

***Arabidopsis* – what can crop breeders learn from a weed?**

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Abstract

The *Arabidopsis* genome sequence was completed in 2000. This has led to a great increase in our understanding of the molecular basis of both plant development and the response to environmental stimuli. Having the genome sequence has enabled genomics approaches which aim to assign a function to each of the predicted 26,000 genes. Knockout mutations generated by insertional mutagenesis or gene silencing with RNAi methodology suggest a function for a gene if the mutants can be linked to a phenotype. Oligonucleotides corresponding to each of the predicted genes can be spotted on a microarray which can be used to determine the pattern of expression of each of the genes, again, suggesting a function. The knowledge of gene function is deepening our understanding of development in *Arabidopsis*, and importantly, it is enabling *Arabidopsis* to be a platform into similar levels of understanding in our major crop plants.

Media Summary

The *Arabidopsis* genome sequence was completed in 2000 and has led to a great increase in our understanding of the molecular basis of both plant development and the response to environmental stimuli. This knowledge is enabling *Arabidopsis* to be a platform into similar levels of understanding in our major crop plants.

Key words

Brassica; rice; genome sequence; functional genomics; gene silencing; chromatin.

Genetics approach to gene function

Plant science was transformed in December 2000 when the complete sequence of the *Arabidopsis* genome was published [The *Arabidopsis* Genome Initiative, 2000]. This sequence was the result of an international collaboration between scientists in the United States, Japan and Europe and delineated, for the first time, the number and type of genes required for all of the processes that, together, specify a plant – how it develops and how it functions [<http://www.arabidopsis.org/info/agi.jsp>].

Many genes, classified according to function, were predicted from the DNA sequence by a combination of computer programs which identified putative start and stop sites of coding regions and possible introns and exons. Data already available from the EST (Expressed Sequenced Tag) Program which had sequenced some 13,000 unique gene transcripts, together with a full-length cDNA database, were used to facilitate gene prediction. These methods suggest that approximately 26,000 protein-coding genes specify the complete life cycle of the *Arabidopsis* plant. Of these 26,000 genes, some 8,000 result from a partial duplication of the genome indicating that 18,000 may be the minimum number of genes necessary for specifying a plant. Duplication of genes may allow for a finer control of expression by allowing independent evolution of the duplicated genes, thereby producing more elaborated biological function. Computer prediction from sequence similarity to genes of known function in other species facilitates our discovery of gene function, and human, *Cenorhabditis elegans* and yeast gene annotation have contributed substantially to the prediction of gene function in *Arabidopsis* (Fig 1).

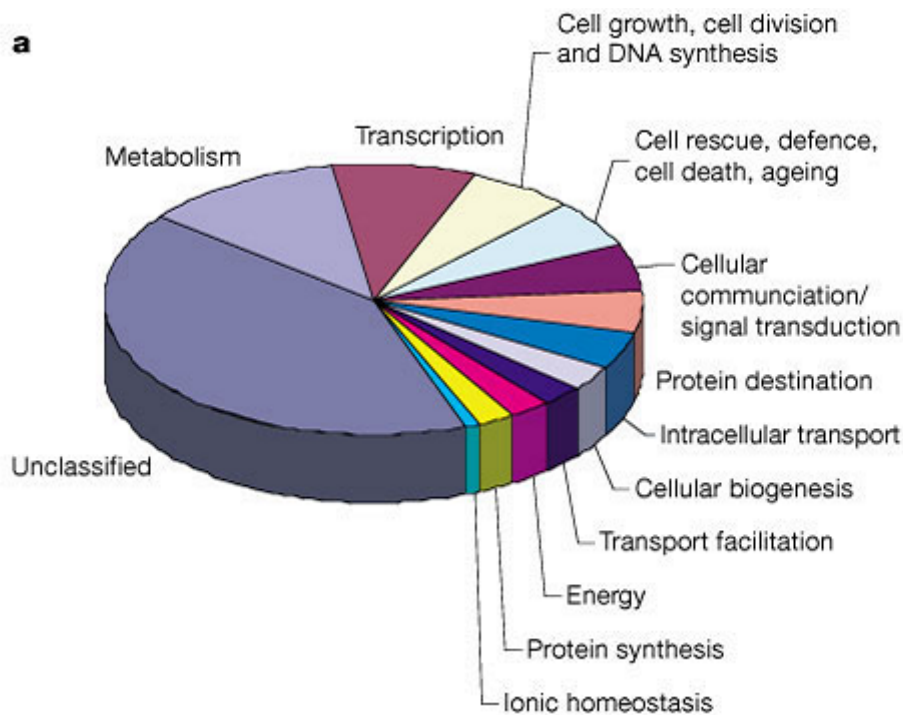


Fig 1. The proportional phenotypic and/or biochemical classes of genes in *Arabidopsis* predicted from the genome sequence.

The complete genome sequence provides the basis for addressing the key question of plant developmental biology - How can a plant develop different tissues and organs, such as roots, leaves and flowers from a DNA complement present in the zygote and which is the same in all cells and contains the same genes? Clearly, the answer lies in differential patterns of gene expression; different genes are expressed at different times and places in the plant and to different extents. Understanding, first of all, what these genes do and, secondly, how they are regulated, will allow us to understand plant development and to understand how plants respond to the biotic and abiotic stresses of the environment.

Knowing the sequence of 26,000 genes in the gene complement is like having a dictionary with all of the words but none of the meanings. The immediate focus of the international *Arabidopsis* community has been to determine what each of the 26,000 genes is doing. This functional approach to the genome, as was the case in the genome sequencing, is a collaborative effort with all information available in the public domain. The aim of the international program, the '2010 Project', is to determine a function for all of the genes of *Arabidopsis thaliana* by the year 2010 [<http://www.nsf.gov/cgi-bin/getpub?bio011>; http://www.arabidopsis.org/info/2010_projects].

Gene function by knockout mutations

The functions of the genes are being determined in several ways. The first is using insertional mutagenesis involving DNA 'tags' of a known sequence being inserted randomly throughout the genome. This generates lines each with an insert in a single gene. These lines are then characterised for altered phenotype under a variety of conditions. The gene into which the DNA tag has been inserted is determined using the known 'tag' sequence to isolate the flanking gene sequences. In this way, phenotype is linked to disruption of a particular gene, in turn leading to the discovery of the normal function of the gene. T-DNA insertion lines are available in public stock centres for about two-thirds of the *Arabidopsis* genes. [http://www.arabidopsis.org/abrc/ecker_frank.jsp]. So, to determine the function of any gene sequence, it is a simple matter of ordering the stock from the stock centre and screening for a phenotype.

Another method of determining gene function is to knock out gene activity by RNAi methods. Once the sequence of a gene is known, RNAi (hairpin) constructs targeting the RNA produced from that gene can be introduced and the resulting phenotype can be determined. One benefit of the RNAi method is that transcripts from a number of members of a gene family can be targeted at the same time. Additionally,

any pre-existing mutant can be used as the target line allowing the testing of the effect of knocking out several genes simultaneously.

Expression profiling

The function of a gene can be suggested by its pattern of gene expression. The sequencing of the genome has allowed the production of microarrays where the whole genome is arrayed on a chip.

Oligonucleotides of about 70 bases corresponding to each of the genes are synthesised. These individual oligonucleotides are spotted on a slide (25,000 spots and upwards). The array can then be used to probe RNAs from different tissues to see the relative level of activity of each of the genes. Thus, by analysing the pattern of gene activity and identifying the particular genes which are more or less active in particular developmental stages or in response to a stress, candidate genes important for the response can be identified. These genes can be assayed further for function using the mutagenesis methods. A productive use of microarrays has been the identification of genes expressed in response to stresses such as drought, cold or low oxygen. Identifying genes critical for a response in *Arabidopsis* may lead to isolation of stress induced genes in crop plants and strategies for alleviating the impact of such stresses. There is a collaborative large scale analysis of *Arabidopsis* microarray data where the data from array experiments are deposited on a data base [<http://www.arabidopsis.org/tools/bulk/microarray/analysis/index.jsp>].

Arabidopsis to crop species

Sequence analysis has shown that most of the *Arabidopsis* genes have homologues in crop plants (Schoof and Karlowski 2003). The genes of Brassicas are approximately 90% identical to those of *Arabidopsis* and about 80% identical to those of cotton and other broad leaved plants making them readily recognisable. *Arabidopsis* and Brassicas show many chromosomal regions with similar genes in the same order (synteny) assisting with map-based cloning in Brassicas. In cereals, most of the *Arabidopsis* genes have homologues and many of the genes have similar functions and similar patterns of expression. About 70% of the *Arabidopsis* genes show significant homology with genes in the rice genome, whereas about 50% of the rice genes have significant homology with genes in *Arabidopsis*. Thus, understanding the function of an *Arabidopsis* gene suggests which gene should be targeted in cereals to either understand a physiological effect or to be used as a DNA marker in breeding.

When genes have been identified with the desired characteristics in crop species, they must be incorporated into breeders' already adapted lines. The candidate genes can be used for transformation of breeders' lines, or used non-transgenically. A new technology developed in *Arabidopsis* is TILLING (target induced local lesions in genomes) where mutations in target genes can be identified without the production of genetically modified organisms [Greene et al, 2003]. A (chemically) mutagenised population is screened with PCR primers to a specific gene, and then using one of a number of techniques which identifies single base mis-matches, an allelic series of point mutations can be identified. This raises the possibility of screening for mutations in a target gene which have the appropriate level of expression. TILLING is also of particular value for essential genes where sub-lethal alleles are required for phenotypic analysis. TILLING can be automated in a high throughput system and mutants with lesions in a specific gene with leaky phenotypes and, perhaps, with higher potential agronomic value can be identified. There is an *Arabidopsis* TILLING Project which is making mutations in all the *Arabidopsis* genes. The technology is being extended to maize and rice [<http://tilling.flcrc.org>].

Arabidopsis and plant science

Before the sequencing of the *Arabidopsis* genome and the genomics approach to plant science, *Arabidopsis* provided much of our understanding of plant processes using a single gene approach. The advantages of *Arabidopsis* – its small genome size, rapid generation time of approximately six weeks seed to seed, ease of self pollination and prolific seed set means that many thousands of plants can be grown in a small area (Fig 2) and readily screened for mutants. An efficient transformation method has been developed where flowering *Arabidopsis* plants are dipped in a culture of *Agrobacterium tumefaciens* containing a gene of interest linked to a gene encoding a selectable marker. Up to 1% of the seed that is set is transformed. Transformed seedlings are selected by growing on a selective antibiotic containing medium. This method is simple and avoids any tissue culture steps and any mutations generated by tissue culture. From the first use of *Arabidopsis*, all information generated has been in the public domain and freely exchanged [<http://www.arabidopsis.org>]. A collection of freely available mutants has been built up, providing the basis for a mutant-based approach to tackling many of the objectives of plant science.

Sequencing of the *Arabidopsis* genome provided not only information about the genes and genome organisation but also provided a model for managing a public collaboration in plant science. The *Arabidopsis* Genome Initiative, *Arabidopsis* Stock Centre and the *Arabidopsis* Functional Genomics effort has set the stage for future genome projects [<http://www.arabidopsis.org>]. The sequencing of the rice genome has progressed in a similar publicly funded effort, although both Monsanto and Syngenta have also funded private genome sequencing projects. The public domain rice sequencing project is an international collaboration coordinated by Japanese laboratories and the functional analysis is also an international collaboration, both being modelled on the *Arabidopsis* projects [The *Arabidopsis* Genome Initiative, 2000; Goff et al, 2002; Japan Rice Genome Project].



Fig 2. *Arabidopsis* can be grown at high density for screening. Each pot has about 100 plants.

Gene isolation from *Arabidopsis* has led to new knowledge in crops

Oil modification

About one third of the calories in our diet come from vegetable oils. Vegetable oils are thought to be healthier than animal oils because of their lower level of saturation. However, many of these are not well suited to food uses because they are polyunsaturated. For many uses, the polyunsaturated oils are chemically modified by hydrogenation – this causes many double bonds to isomerise from cis to trans form with unknown medical effects. The genes for most of the fatty acid desaturases which catalyse the steps providing polyunsaturated oils have been cloned from *Arabidopsis* using a combination of mutants and high throughput GCMS to identify the step at which the mutant is blocked. These *Arabidopsis* genes have been used to clone the corresponding genes from soybean, canola and other crop species as well as to engineer crop plants with reduced levels of polyunsaturation [Mekhedov et al, 2000]. The ideal oil is monounsaturated and manipulation of the levels of the desaturase enzymes can produce oil with the desired composition.

Light signal transduction

The light signal transduction pathways critical to all plants have been elucidated in *Arabidopsis*. Distinct but overlapping roles for five phytochromes (A through to E) in the control of light regulated responses have been determined [Casal, 2000]. Mutants for each of the phytochromes have been recovered allowing assignment of specific functions of each of the phytochromes. Phytochrome A perceives far red light and Phytochrome B red light. Different members of the phytochrome family act in an organ-specific fashion in regulating the de-etiolation. The chemical nature of blue light receptors has been revealed by cloning the CRY genes [Lin, 2000]. *CRY2* is a photolyase which is a flavin binding protein [Jarillo et al, 2001].

Initiation of flowering

The genes involved in the control of flowering initiation have been identified in *Arabidopsis*. Extensive mutagenesis has shown at least eighty genes are involved. A number of pathways to flowering have been

defined and many genes placed in these pathways. These routes to flowering include the day-length pathway which is dependent on growth in long days, the autonomous or developmental pathway, the vernalisation pathway (dependent on exposure to low temperatures) and the Gibberellin (GA) pathway [Mouradov et al, 2003]. Molecular mechanisms by which a plant can measure the number of hours of light to which it is exposed, have been uncovered, leading to the genes controlling the day length pathway. The molecular basis of the response to cold during vernalisation has been better understood because of the identification of the *FLC* gene which is a key regulatory gene mediating the response to cold temperature [Sheldon et al, 2000]. The way in which these pathways come together to effect the transition from vegetative to reproductive stages so the apical meristem no longer makes vegetative structures such as leaves and shoots but instead is making floral structures such as anthers, carpels and petals, has been worked out in *Arabidopsis* and a number of so-called integrative genes identified [Mouradov et al, 2003].

Many of the *Arabidopsis* flowering time genes have homologues in Brassicas and, apparently, the pathways to flowering are similar; for example the vernalisation pathway is mediated by *FLC*-like genes in both [Tadege, 2001]. Knowledge of the genes involved in the pathways to flowering in *Arabidopsis* led to a search for similar genes controlling flowering time in cereals. The genes involved in the day length control of flowering are conserved at the sequence level between *Arabidopsis* and rice (Izawa et al, 2003). Sometimes these genes work in a different mode in rice; for example the *CONSTANS (CO)* gene, which is important for long day mediated flowering in *Arabidopsis* corresponds to the *HEADING DATE1 (Hd1)* gene in rice which is important for short day mediated flowering. *Hd1* inhibits flowering under long day conditions in rice. In *Arabidopsis*, *CO* is an activator of another gene, *FT*. In rice, there are *FT*-like genes (*Hd3*) but there appear to be different modes of action depending on the phytochromes present. However both *CO* and *Hd1* mRNAs are mainly regulated by the circadian clock [Yano et al, 2003; Izawa et al, 2003].

In the vernalisation pathway to flowering in *Arabidopsis*, the MADS-box *FLC* gene is a repressor of flowering (Fig 3) which represses another MADS-box gene, *SOC1*, which is a promoter of flowering [Hepworth et al, 2002]. No homologues of *FLC* can be found in rice, wheat or barley. In wheat and barley a MADS-box gene, *VRN1*, which is a promoter of flowering induced by vernalisation [Yan et al, 2003; Trevaskis et al, 2003], has been isolated. It is the major gene controlling vernalisation responsiveness in wheat. However, *VRN1*, although a MADS-box promoter of flowering, is not an orthologue of *SOC1*. *SOC1* homologues do exist in cereals but their functions are still being determined. The analysis of flowering initiation has shown that the *Arabidopsis* data sometimes do not translate directly to a system in crop plants (cereals), but in other cases, they can lead to the genes which are important for floral initiation. Certain classes of genes may have related but different roles in monocots and dicots. Knowledge of the *Arabidopsis* system suggested the classes of genes which are important in controlling flowering time, sometimes enabling the direct orthologue to be identified and at other times providing clues as to other genes which may be important.



Fig 3. Left: Plants showing high levels of *FLC* and not flowering; Right: Plants with silenced *FLC* flower early.

Flower development

Another important contribution to understanding developmental processes in crop plants that has come from *Arabidopsis* research is knowledge of the genes involved in flower development. A model (the ABC model) for floral development was formulated on the basis of work in *Arabidopsis* and *Antirrhinum* [Coen and Meyerowitz, 1991]. Mutants with altered floral development were selected and the corresponding genes isolated. Three patterns of gene expression were seen (A, B & C type genes). The outer whorl, the sepals, has the A genes expressed, the next whorl, the petals, has A + B, the next whorl, the stamens, has B + C and the inner whorl, the carpel, has C. Thus, four whorls are generated by differential expression of three types of genes. These genes are generally MADS-box transcription factors and the way in which they interact with each other to control the expression of associated genes has been established in *Arabidopsis*. In most flowering plants, including rice, similar genes are involved in the formation of the flowers although the morphology of grass flowers is very different [Shimamoto and Kyojuka, 2002]. The *Arabidopsis* genes and the rice homologues show the same inflorescence-specific expression in their respective species [Krizek and Meyerowitz, 1996].

Hormone biosynthesis

In biosynthetic pathways such as the hormone biosynthetic pathways, enzymatic reactions are conserved among all plants. For example, this conservation has been clearly demonstrated for the Gibberellin (GA) pathway. Genes for the biosynthesis and breakdown of GA have been cloned from *Arabidopsis* by analysing mutants, particularly dwarf mutants, blocked in GA biosynthesis. The step at which the mutant is blocked in the pathway was determined through chemical analysis of intermediates in the GA pathway. These genes have homologues which can be identified in cereals on the basis of sequence conservation and the corresponding genes isolated. However, the number of genes encoding an enzyme catalyzing a particular step is often different between *Arabidopsis* and crop plants. For example, in *Arabidopsis*, there is a single gene for kaurene synthase whereas in rice there are between three and six genes. In *Arabidopsis* there are two genes for kaurenoic acid oxidase whereas in barley there is only one gene. Gene duplications which occur differentially in different lineages of plants probably allow for fine control of gene activity in a particular tissue. The duplicated genes are likely to have somewhat different activity patterns in the tissues of a plant [Helliwell et al, 2001].

The molecular basis of resistance to pathogens

Four laboratories studying different host/pathogen systems identified resistance genes for a virus, a rust, a fungus and a bacterium at approximately the same time [Staskawicz et al, 1995]. The surprising finding was that these genes all had similar structures and the proteins they encode belonged to the leucine rich repeat class. The signal transduction pathway from the pathogen interacting with the plant to a resultant change in gene expression has been worked out in *Arabidopsis*. We now understand the classes of genes that are involved in responding to a pathogen, not only the resistance gene of the plant and the avirulence gene of the pathogen but genes that are involved in mediating the response. Mutants in *Arabidopsis* blocked in the response were used to identify the corresponding genes. Given the similarities to the response in cereals it is likely similar genes will be identified in crop plants. This work raises the possibility that some of these pathway genes could be increased in activity to give resistance to pathogens [Tor et al, 2002; Jones, 2001].

Chromatin structure and gene regulation

All genes are controlled by transcription factor proteins which bind to short DNA motifs in the upstream promoter region of a gene causing it to become active. An understanding of the *Arabidopsis* genome has identified families of plant-specific transcription factors such as *MADS-box* genes which regulate many other genes. The *MADS-box* family of transcription factors are mainly involved in regulating genes associated with the reproductive pathways of development. Other families of transcription factor genes such as *WRKY* and *MYB* are either plant specific or show a much greater level of diversity and control many more genes than the corresponding family in animal systems. Recent work in *Arabidopsis* has shown that many genes, particularly those involved in plant development have another level of control; this is at the level of chromatin structure [Steimer et al, 2004]. Chromatin is the DNA molecule packaged with its associated proteins and histones; the packaging ensures that up to many metres of DNA is compacted to a manageable size in the chromosomes of every cell. The state of packaging of the chromatin, making a gene more or less accessible to binding of transcription factors, can regulate a gene causing it to be more or less active. If the cytosine residues of DNA are methylated then this affects the state of packaging of the DNA and, hence, gene expression. In the same way, if the histone proteins binding to the DNA are modified by acetylation or methylation of lysine groups of the histones, the packaging of the chromatin is affected and the genes become inaccessible to transcription factors preventing gene activity (Fig 4).

Changes in the state of chromatin, and hence gene expression pattern, can be inherited from cell to cell or, sometimes, from generation to generation, but do not involve any changes in the DNA sequence. These are called epigenetic changes. A gene's state of activity can be altered so the plant appears to be mutant but this may not be a consequence of changes in the DNA sequence but, rather, through changes in the chromatin structure causing a change in gene expression. Many of the major developmental changes in *Arabidopsis* are controlled by changes in the state of the chromatin. For example, in the low temperature promotion of flowering (vernalisation) the regulation of the *FLC* gene is affected by changes in DNA methylation status or by cold-induced changes in chromatin state of the gene. Following vernalisation, *FLC* activity is dramatically reduced; this reduced activity is inherited mitotically but must be reset in the next generation [Sheldon et al, 2000]. Accompanying the change in *FLC* activity is a change in chromatin structure and changes in the acetylation and methylation of the histones associated with the *FLC* gene [Sung and Amasino, 2004 – A, B]. In a similar way, the genes controlling early seed development are held inactive until fertilization occurs. Following fertilization there is a change in the chromatin structure surrounding these genes, the genes become active and development proceeds. Flowering, seed development and, possibly, germination are all regulated in a similar epigenetic manner. This is another layer of gene control which has been elucidated in *Arabidopsis*; all indications are that it will also apply in other plants [Kohler et al, 2002].

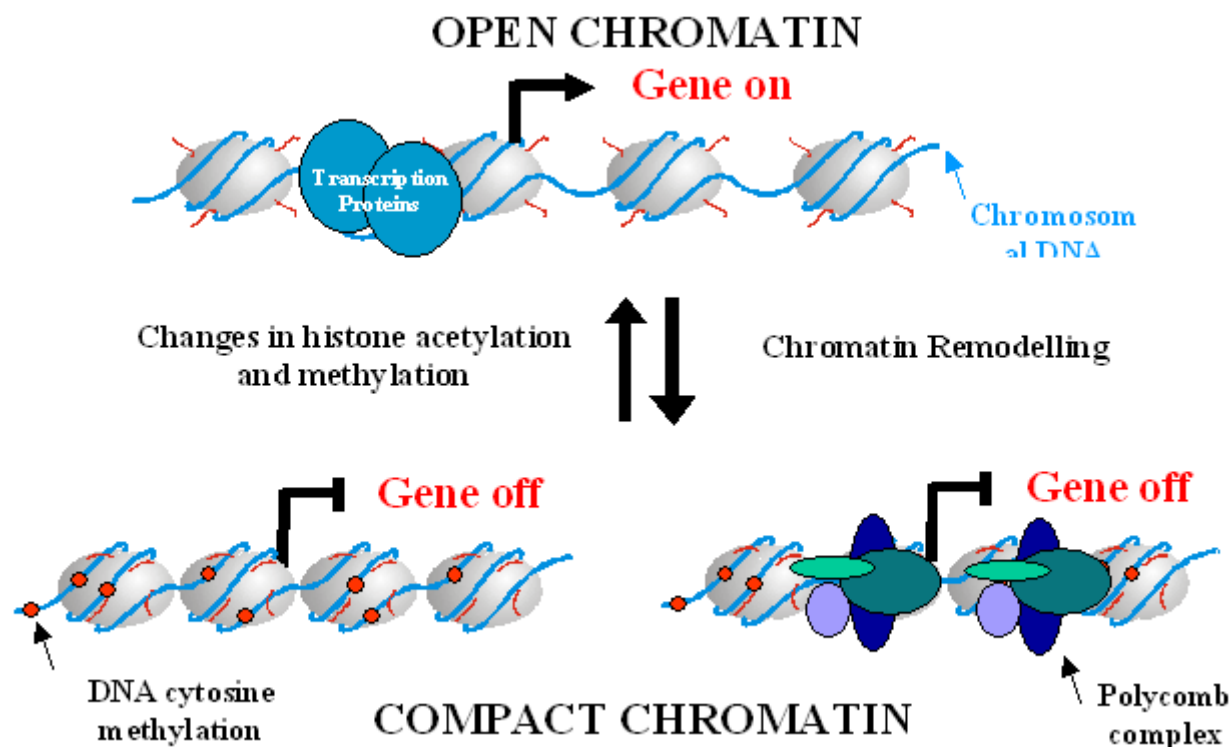


Fig 4. Changes in chromatin structure affect gene expression: Open chromatin genes are on; compact chromatin genes are off

RNA – yet another key function

One of the most exciting and novel findings in the control of gene expression is the fact that small RNAs of approximately 21-24 nucleotides play an important role in controlling gene expression in biological processes, including developmental timing and patterning and in pathogen defence. These RNAs are of two types, miRNA and siRNA [Xie et al, 2004]. miRNA is synthesised from non-protein coding genes through formation of a precursor transcript followed by nucleolytic processing steps. Part of the precursor forms a fold-back structure (resembling a double stranded RNA) which interacts with the DICER enzyme to produce the mature miRNA. miRNAs negatively regulate target genes controlling developmental events. In general, the targets of the miRNAs are transcription factor transcripts which regulate other proteins [Palatnik et al, 2003]. Sequences which are targets for miRNAs are conserved between cereals and *Arabidopsis* suggesting that miRNAs will also regulate crop plant genes. Much of the sequence of the genome is thought not to code for proteins and perhaps be of no importance but some of the unknown portion of the genome is involved in coding for these small RNAs. By isolating and sequencing small RNA molecules, several hundred gene targets have been identified in *Arabidopsis* as being controlled by these RNAs but, because the genes that are controlled are generally transcription factors, which in turn control other genes, the number of genes that are directly or indirectly regulated by small RNAs is large. As this is a rapidly progressing area it is not yet clear how many genes are regulated by small RNAs and what controls these regulators.

The second type of small RNAs, siRNAs, are also between 21-24 nucleotides and are associated with post transcriptional RNA silencing (RNA interference or RNAi) and transcriptional silencing. They are processed from double stranded RNA precursors. siRNA probably arose as a mechanism to overcome viral infection because plant viruses are, in general, RNA viruses which have double stranded RNA replication intermediates. Double stranded viral RNA is cut up by DICER into siRNA which directs the silencing of homologous viral genes concerned with viral replication. This virus-induced mechanism has also been utilised to deliberately silence genes in *Arabidopsis* and also in crop plants [Waterhouse and Helliwell, 2003]. For example, the composition of fatty acids can be modified by silencing fatty acid desaturase enzymes, flowering time can be altered by targeting *FLC* and immunity against Barley Yellow Dwarf virus conferred by making hairpin RNAs against the virus (Fig 5) [Wang et al, 2002; Stoutjesdijk et al, 2002]. Unravelling the mechanism and rules for RNAi action and its application to biological

problems have been dependent on *Arabidopsis* and the ability to isolate mutants in key genes in the silencing pathway.

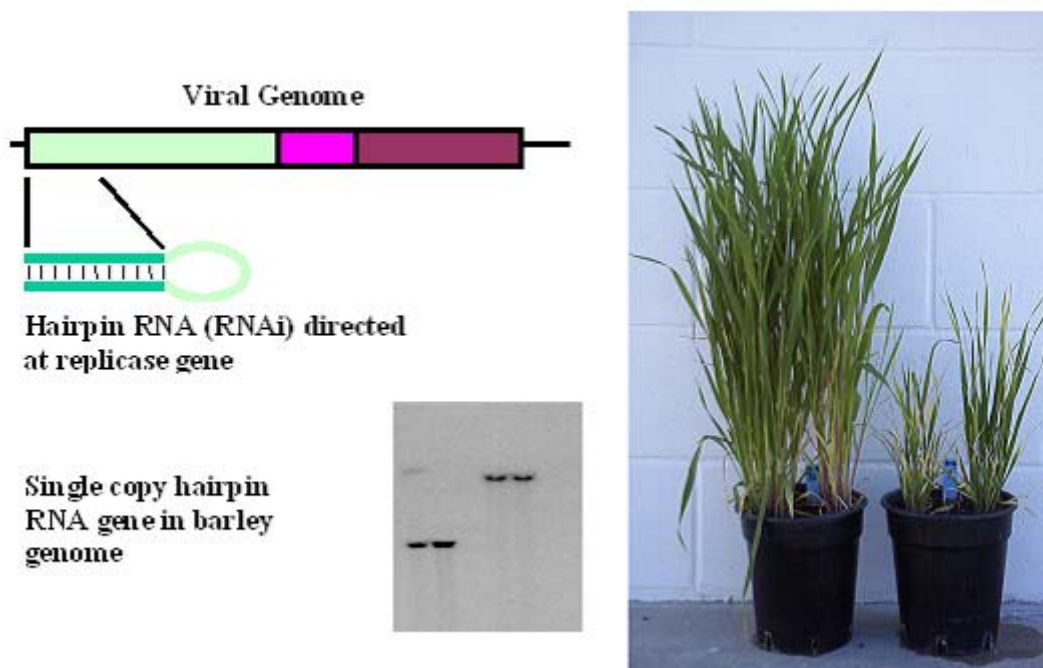


Fig 5. Hairpin construct used to protect against Barley Yellow Dwarf virus. Plants on the left contains RNAi construct directed against Barley Yellow Dwarf virus. Southern analysis showed that the transgenic line contained a single copy of the hairpin RNA gene. Lanes: 1,2,4,5 show DNA from the transgenic lines; 4,6 from non transgenic controls. Probe is Barley Yellow Dwarf virus DNA.

Conclusion

Arabidopsis has proved an essential weapon in the armory of modern plant scientists whether they study crop plants or model systems. It has made fundamental contributions to our understanding of gene regulation, both genetic and epigenetic, in development. It has identified the suites of genes activated in response to biotic and abiotic stress and has led to the discovery of genes useful for genetic manipulation of crop plants.

1 2 3 4 5 6

The sequencing of the *Arabidopsis* genome has allowed the totality of a plant genome to be explored and provided a model for other genome projects. *Arabidopsis* has joined *E.coli*, yeast and *Drosophila* as a model organism of biology.

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