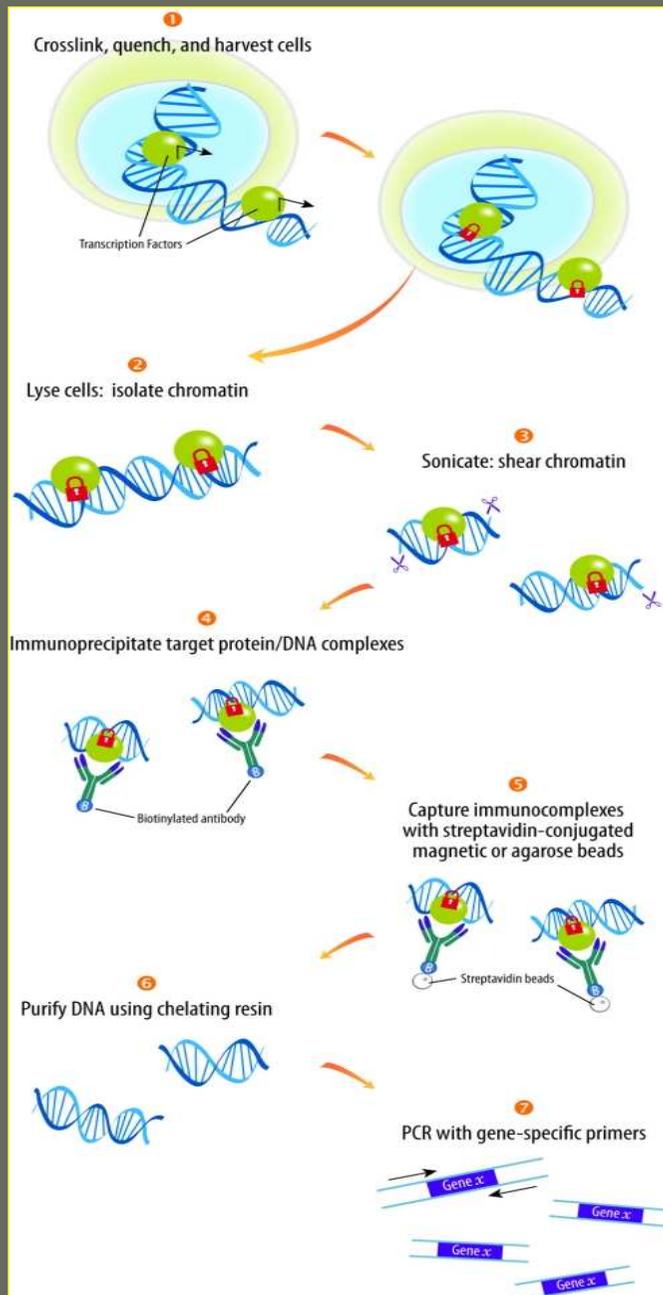
Conventional sodium bisulphite treatment of genomic DNA, followed by PCR amplification of regions of interest (300-400 bp in size).

These products are hybridized to custom microarrays that contained probes to discriminate converted versus unconverted cytosines at the CpG site of interest.

## ➤ Sequencing-based methylation profiling

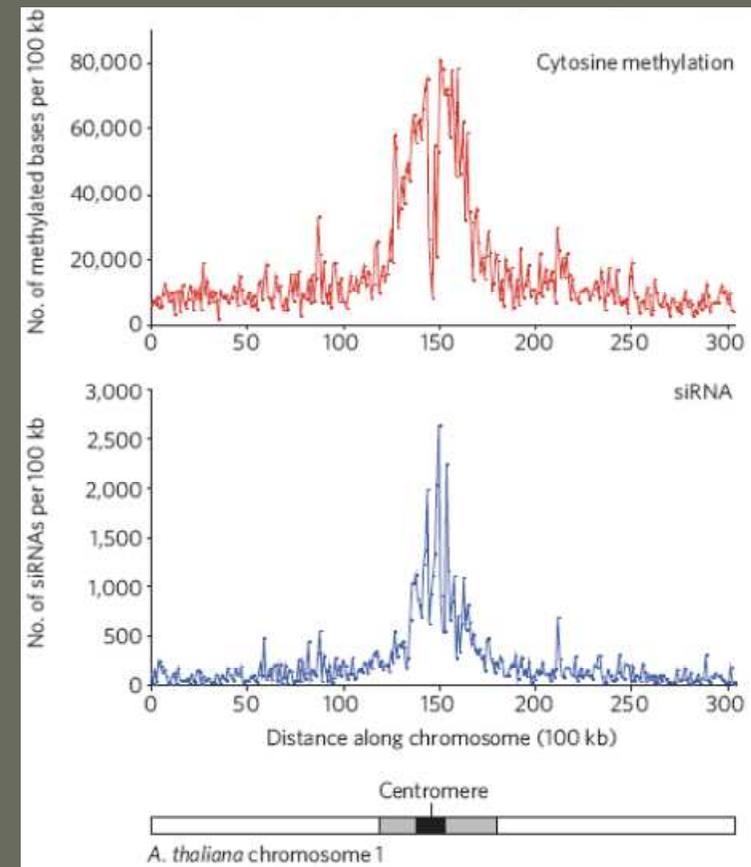
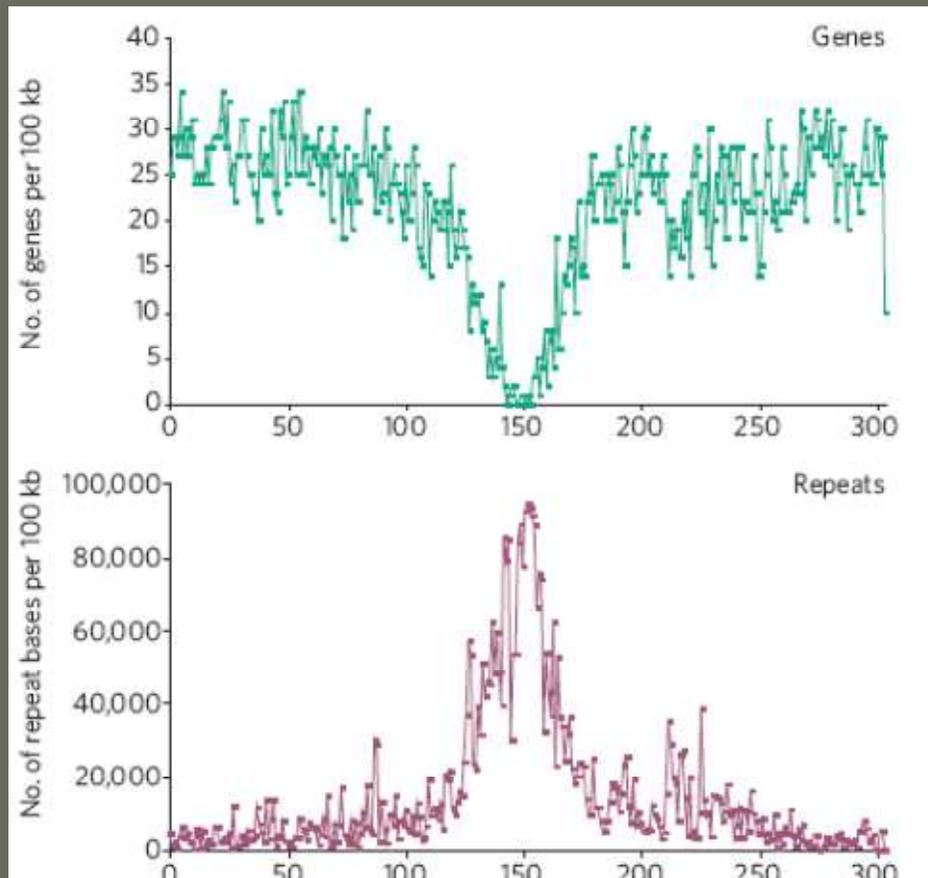
Bisulphite sequencing has been adapted for pyrosequencing, which is based on quantitative, real-time sequencing-by-synthesis. In this approach, genetic polymorphisms such as single nucleotide polymorphisms (SNPs) and epigenetic polymorphisms such as methylation variable positions (MVPs) can be analysed in a single assay.

# Chromatin Immunoprecipitation (ChIP)



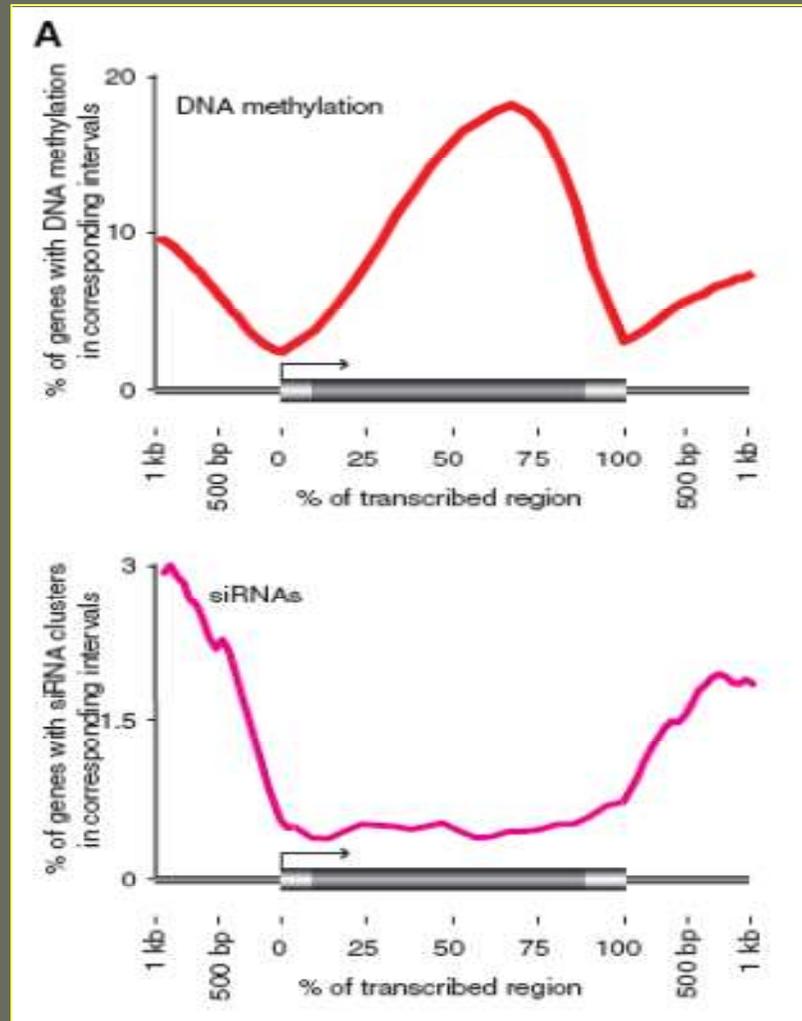
- The chromatin state of a given DNA sequence influences transcriptional activity and is regulated by potentially reversible **covalent modifications of DNA and histones**.
- Histone modifications at conserved lysine and arginine residues within the flexible N-terminal tails, such as **phosphorylation, acetylation and methylation**, specify a code which serves as an interaction platform with specific domains of chromatin-associated proteins.
- The immunoprecipitation (IP) of crosslinked chromatin with **antibodies specific for certain histone modifications** (chromatin immunoprecipitation; ChIP), followed by PCR to detect a potential enrichment or depletion of a DNA sequence of interest within IP fractions, constitutes an **elegant and direct method to query specific chromatin states** of individual genes

# The epigenetic landscape of *A. thaliana*

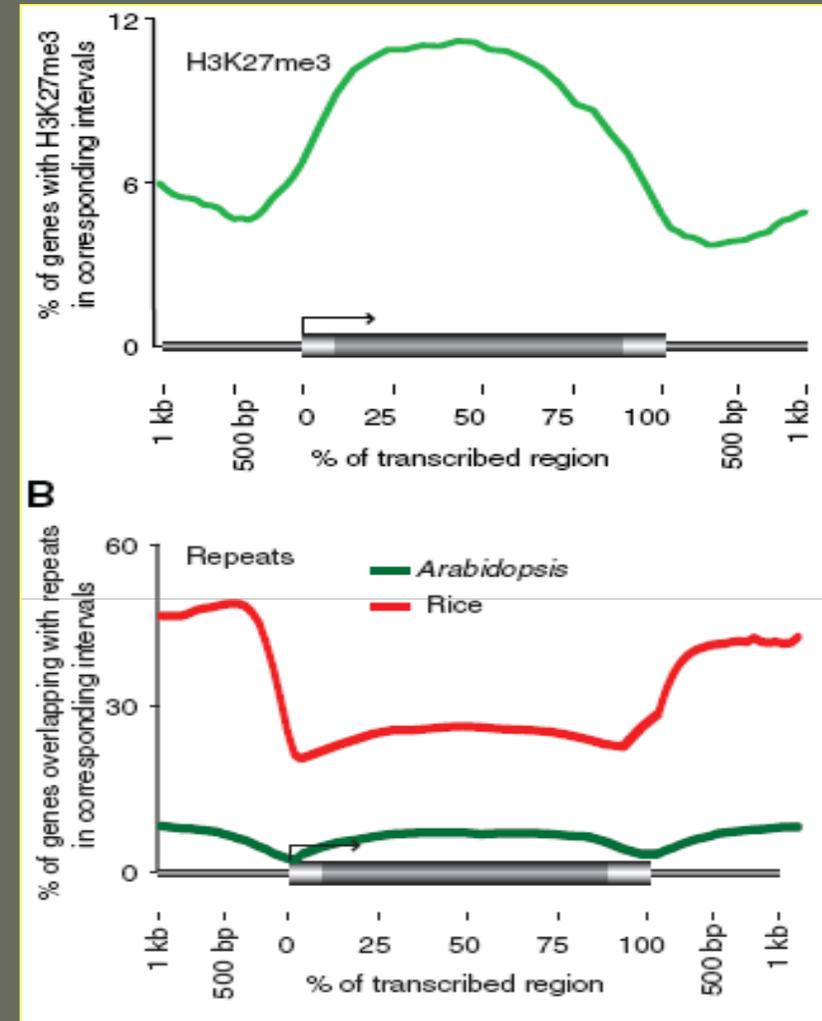


The relative abundance of genes, repeats, cytosine methylation and siRNAs is shown for the length of *A. thaliana* chromosome 1, which is ~30 Mb long.

# Positioning relative to *Arabidopsis* genes



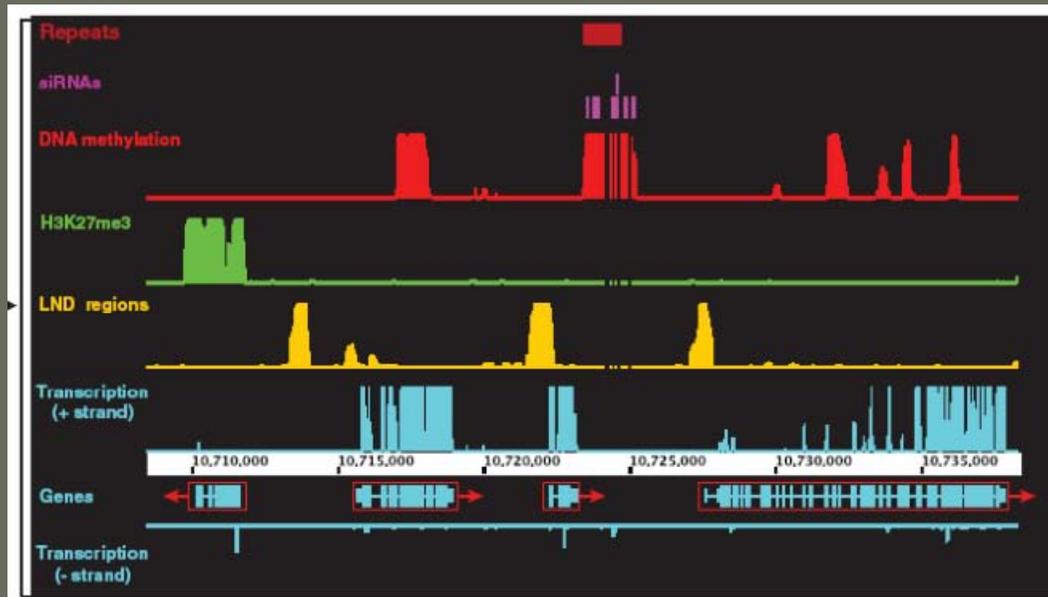
(A) Distribution of DNA methylation, siRNAs, and H3K27me3 relative to *Arabidopsis* genes.



(B) Distribution of repetitive sequences relative to genes in *Arabidopsis* (green) and rice (red).

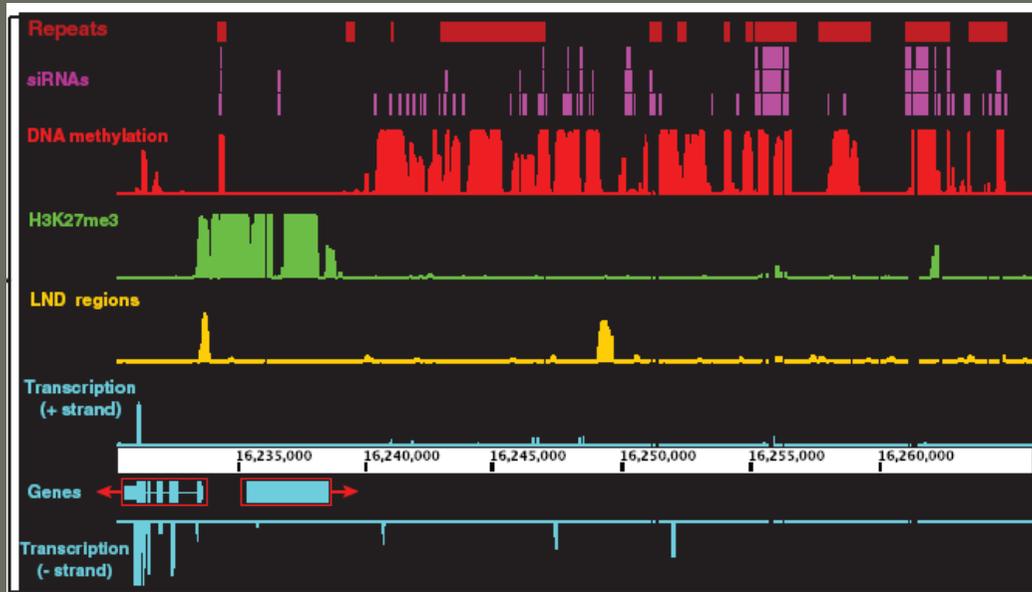
Thick and thin horizontal bars represent genes and intergenic regions, respectively.

# Distribution patterns and transcription activity



A

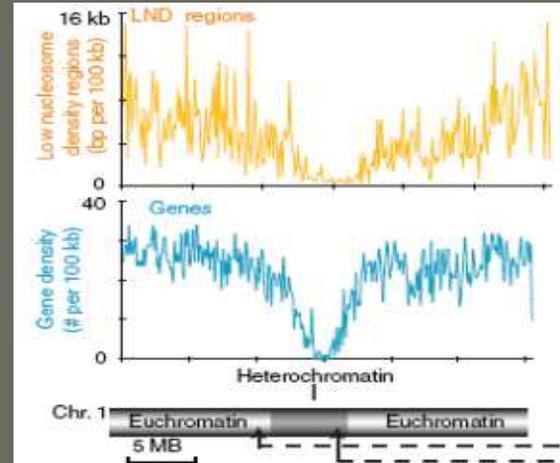
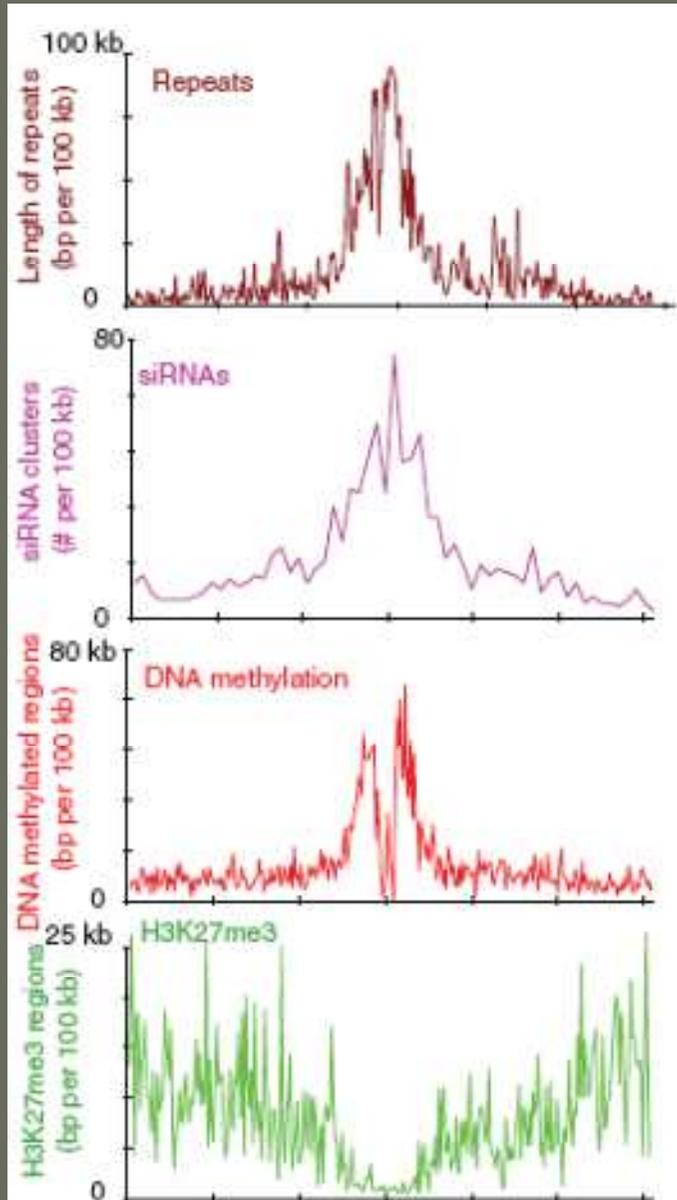
Detailed distribution patterns and transcription activity (vertical blue bars) in a **gene-rich region (A)** and a **repeat-rich region (B)**.



B

Red boxes: genes;  
Arrows indicate the direction of transcription.

# Motiv density along chromosome



Distribution of **genes**, **repetitive sequences**, **DNA methylation**, **siRNAs**, **H3K27me3**, and **low nucleosome density** (LND) regions in Arabidopsis.

The chromosomal distributions use chromosome 1 as an example. The x axis shows chromosomal position.

Breakthrough Online  
For an expanded version  
of this section, with refer-  
ences and links, see www.  
sciencemag.org/content/  
vol.298/issue5502/special

## Breakthrough

#1 **The Winner**

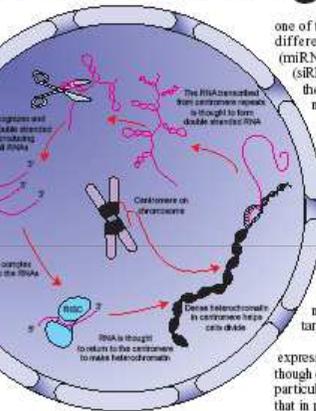
Just when scientists thought they had deciphered the roles played by the cell's leading actors, a familiar performer has turned up in a stunning variety of guises. RNA, long upstaged by its more glamorous sibling, DNA, is turning out to have star qualities of its own.

### Small RNAs Make Big Splash

For decades, RNA molecules were dismissed as little more than drones, taking orders from DNA and converting genetic information into proteins. But a string of recent discoveries indicates that a class of RNA molecules called small RNAs operate many of the cell's controls. They can turn the tables on DNA, shutting down genes or altering their levels of expression. Remarkably, in some species, truncated RNA molecules literally shape genomes, carving out chunks to keep and discarding others. There are even hints that certain small RNAs might help chart a cell's destiny by directing genes to turn on or off during development, which could have profound implications for coating cells to form one type of tissue or another. *Science* hails these electrifying discoveries, which are prompting biologists to overhaul their vision of the cell and its evolution, as 2002's Breakthrough of the Year.

These astonishing feats are performed by short stretches of RNA ranging in length from 21 to 28 nucleotides. Their role had gone unnoticed until recently, in part because researchers, focused on the familiar larger RNA molecules, tossed out the crucial small ones during experiments. As a result, RNA has long been viewed primarily as an essential but rather dull molecule that ferries the genetic code from the nucleus to the ribosomes, the cell's protein factories, and helps assemble amino acids in the correct order during protein synthesis.

Signs that RNA might be more versatile came in the early 1990s, when biologists determined that some small RNAs could quash the expression of various genes in plant and, later, animal cells. But they didn't appreciate the molecules' true powers until 1998. That's when Andrew Fire of the Carnegie Institution of Washington in Baltimore, Maryland, Craig Mello of the University of Massachusetts Medical School in Worcester, and



Life cycle. With a helping hand from proteins RISC and Dicer, small RNAs are born. We now know that these molecules keep DNA in line and ensure a cell's good health.

their colleagues injected stretches of double-stranded RNA into worms. Double-stranded RNA forms when a familiar single strand kinks back in a hairpin bend, putting two complementary sequences alongside each other. To the researchers' surprise, double-stranded RNA dramatically inhibited genes that had helped generate the RNA in the first place. This inhibition, which was later seen in flies and other organisms, came to be known as RNA interference (RNAi). It helped prove that RNA molecules were behind some gene silencing.

Another crucial step came last year, when Gregory Hannon of Cold Spring Harbor Laboratory in New York and his colleagues identified an enzyme, appropriately dubbed Dicer, that generates the small RNA molecules by chopping double-stranded RNA into little pieces. These bits belong to

one of two small RNA classes produced by different types of genes: microRNAs (miRNAs) and small interfering RNAs (siRNAs). siRNAs are considered to be the main players in RNAi, although miRNAs, which inhibit translation of RNA into protein, were recently implicated in this machinery as well.

To bring about RNAi, small RNAs degrade the messenger RNA that transports a DNA sequence to the ribosome. Exactly how this degradation occurs isn't known, but scientists believe that Dicer delivers small RNAs to an enzyme complex called RISC, which uses the sequence in the small RNAs to identify and degrade messenger RNAs with a complementary sequence.

Such degradation tatchets down the expression of the gene into a protein. Although quashing expression might not sound particularly useful, biologists now believe that in plants, RNAi acts like a genetic "immune system," protecting against harmful DNA or viruses that could disrupt the genome. Similar hints were unearthed in animals this year. In labs studying gene function, RNAi is now commonly used in place of gene "knockouts": Rather than delete a gene, a laborious process, double-stranded RNA is applied to ramp down its expression.

The year's most stunning revelations emerged in the fall, in four papers examining how RNA interference helps pilot a peculiar—and pervasive—genetic phenomenon known as epigenetics. Epigenetics refers to changes in gene expression that persist across at least one generation but are not caused by changes in the DNA code.

In recent years, researchers have found that one type of epigenetic regulation is caused by adjustments in the shape of complexes known as chromatin, the bundles of DNA and certain fundamental proteins that make up the chromosomes. By changing shape—becoming either more or less compact—chromatin can alter which genes are expressed. But what prompts this shape-

ILLUSTRATION: C. ZAVITZ/SCIENCE

- These tiny RNAs (~21-26 nt) induce silencing through homologous sequence interactions

- They can control mRNA stability or translation, or target epigenetic modifications to specific regions of the genome.

- Small RNAs and evolutionarily conserved RNA-mediated silencing pathways have established a new paradigm for understanding eukaryotic gene regulation and revealed novel host defenses to viruses and transposons.

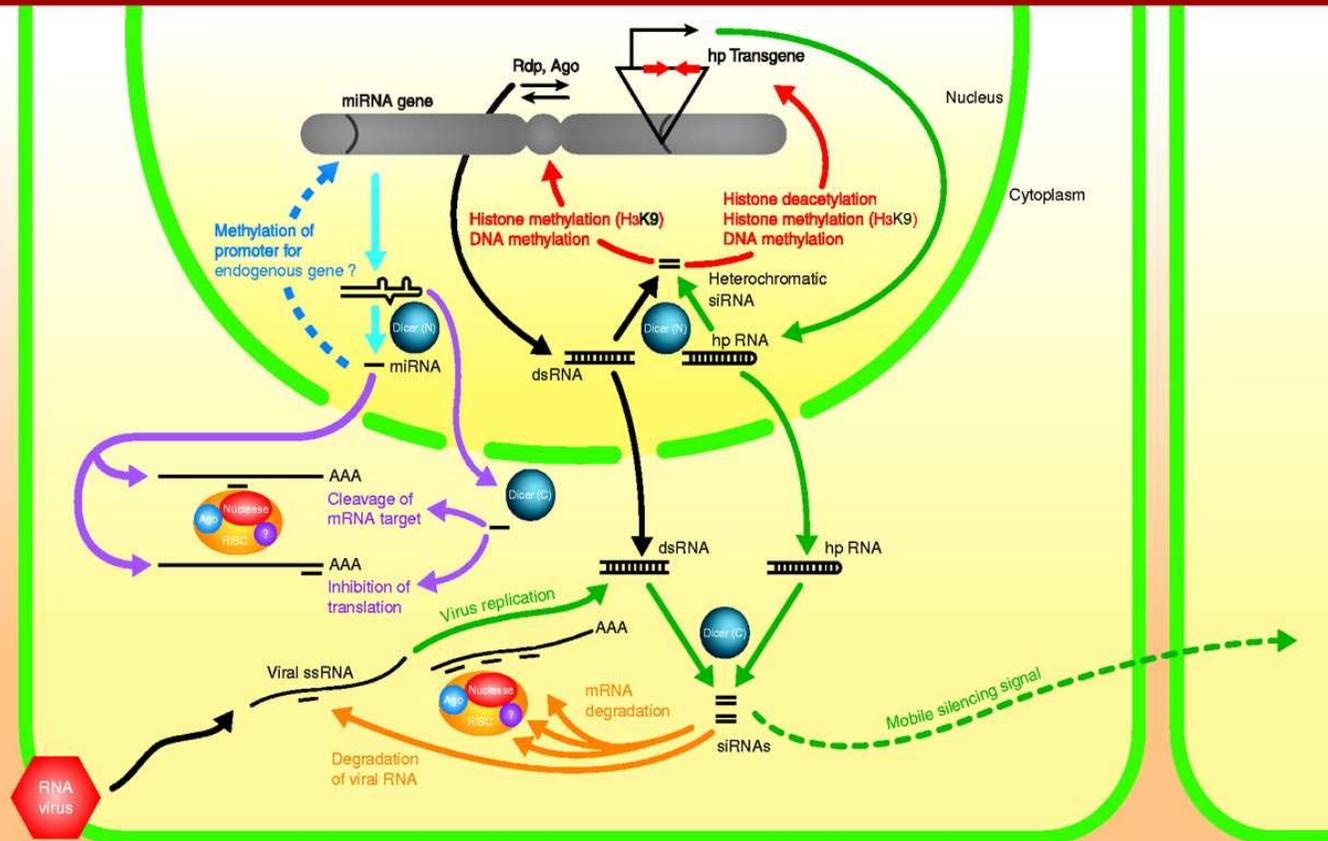
## Function of small RNAs

- MicroRNAs (miRNAs) and transacting siRNAs (tasiRNAs) are primarily involved in regulating gene expression and plant development,
- siRNAs play a major role in defending the genome against the proliferation of invading viruses and endogenous transposable elements.
- The function of the fourth type of sRNAs, natural-antisense siRNAs (nat-siRNAs), is not entirely clear but is likely related to plant stress responses

# Deciphering the small RNAs machinery

Journal of  
Cell Science

The Small RNA World  
E. Jean Finnegan and Marjori A. Matzke



RNA-directed DNA methylation (RdDM), first discovered in plants requires a dsRNA that is processed to 21-24 nt small RNAs.

In *Arabidopsis*, links between locus specific small RNAs, DNA methyltransferases, and histone modifications, including deacetylation and histone H3 lysine 9 (H3K9) methylation have been identified (see red pathway).

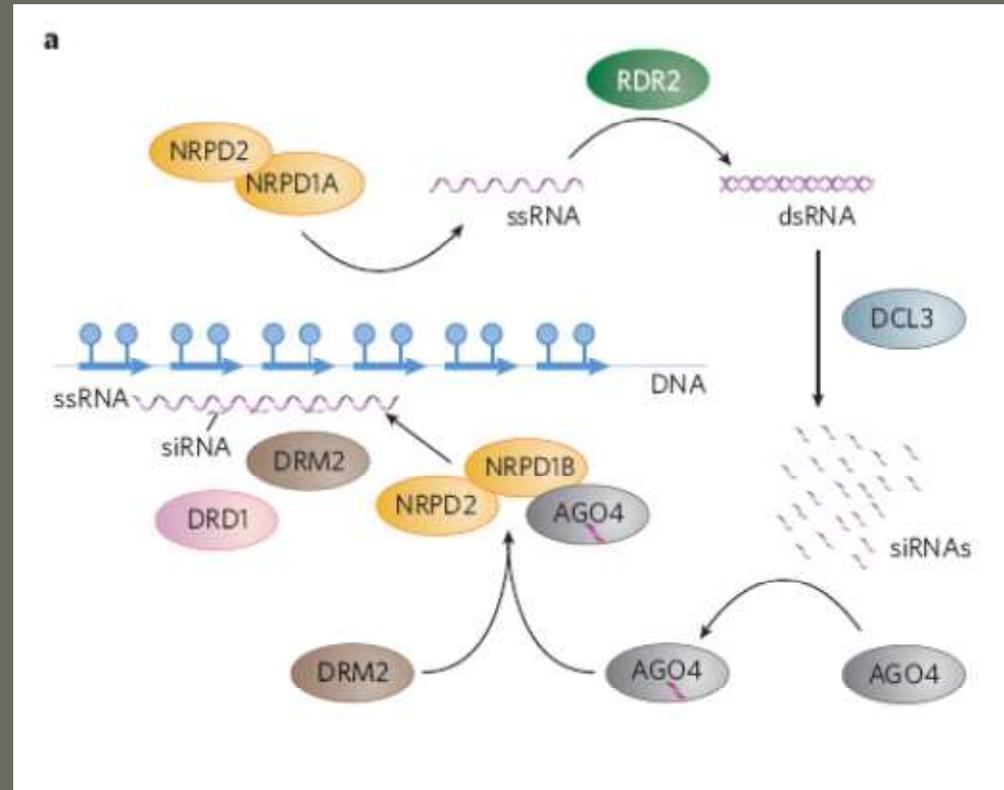
Some small RNAs might target native promoters of endogenous genes (see dashed blue pathway).

© Journal of Cell Science 2003 (116, pp. 4689-4693)

RNAi and epigenetic alterations of the genome, such as DNA methylation and histone modifications

# RNA-directed DNA methylation

Putative pathway for RNA directed DNA methylation in *A. thaliana*. Target loci (in this case tandemly repeated sequences; coloured arrows) recruit an RNA polymerase IV complex consisting of NRPD1A and NRPD2 through an unknown mechanism, and this results in the generation of a single-stranded RNA (ssRNA) species. This ssRNA is converted to double-stranded RNA (dsRNA) by the RNA-dependent RNA polymerase RDR2.



The dsRNA is then processed into 24-nucleotide siRNAs by DCL3. The siRNAs are subsequently loaded into the protein AGO4, which associates with another form of the RNA polymerase IV complex, NRPD1B-NRPD2. AGO4 that is 'programmed' with siRNAs can then locate homologous genomic sequences and guide the protein DRM2, which has *de novo* cytosine methyltransferase activity. Targeting of DRM2 to DNA sequences also involves the chromatin remodelling protein DRD1.

# Conclusions

- ✓ The '**epigenetic code**' considerably extends the information potential of the genetic code.
- ✓ Thus, **one genome can generate many 'epigenomes'** as the fertilised egg progresses through development and translates its information into a multitude of cell fates.
- ✓ The **transcriptomes of an organism are continually changing** in response to developmental and environmental cues.
- ✓ The **epigenome is not static** and can be molded by developmental signals, environmental perturbations, and disease states.
- ✓ Therefore, **many epigenomes will need to be sequenced for a single organism**, making epigenome sequencing perhaps even more challenging than genome sequencing.

# The search for epigenetic factors of flower development in oil palm



Alain Rival (Cirad)

Sylvie Doubeau (IRD), Frédérique Aberlenc (IRD), James Tregear (IRD), Estelle Jaligot (Cirad), Pascal Ilbert (Cirad), Thierry Beulé (Cirad)

## *Rationale*

- The commercial development of large scale propagation of oil palm is hampered by the generation of **somaclonal variants affecting the floral architecture**: the « mantled » genotype.
- Field observation of clonal plantings on the long term and results from DNA markers analysis (Rival et al, 1998) are consistent with an **epigenetic origin** for somaclonal variation.
- The “mantled” phenotype is correlated with a **global hypomethylation of genomic DNA**, which was revealed through a **whole genome approach** (Jaligot et al, 2000) and the use of **Methylation-Sensitive DNA markers**, such as MS-RFLPS and MSAPs (Jaligot et al, 2002; 2004).

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doi:10.1093/jxb/ern178 Advance Access publication 17 July, 2008

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Experimental  
Botany  
  
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RESEARCH PAPER

## Isolation and expression analysis of genes encoding MET, CMT, and DRM methyltransferases in oil palm (*Elaeis guineensis* Jacq.) in relation to the ‘mantled’ somaclonal variation

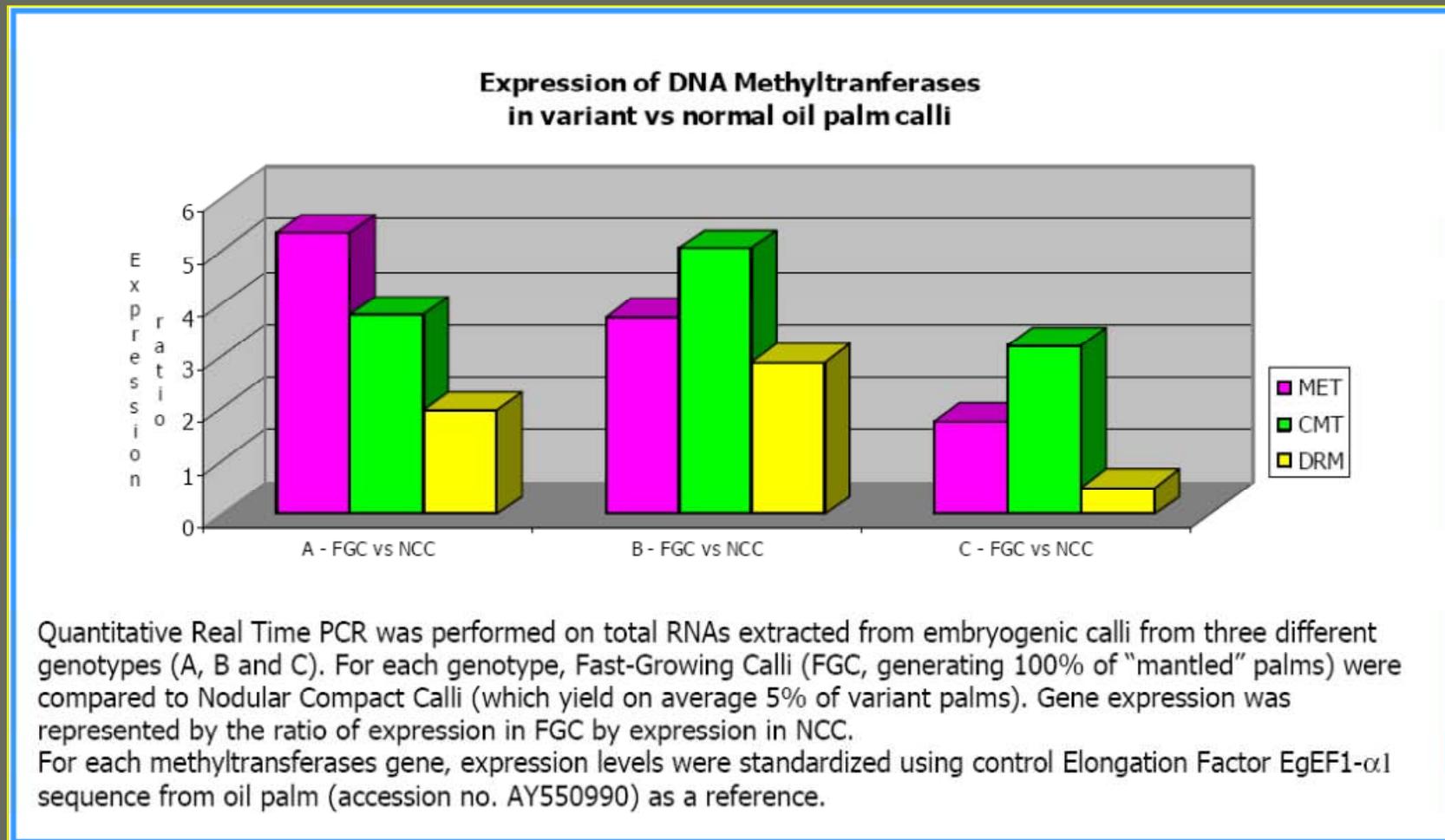
Alain Rival<sup>1,\*†</sup>, Estelle Jaligot<sup>1,\*</sup>, Thierry Beulé<sup>1</sup> and E. Jean Finnegan<sup>2</sup>

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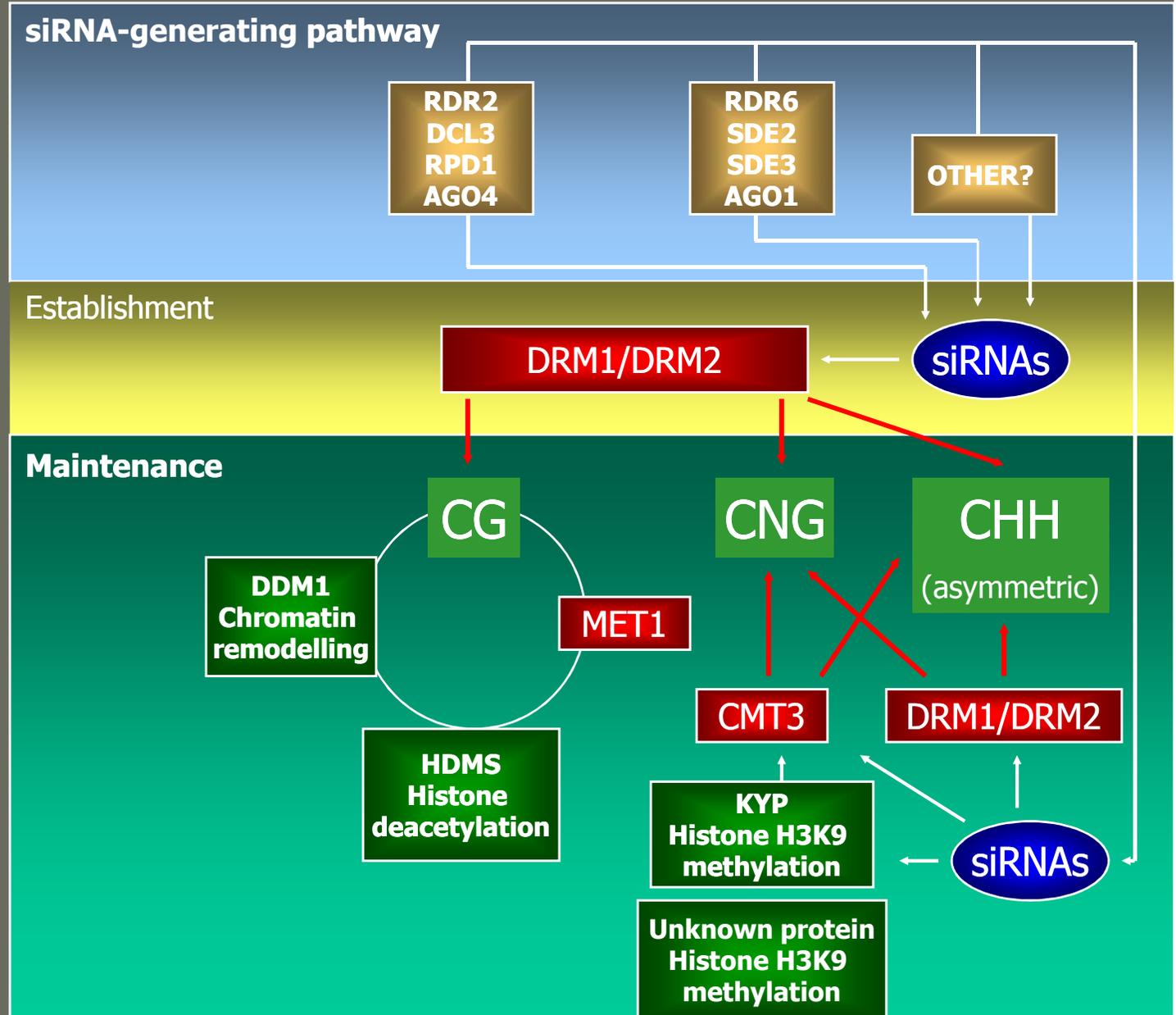
# Real Time qPCR analysis on embryogenic calli



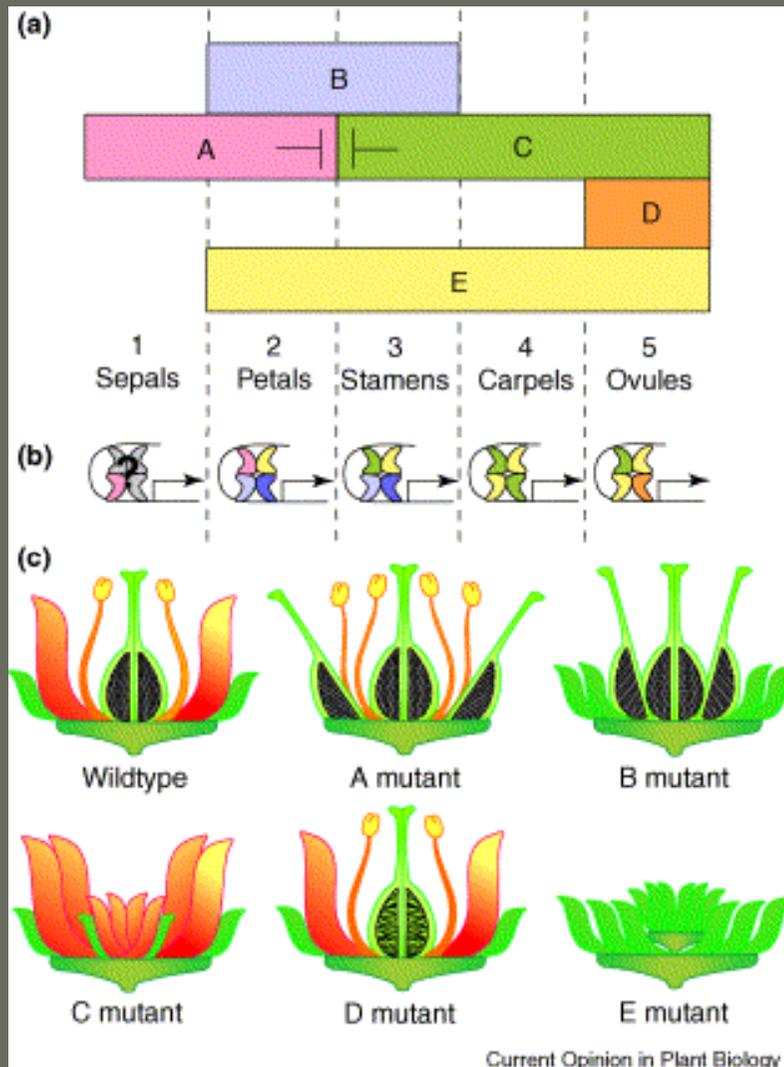
# Establishment and Maintenance of DNA methylation

GARDENING THE GENOME  
DNA METHYLATION IN  
*ARABIDOPSIS THALIANA*

*Simon W.-L. Chan\*, Ian R. Henderson\* and Steven E. Jacobsen*



# Methylation around MADS Box candidate genes

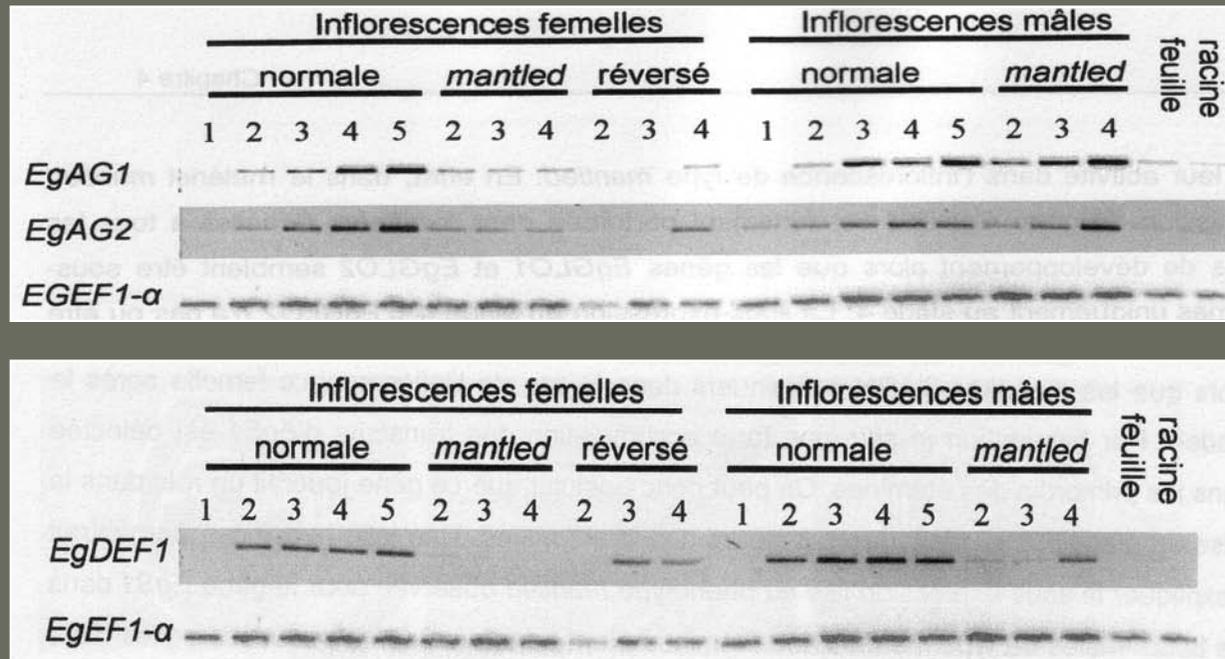


Several oil palm MADS box genes have shown differential expression patterns according to the presence of *mantled* abnormality

Alterations in expression affect not only B-type, but also C, D and E-type genes

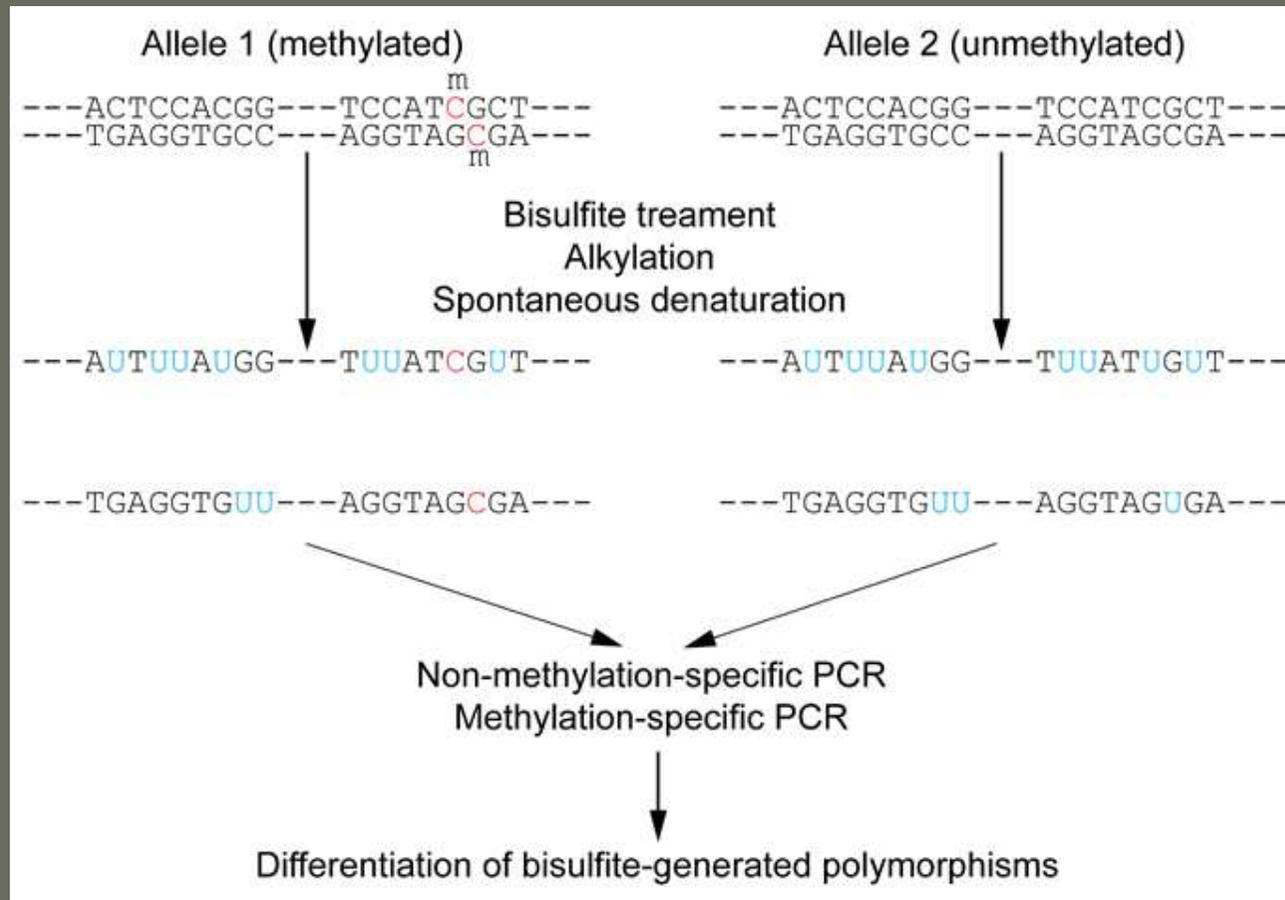
Whorls 2, 3 and 5 are affected by homeotic changes

# Methylation around MADS Box candidate genes



*Reduced expression of genes Eg DEF1 and EgGLO2 (B type), EgAG2 (C and D type) and EgS1 (type E) in abnormal oil palm flowers*

# Methylation-specific PCR: towards MS markers...



## Relevant literature

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Thank you for your kind attention ...

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