

# THE ART AND DESIGN OF GENETIC SCREENS: *ARABIDOPSIS THALIANA*

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Molecular genetic studies rely on well-characterized organisms that can be easily manipulated. *Arabidopsis thaliana* — the model system of choice for plant biologists — allows efficient analysis of plant function, combining classical genetics with molecular biology. Although the complete sequence of the *Arabidopsis* genome allows the rapid discovery of the molecular basis of a characterized mutant, functional characterization of the *Arabidopsis* genome depends on well-designed forward genetic screens, which remain a powerful strategy to identify genes that are involved in many aspects of the plant life cycle.

The plant kingdom constitutes most of the biomass of our planet and is crucial in the ecosphere. Owing to the huge diversity of plants, and their ability to adapt to a wide variety of environments, it was thought for a long time that conclusions drawn from a plant model system could not easily be applied to other plant species. Indeed, studying plant model organisms is a relatively new approach compared with studies on animals. Until the 1980s, experiments on plants mainly focused on economically important species, providing the potential for increased yield through crop improvement. But many plants, including some principal crops, have large, polyploid genomes that often contain highly repetitive DNA. These disadvantages make genetic and molecular studies very difficult, and hinder the analysis and manipulation of these plant genomes. Therefore, the ideal experimental model system for the plant community would need to be an organism with a simple genome that is amenable to both classical and molecular genetic studies.

## ***Arabidopsis* as an experimental system**

The small mustard weed *Arabidopsis thaliana* (family Brassicaceae) was known to botanists for at least four centuries, and had been used in experimental research for about half a century (reviewed in REF. 1), before Friedrich Laibach outlined the advantages of using it in genetic experiments<sup>2</sup>. He also suggested that it could

be used as a plant model system in 1943. Several features make *Arabidopsis* amenable to classical experimental genetics: a small size, a rapid generation time (5–6 weeks under optimum growth conditions), the ability to grow well under controlled conditions (either in soil or in defined media), high fecundity (up to 10,000 seeds per plant), and the ease with which a mutant line can be maintained (by self-fertilization) and out-crossed.

As well as having all the key features of a model system for classical genetics, *Arabidopsis* has the smallest known plant genome (125 Mb)<sup>3</sup>, with fewer repetitive sequences than any known higher plant, greatly facilitating molecular studies and map-based cloning<sup>4,5</sup>. In addition, *Arabidopsis* can easily be transformed by *Agrobacterium*-mediated gene transfer (BOX 1), which is a prerequisite for many molecular genetic experiments. The advantages of using *Arabidopsis* in such experiments have been reviewed extensively<sup>6–8</sup>. Although *Arabidopsis* had been used for classical genetic studies for a long time, its role was not comparable with that of *Drosophila* in the systematic analysis of developmental and metabolic processes. Only once it was recognized that *Arabidopsis* had a small genome and, therefore, the potential to combine classical genetics with molecular studies, did it advance to being the most studied higher plant.

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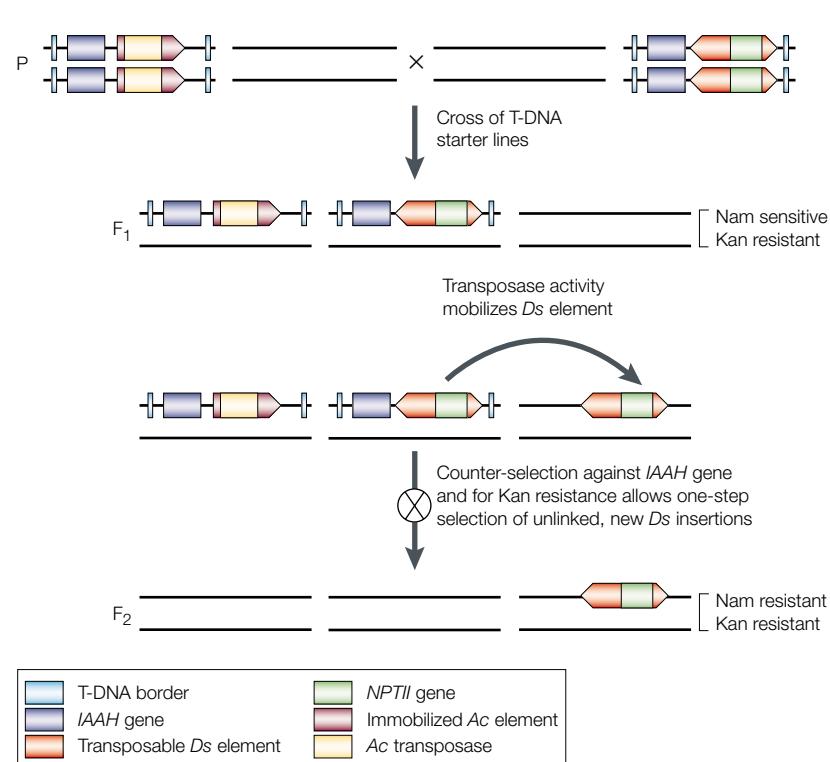
The success of *Arabidopsis* as a model organism is mainly due to its amenability to forward genetic screens, by which genetic variation is artificially induced and mutagenized plants are screened for phenotypes of interest. Mutagenesis in *Arabidopsis* (BOX 2)

mainly relies on ethylmethane sulphonate (EMS) and, to a much lesser extent, on fast neutron radiation (FNR). The study culminates in the molecular isolation of the mutated gene, which causes the phenotype; so, a function can be assigned to a molecular change in the

### Box 1 | Systems for insertional mutagenesis in *Arabidopsis*

Insertional mutagenesis offers a unique tool for both forward and reverse genetic approaches. For *Arabidopsis*, two principal systems are used successfully as biological mutagens: *Agrobacterium*-mediated T-DNA (transferred DNA) transformation and two transposable element systems that originate from maize (*Ac/Ds* and *En/I*).

**T-DNA mutagenesis**  
*Agrobacterium*-mediated transformation results in the random and stable integration in the plant nuclear genome of a DNA sequence (T-DNA), which is part of a large bacterial plasmid. The availability of highly efficient T-DNA transformation methods, such as FLORAL DIPPING, has made it feasible to generate tens of thousands of



**FLORAL DIPPING**  
A T-DNA transformation method in which plants are simply dipped into an *Agrobacterium* suspension.

**OVULE**  
The organ that develops into a seed after fertilization.

**ENHANCER DETECTION**  
A method originally developed in *Drosophila* that allows the identification of genes on the basis of their expression patterns. Engineered insertion elements carry a reporter gene construct under a minimal promoter that can respond to *cis*-acting regulatory elements near the insertion site.

**GENE TRAPPING**  
A modification of enhancer detection first used in mice. The minimal promoter driving the reporter gene is replaced by splice acceptor sites such that expression depends on integration within a gene.

transformants relatively easily. A single treatment generates many independent T-DNA insertion mutants. Recessive T-DNA-induced mutants are not observed in the progeny from the transformed plant (T<sub>1</sub>) but rather in the subsequent generation (T<sub>2</sub>). This indicates that integration might occur late in flower development, after the lineage separation of male and female gametes. Recently, it was shown that the OVULE, or more precisely the female gametophyte, is the target of T-DNA integration<sup>67,68</sup>. The main drawbacks of T-DNA mutagenesis are the often complex integration patterns of T-DNA, including transfer of vector sequences that flank the T-DNA, multiple insertions and the high frequency of concatemeric insertions, which can complicate the identification of flanking sequences<sup>81</sup>.

#### Transposon mutagenesis

After Barbara McClintock's discovery of transposon activity in maize, transposable elements have been found in essentially all organisms examined. Transposons are believed to generate evolutionary diversity by inducing mutations and genome rearrangements. The wide distribution and the mutagenic potential of these elements have led to their exploitation for molecular genetic studies. The maize transposon systems *Ac/Ds* and *En/I* have been shown to transpose at sufficiently high rates in *Arabidopsis* to allow gene tagging and cloning<sup>82–85</sup>.

Transposon mutagenesis involves several steps illustrated here by the *Ac/Ds* transposon system developed by Sundaresan *et al.*<sup>86</sup>. Some other systems do not select for unlinked transpositions nor do they stabilize the insert (that is, select against the transposase). The two starter lines are transgenic plants that carry T-DNA that contains the *Ac* and the *Ds* element, respectively. When the starter lines are crossed, *Ac* transposase activity allows the mobilization of the *Ds* element, generating genetically mosaic F<sub>1</sub> plants. *Ds* elements preferentially transpose to sites that are closely linked to the donor site<sup>82,84</sup>, so starter lines were designed that allow positive selection for the presence of a newly transposed *Ds* element and negative selection against the *Ac* and the *Ds* donor locus<sup>86</sup>. The immobilized *Ac* transposase source in the F<sub>1</sub> is counter-selected in the F<sub>2</sub>. The *Ac* T-DNA vector contains an indole acetamide hydrolase (*IAAH*) gene that confers sensitivity to naphthalene acetamide (NAM). *Ds*, which carries the *NPTII* gene that confers kanamycin resistance, can be positively selected using kanamycin.

To select against *Ds* elements linked to the donor *Ds*, the donor *Ds* T-DNA construct also contains the counter-selectable marker gene *IAAH*. This combination selects against *Ac* transposase, the donor *Ds* and *Ds* insertions that are linked to it, whilst selecting for unlinked *Ds* elements. The new transposon lines are maintained by self-pollination and are screened for phenotypes, or reporter gene expression patterns in the case of ENHANCER DETECTION and GENE TRAPPING.

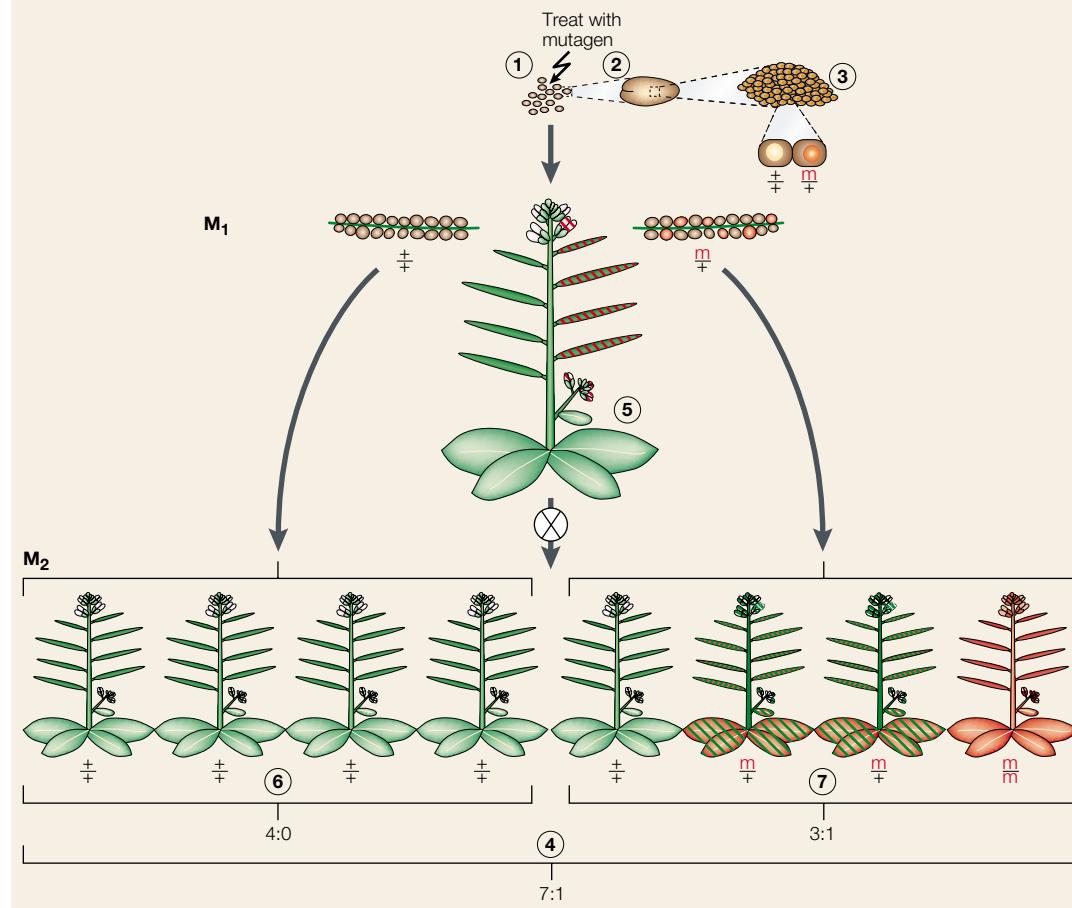
inherited material of an individual. As more scientists joined the *Arabidopsis* community, experience was gained and tools were developed to carry out these screens with the aim of isolating the genes that are involved in all aspects of plant life.

The application of forward genetics allows the directed analysis of a given process of interest. In this review, we focus on the design of forward genetic screens, which provide a very powerful means to characterize aspects of the plant life cycle. We illustrate

#### Box 2 | The basis of seed mutagenesis in *Arabidopsis*

The most practicable way to induce heritable mutations in *Arabidopsis* is by seed mutagenesis (1). The targets of seed mutagenesis are the diploid cells of the fully developed embryo (2) covered by the seed coat. To assess the effectiveness of mutagenesis, it is crucial to know how many of the targeted cells will eventually contribute to the next generation. Although plants do not have a determined germ line, in practice, the functional germ line can be defined as the number of cells in the shoot meristem of the embryo (3) that will contribute to the seed output (the genetically effective cell number (GECN))<sup>87</sup>. The GECN is determined genetically and might vary between different species and for different developmental stages. If the segregation in the M<sub>2</sub> — the generation derived from mutagenized M<sub>1</sub> plants — is ~3:1, the functional germ line consists of a single cell at the time of mutagenesis (GECN = 1). In *Arabidopsis*, recessive mutants segregate in a ratio of 7:1 in an M<sub>2</sub> population (4), therefore, GECN = 2. Because the functional germ line consists of two cells at the time of mutagenesis, the developing M<sub>1</sub> (which develops from the mutagenized seed) is chimeric and consists of two sectors, which might vary in size (5). Mutations segregate 4:0 (6) or 3:1 (7), depending on the sector from which the M<sub>2</sub> seeds derive. To recover a potential mutation, it is therefore necessary to analyse M<sub>2</sub> seeds from different branches of the M<sub>1</sub> plant.

The number of M<sub>1</sub> plants to be screened to obtain at least one allele at a given locus, with 99% probability, depends on the induced mutation frequency per locus, which in turn depends on the mutagenesis treatment and the locus of interest. Using various EMS-based protocols, mutation frequencies ranging from 1/300 to 1/30,000 per locus have been reported. On the basis of these mutation rates, a GECN value of 2 and the Poisson distribution (see REF. 88 for details), the number of M<sub>1</sub> plants required is ~700 and 70,000, respectively. In practice, many screens are based on 2,000–3,000 M<sub>1</sub> plants after exposure to 15–20 mM EMS for 12 h, which is at sub-saturation level but typically yields three or more alleles per locus for easily scorable floral mutants (J. Bowman, personal communication). This provides a reasonable balance between recovering as many mutants as possible and diminishing returns while approaching saturation.





**Figure 1 | *Arabidopsis thaliana*, the model for molecular genetic studies in higher plants.**

**a** | Mature *Arabidopsis thaliana* plant, Landsberg erecta (Ler) accession. **b** | Mature wild-type *Arabidopsis* flower. **c** | *agamous-1* mutant flower. The homeotic flower mutant *agamous* was obtained in one of the pioneering forward genetic screens for developmental mutants. Early screens identified many mutants that affect flower morphology and flowering time<sup>9</sup>, several of which were characterized later in the Meyerowitz laboratory. This analysis led to the formulation of the ABC model for flower development, which explains the determination of floral organ identity based on three overlapping and combinatorially acting functions (reviewed in REF. 89). Panels **b** and **c** provided by John Bowman, UC Davis, California.

how complex developmental pathways can be analysed by powerful second- and third-site modifier screens, and how highly specific processes, such as gene silencing or RNA decay pathways, can be investigated using specifically tailored genetic backgrounds for mutagenesis. Moreover, we show how the use of insertional mutagenesis approaches has renewed interest in the elusive GAMETOPHYTIC phase of the plant life cycle, and how the exploitation of natural variation provides new insights into complex regulatory processes.

#### Considerations for classical genetic screens

The pioneers of *Arabidopsis* genetics carried out screens in strains that were collected from the wild and were highly successful in identifying mutants with marked phenotypic changes (FIG. 1). For instance,

mutants were isolated that had altered leaf shape<sup>9</sup>, altered flowering time and flower morphology<sup>10,11</sup> or that were characterized by embryo<sup>12,13</sup> or seedling<sup>14</sup> lethality. In addition, screening on defined media allowed the recovery of mutants that were defective in PHYTOHORMONE synthesis and perception<sup>15–19</sup>, and other biosynthetic pathways<sup>20</sup>. Brute force screens that use biochemical assays led to the analysis of the lipid biosynthesis pathway<sup>21–23</sup>, and screening under specific biotic and abiotic environmental conditions led to the genetic analysis of photomorphogenesis<sup>24</sup> and plant-pathogen interactions<sup>25</sup>. After screening for obvious phenotypes that can easily be assayed, genetic screens became more sophisticated through the design of particular genetic backgrounds in which more precise questions can be addressed.

The success of a forward genetic screen depends mainly on two factors: first, the definition of a suitable genetic background; and second, an easy and tight procedure to identify the mutants of interest. Whereas a wild-type background is suitable for recovering mutants with striking phenotypes, it is not suitable for detecting additional loci that are redundant to a previously identified gene. A redundant gene is one the function of which can be performed by another gene in the same or a parallel pathway and, therefore, only the double mutant reveals the roles of these genes in a particular process. Neither does a wild-type background allow the identification of mutants that are involved in processes that do not immediately lead to a morphological or easily scorable biochemical phenotype. At present, the establishment of transgenic approaches that use drug or herbicide resistance genes for selection, and reporter genes to construct visible phenotypes, allows *Arabidopsis* researchers to engineer a scorable phenotype for virtually any process in the life cycle of a plant (see below).

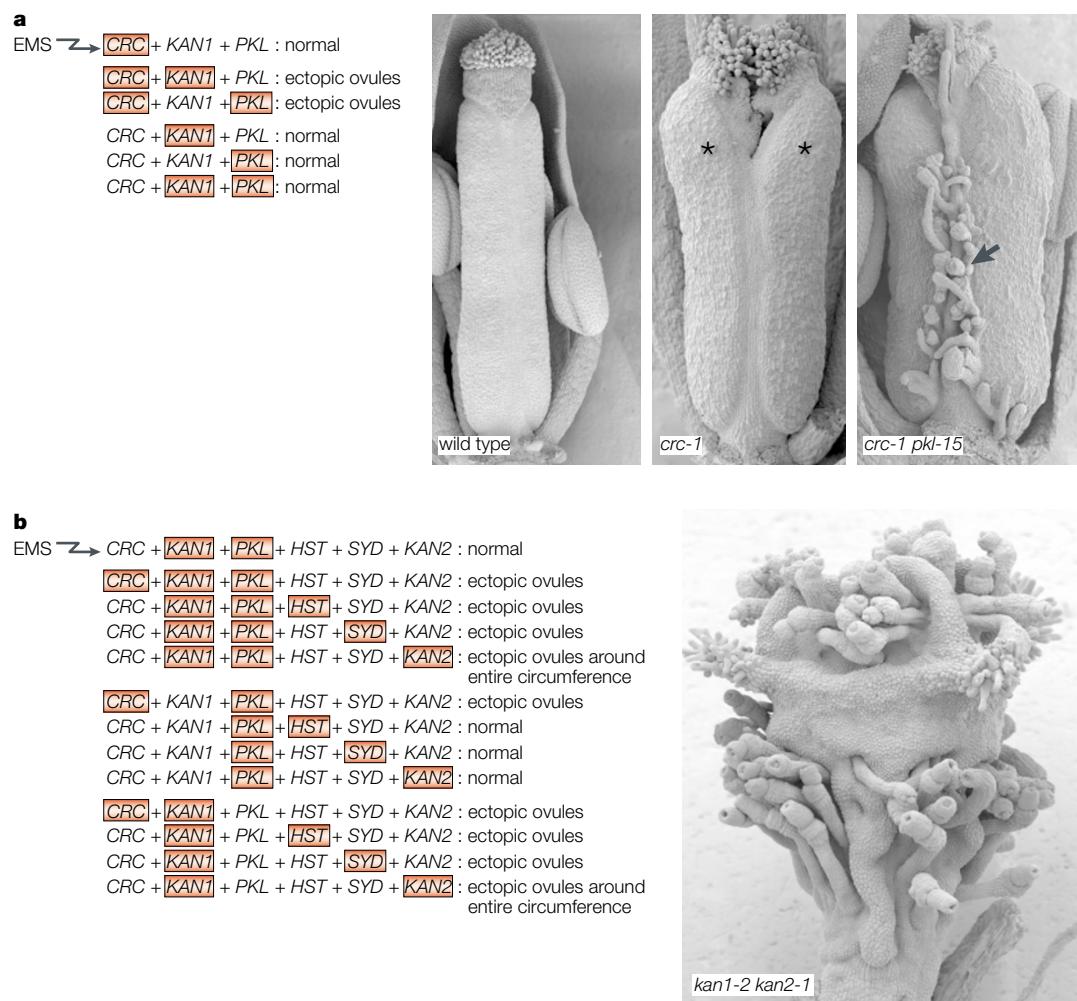
The simpler the screening procedure, the better suited it will be to screening large numbers of plants, so increasing the chances of identifying mutants of interest. Ideally, if a selection method is available — which allows only the mutants of interest to grow (for example, see REF. 26) — then this is preferable to screening, as it is less laborious. If screening cannot be avoided, it should be easy and tight; that is, the fraction of false positives should be low. If neither selection nor an easy screening procedure is possible, the assay should at least be very informative, as is the case for enhancer detection and gene trapping, which provide detailed information about the expression of a gene. However, such procedures are very time consuming and, because they will only identify a small number of genes that are involved in a process, they are not suitable for mutagenic approaches that aim to saturate the genome. Two-step screens offer a successful way to isolate mutants that are not easy to identify (for example, see REFS 27,28). In this design, the first step consists of screening for an easily scorable trait that can be expected to alter in the mutant candidates, as well as for a secondary phenotype that is characteristic of the mutants targeted. For example, a mutation might cause a morphological or biochemical defect that is difficult to assay but also an easily scorable

#### GAMETOPHYTE

The plant generation that produces the gametes and usually has a reduced chromosome number.

#### PHYTOHORMONE

A plant hormone that controls, or regulates, germination, growth, metabolism or other physiological functions.



**Figure 2 | Modifier screens.** Schematic description of an enhancer screen that identified the genes involved in establishing polarity in *Arabidopsis* CARPELS, and illustration of wild-type and mutant carpel phenotypes. Unshaded genes represent active genes, and genes shaded red represent loss-of-function mutant alleles. **a** | A second-site mutagenesis screen for enhancers of the *crabs claw* (*crc*) mutant carpel phenotype identified two genes, *KANADI1* (*KAN1*) and *PICKLE* (*PKL*, also known as *GYMNO*). The *crc* mutant (middle panel) shows defects in gynoecium development: it has partially unfused carpels (asterisks) but no obvious loss in defining polarity in the carpels. Loss-of-function alleles of either *KAN1* or *PKL* in a *crc* mutant background cause the formation of ectopic ovules (arrow). The right image panel shows *crc;pk1* double mutant carpels, which develop ectopic ovules medially, where the carpels fuse. Reproduced with permission from REF. 31 © (1999) Elsevier Science. **b** | Third-site mutagenesis of a *kan;pk1* background, which does not have ectopic ovules, identified three more genes that cause ectopic ovule formation: *hst* (*hasty*), *syd* (*splayed*) and *kan2*. The photo shows *kan1;kan2* double mutant carpels, which show a new phenotype in which polarity is completely lost and ovules develop radially. EMS, ethylmethanesulphonate. Reproduced with permission from REF. 36 © (2001) Elsevier Science.

secondary phenotype, such as fluorescence under ultraviolet (UV) light or a distorted segregation ratio. Screening first for the easily scorable phenotype allows potentially interesting mutants to be identified more rapidly. However, as the secondary phenotype could also result from phenomena that are unrelated to the biological process of interest, the mutant candidates must be further characterized in a second step using the more laborious assay to select the informative mutants.

These types of genetic screen have ‘co-evolved’ with the growing knowledge of the model system. In the following sections, we present the ‘evolution’ of forward genetic screens in *Arabidopsis*, highlighting representative screens in which genetic strategies have a great potential for future applications.

### Second-site modifiers

Once a mutation that affects a particular developmental or metabolic pathway has been identified, secondary screens allow further dissection of the pathway that is affected by the mutation. One of the most powerful ways to achieve this is to screen for second-site mutations (modifiers), which either enhance (worsen) or suppress (alleviate) the primary phenotype<sup>29</sup>. Enhancer mutations identify genes that act redundantly with the primary mutation or possibly interact physically with the mutant gene product. Many cases of genetic redundancy can be explained by the action of genes that encode proteins with similar biochemical functions<sup>30</sup>. Alternatively, enhancer mutations might uncover a partially redundant parallel pathway, whereas suppressor

#### CARPEL

A leaf-like structure that encloses the ovules and is the defining character of flowering plants. In some species, multiple carpels might be present in a compound structure, called an ovary.

mutations might also identify interacting proteins or alternative pathways that become activated by the second-site suppressor. Modifier screens are unbiased and are, therefore, likely to lead to new insights into interactions between genes, be it because they are biochemically similar, or, more importantly, active in a parallel pathway.

A successful modifier screen was carried out to look for genetic enhancers of the *crabs claw* (*crc*) mutation and led to a better understanding of how polarity is established in *Arabidopsis* carpels<sup>31</sup>. An obvious aspect of carpel polarity is the formation of PLACENTAL tissue and ovules on the adaxial (internal) but not the abaxial (external) side of the carpel. *CRC* is the founding member of the YABBY gene family, which encodes putative transcription factors<sup>32</sup>. At least three of its members — *FILAMENTOUS FLOWER* (*FILE*), *YABBY2* and *YABBY3* — are involved in establishing polar differentiation of lateral organs such as leaves and floral organs<sup>33,34</sup>. Loss-of-function *crc* alleles lead to defects in carpel and NECTARY development, but none of the phenotypes immediately indicate a defect in polar differentiation. Homozygous secondary mutations in a *crc* background, however, lead to a marked morphological change and to new carpel phenotypes with severe defects in polarity (FIG. 2a). Two genes were identified in this screen: *PICKLE* (*PKL*, also known as *GYMNOS*) and *KANADI1* (*KAN1*). When either of these two genes is mutated in a *crc* background, they lead to a new mutant phenotype: ectopic placentae and ovules on the abaxial (external) side of *Arabidopsis* carpels<sup>31</sup> (FIG. 2a). Because *PKL* encodes a putative CHD3 chromatin remodelling factor<sup>31,35</sup>, this protein was suggested to be functionally rather than biochemically redundant with *CRC*, indicating that distinct mechanisms are responsible for the proper establishment of polarity in *Arabidopsis* carpels<sup>31</sup>. The placental duplications on the abaxial side of medial carpel regions in plants that are mutant for both *crc*, and either *kan1* or *pkl*, together with evidence that none of the single mutants show these abnormalities, led the authors to conclude that *CRC* promotes abaxial cell fate in the carpels. This function is masked either by other abaxial-promoting genes, such as *KAN1*, or by genes that fine-regulate MERISTEMATIC activity, such as *PKL*<sup>31</sup>.

Eshed *et al.*<sup>36</sup> addressed the question of redundancy in this developmental pathway by screening for genetic enhancers in a *kan1-2; pkl-12* double mutant background, which does not produce ectopic ovules (FIG. 2b). Screening for ectopic ovule formation in this background should identify the *crc* mutation and uncover additional genes that act redundantly with *KAN1* or *PKL* in establishing carpel polarity. Four different enhancer loci were identified: *crc*, *hasty* (*hst*)<sup>37</sup> and *splayed* (*syd*) (D. Wagner and E. Meyerowitz, personal communication), and the novel locus *kanadi2* (*kan2*). A *kan1; kan2* double mutant shows a new, pronounced phenotype in which carpel polarity is almost completely lost and floral organs have lost much of their bilateral symmetry (radIALIZED). The fact that a *kan2* single mutant does not result in any visible phenotype, but has a marked effect in a *kan1* background, indicates not only functional but also

biochemical redundancy between the two genes. By comparing the genomic regions to which *KAN1* and *KAN2* map, duplicate genes that correspond to *KAN1* and *KAN2* were identified. In summary, two rounds of enhancer screens and a detailed analysis of single, double and triple mutant phenotypes proved to be an extremely straightforward and powerful strategy to understand how polarity is established in *Arabidopsis* carpels at the molecular genetic level. Similar strategies will certainly prove valuable in analysing other complex developmental pathways.

### Endogenous reporter genes

Like genetic variants, methylation profiles are also heritable features that contribute information for controlling plant growth and development. Various screening strategies have been developed to identify genes that affect such epigenetic information; for example, genes that affect the methylation profile of a locus<sup>26,38,39</sup>. *Arabidopsis* is particularly suited for studying epigenetic phenomena. Unlike other model systems, such as mouse, *Arabidopsis* tolerates large defects in epigenetic regulation: the *ddm1* mutant (*decrease in DNA methylation 1*) for instance, which reduces genome-wide cytosine methylation by 70%, is viable and fertile<sup>38,40,41</sup>.

In a screen for mutants that alter methylation and silencing of a densely methylated endogenous reporter gene, Bartee *et al.*<sup>28</sup> identified many second-site suppressor mutations on the basis of an easily scorable phenotype. They made use of a previously described mutant (*pai1C251Y*)<sup>42</sup> that is deficient in PHOPHORIBOSYLANTHRANILATE ISOMERASE (*PAI*), an enzyme of the tryptophan biosynthesis pathway. The *Arabidopsis* Wassilewskija (Ws) accession contains four *PAI* genes (*PAI1–4*) at three loci (FIG. 3a): only two (*PAI1* and *PAI2*) encode functional proteins, and two loci (*PAI1* and *PAI4*) are arranged as a tail-to-tail inverted repeat. The functional singlet (*PAI2*) is silenced by methylation<sup>43</sup>. Despite dense methylation in the coding region, the functional *PAI1* gene in the inverted repeat is expressed and provides PAI enzyme activity.

In the *pai1C251Y* mutant used for mutagenesis, the *PAI1* gene is made non-functional by a missense mutation. So, *PAI2* is the only functional *PAI* gene — but it is silenced by methylation. Release of this methylation should lead to activation of *PAI2* and to PAI enzyme activity. Owing to its PAI deficiency, the *pai1C251Y* mutant accumulates fluorescent intermediates, with the intensity of blue fluorescence under UV irradiation acting as a direct readout of the degree of silencing of the methylated *PAI2* gene. The mutant also has other phenotypes, such as reduced size and fertility, as well as increased bushiness. Suppressed fluorescence and suppressed PAI-deficient phenotypes offer an easily scorable phenotype to identify second-site mutations that relieve *PAI2* silencing. In a second step, the effect of these mutations on methylation at *PAI2* was analysed. This screening strategy allowed the isolation of 11 loss-of-function alleles of the *CHROMOMETHYLASE3* (*CMT3*) gene, which is crucial for CpNpG METHYLATION<sup>28</sup>.

#### PLACENTA

The tissue in the female reproductive organ of a plant that produces the ovules.

#### NECTARY

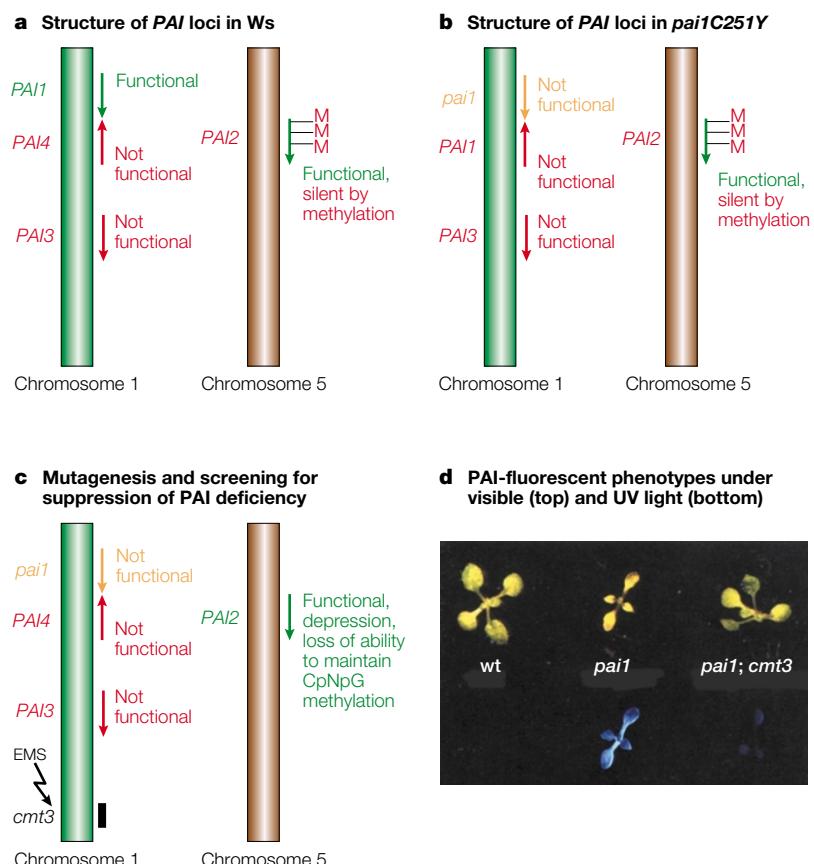
The nectar-secreting gland in a flower.

#### MERISTEMATIC CELL

Totipotent, undifferentiated cells in the meristems.

#### CpNpG METHYLATION

In contrast to animals, in which 5-methylcytosine that is found in the sequence CpG is the main site of DNA methylation, both CpG and CpNpG (where N = A, T, C or G) are methylated in plants.



**Figure 3 | Screen for the altered activity and methylation pattern of an endogenous gene.** **a** | Structure and map position of PHOSPHORIBOSYANTHRANILATE ISOMERASE (PAI) loci in the Wassilewskija (Ws) accession. Only PAI1 provides PAI activity. Although PAI2 encodes a functional protein, this locus is silenced by methylation. **b** | Structure of PAI loci in the *pa1C251Y* mutant background used for mutagenesis. In this mutant line, the *PAI1* gene is inactivated by a missense mutation, so PAI activity will only be detected if *PAI2* is relieved from epigenetic silencing. PAI-deficient plants can be assayed easily because they emit blue fluorescence under ultraviolet (UV) light. Mutants that have lost the ability to silence *PAI2* no longer fluoresce. **c** | Mutagenesis of the *pa1C251Y* line identifies *cmt3* (*chromomethylase3*) as a suppressor of PAI deficiency, using the UV-screening strategy. **d** | Wild-type (wt), *pa1* and *pa1; cmt3* mutant phenotypes under visible and UV light are shown. Reproduced with permission from REF. 28 © (2001) Cold Spring Harbor Laboratory Press.

**STAMEN**  
The male, pollen-bearing organ of the flower.

**GYNOCIUM**  
The seed-bearing organ of the flower, which consists of the ovary, stigma (a sticky surface to which pollen grains attach and germinate) and style (which connects the stigma to the ovary).

**CO-SUPPRESSION**  
Silencing of an endogenous gene due to the presence of a homologous transgene or virus.

Lindroth *et al.*<sup>44</sup> followed a comparable screening approach that also led to the identification of *cmt3* alleles. The *Arabidopsis* floral development gene SUPERMAN (*SUP*) becomes densely hypermethylated and silenced in several unlinked mutants that show genome-wide hypomethylation<sup>45</sup>. Similar epigenetically silenced *sup* alleles (*clark kent* alleles) had been identified and characterized previously. They are recessive and heritable, and lead to a phenotype that is similar to loss-of-function *sup* alleles, with an increased number of STAMENS and a defective GYNOCIUM<sup>46</sup>. However, heritable epigenetic *clark kent* alleles are not fully stable and ~1–5% of phenotypic revertants appear per generation<sup>46</sup>. This reversion of the *sup* phenotype, which results in wild-type flowers, is an easily scorable trait for identifying mutants that derepress the silent state of the hypermethylated *SUP* locus. A spontaneous reversion rate of 1–5%, however, does not fulfil the criteria of a stable

background and a tight phenotype that are required for a genetic screen. Lindroth *et al.* overcame this problem by using a line with a non-reverting *clark kent* allele created by introducing an additional copy of the *SUP* gene into *clark kent3* plants<sup>44</sup>. The additional *SUP* copy leads to CO-SUPPRESSION of *SUP* activity, which results in stable, non-revertible EPIALLELES of *sup*. A screen for the suppression of the *sup* phenotype in this background also identified the *CMT3* gene that is responsible for maintaining CpNpG methylation at the *SUP* locus<sup>44</sup>.

#### Screens for altered reporter gene expression

The availability of reporter genes, in combination with efficient transformation methods, make biological processes with subtle or hidden phenotypes accessible to forward genetic approaches. The use of a reporter gene allows the engineering of a highly specific background to dissect virtually any process of interest. Typically, a reporter gene encoding firefly luciferase (LUC), β-glucuronidase (GUS) or green fluorescent protein (GFP) is fused to the promoter of a specifically regulated or inducible gene. In this way, the wild-type expression pattern of the reporter gene can be observed. Subsequent mutagenesis of such lines allows screening for a deviation from the wild-type expression pattern and the identification of genetically interacting loci. This type of screen has been used since the late 1980s and early 1990s, and has allowed the identification of important genes in the circadian system<sup>47</sup>, the hormone signalling pathways<sup>48</sup>, and in plant responses to biotic<sup>49</sup> and abiotic<sup>50</sup> stresses. All of these are extremely difficult to detect in conventional phenotypic screens.

An impressive example of how tightly a genetic background can be engineered using exogenous reporter constructs was published by Johnson *et al.*<sup>51</sup> who identified mutants that are defective in a specific RNA decay pathway — an effective way for a cell to rapidly and tightly regulate gene expression. mRNA instability sequences that mediate rapid mRNA decay have been identified in multicellular organisms. In plants, the small auxin-up RNA (SAUR) transcripts, which are among the most unstable mRNAs in plants, seem to be constitutively unstable such that their abundance can be rapidly altered in response to transcriptional control by the plant hormone auxin<sup>52</sup>. A downstream element (DST) that is found in the 3' untranslated region of SAUR transcripts mediates this destabilization. After showing that the effect of DST can destabilize other RNA molecules, Johnson *et al.* engineered a genetic background that contains reporter genes that are destabilized by the DST element. To isolate mutants that are defective in DST-mediated RNA degradation, the authors carried out a two-step mutant screen in a transgenic line in which the transcripts of the *HPH* gene, which confers resistance to the antibiotic hygromycin, and the *GUS* reporter gene were destabilized by a tetrameric DST element<sup>44</sup>. On the basis of the hypothesis that mutants that are defective in the DST-mediated RNA-degradation pathway might increase RNA levels of the *HPH*



**Figure 4 | Screening for silencing mutants.** Mutagenized seedlings that have reactivated the expression of otherwise stably silenced hygromycin phosphotransferase (*HPT*) transgenes are selected for hygromycin-resistance after eight days of selection on 15 mg l<sup>-1</sup> hygromycin. Only the desired mutants are able to grow on selective medium. Reproduced with permission from REF. 26 © (1998) National Academy of Sciences, USA.

#### EPIALLELES

Epigenetic variant that can confer a phenotype similar to a mutant variant of the same locus, but that has no associated changes at the DNA level.

#### QUELLING

Specifically used to describe transgene-induced silencing in *Neurospora crassa*.

#### RNA INTERFERENCE

(RNAi). The process by which double-stranded RNA specifically silences the expression of homologous genes through degradation of their cognate mRNA.

#### SPOROPHYTE

The multicellular diploid form in plants that undergo alternation of generations. The sporophyte results from a union of haploid gametes and meiotically produces haploid spores that grow into the gametophyte generation.

#### CHALAZA

The region of the ovule that surrounds the proximal pole of the female gametophyte and that gives rise to the integuments (protective coats) of the ovule.

#### MICROPYRE

An opening at the distal pole of the ovule through which the pollen tube enters to effect double fertilization.

and *GUS* genes, EMS mutagenesis was carried out and putative mutants recovered. Increased resistance to hygromycin served as the basis for a first selection, and the primary mutants were rescreened for increased *GUS-DST* mRNA. In this way, spurious mutations (for example, those that alter hygromycin uptake) could be excluded. The two independent mutants that have these phenotypes were subsequently shown to elevate an endogenous DST-containing transcript as well<sup>51</sup>.

#### Tailored genetic backgrounds

Plants were the first organisms in which silencing of exogenous DNA was observed<sup>53</sup>. Since then, numerous examples have confirmed the specific inactivation of a transgene either at the transcriptional level (transcriptional gene silencing, TGS) or the specific degradation of transgenic and, if present, homologous mRNA after transcription (post-transcriptional gene silencing, PTGS)<sup>54</sup>. Gene silencing, initially perceived as an uncontrollable and unwanted source of instability of transgene expression, offered an entry point for the genetic analysis of silencing mechanisms, and different laboratories took advantage of plant lines that harbour a silent transgene. In a way, the starter line was engineered by the unknown process under investigation, and mutagenesis of these plant lines immediately established whether it was possible to identify modifiers of silencing that reactivate the silent locus under study.

The power of screens that are similar to the one done by Johnson *et al.* lies in the ease of screening: only plants that break the silent state of the transgene will be able to grow on a selective medium<sup>26</sup>, produce green fluorescence<sup>55</sup>, or show *GUS* activity<sup>56</sup>. The first successful screen for second-site mutations that interfere with the maintenance of the silent state of a drug resistance gene was reported by Mittelsten-Scheid *et al.*<sup>26</sup>. EMS- and FNR-mutated transgenic *Arabidopsis* seedlings, which carry several integrated hygromycin phosphotransferase (*HPT*) genes that are stably

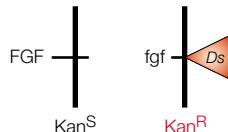
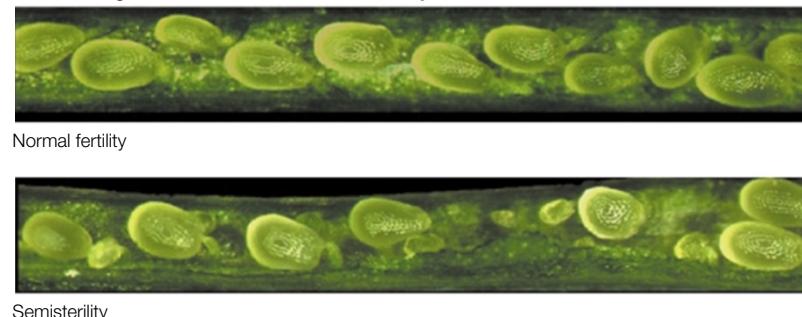
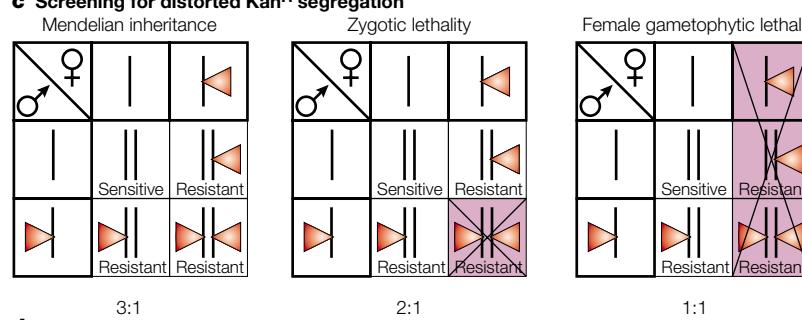
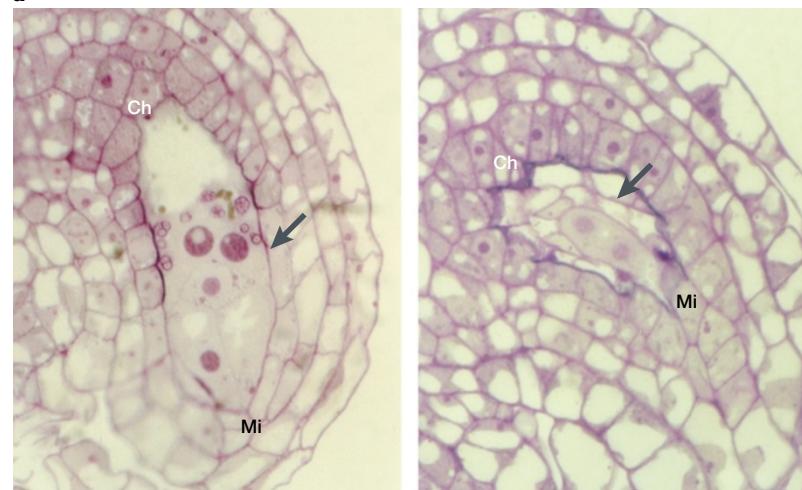
silenced at a single locus, were selected on hygromycin-containing medium (FIG. 4). This led to the identification of eight mutants that activated the normally silenced transgenes and that turned out to be allelic to *ddm1*. This gene, which encodes a putative SWI2/SNF2 chromatin remodelling factor, had been identified previously in a laborious screen for altered DNA methylation patterns at a repetitive centromeric region<sup>38,41</sup>. Loss-of-function *ddm1* alleles lead to genome-wide hypomethylation and accumulation of developmental abnormalities after successive rounds of inbreeding. Suppressors of TGS provided the first molecular link between epigenetic changes, such as chromatin structure and DNA methylation, and transgene silencing in plants.

Using the same line, Amedeo *et al.* conducted a T-DNA (transferred DNA) insertion mutagenesis experiment<sup>57</sup> (BOX 1). Interestingly, although they did not find any additional *ddm1* alleles, they identified a single allele of a gene named *MORPHEUS' MOLECULE1* (*MOM1*), which is essential for TGS but does not affect DNA methylation at the transgene locus. The reason no *mom1* alleles were recovered in the earlier EMS/FNR screen is speculative: reactivation of the *HPT* gene by the *mom1* mutation is sufficient for selection on hygromycin, but it seems to be significantly lower than reactivation by the *ddm1* mutation. This might explain why, in an initial screen for modifiers of silencing, in which most attention had been given to strongly reactivating candidates, the weakly reactivating *mom1* and possibly other mutations were overseen (O. Mittelsten-Scheid, personal communication). The mechanism of PTGS has been studied using similar approaches and led to a better understanding of the regulatory mechanisms that involve RNA degradation and small interfering RNAs. These screens led to the establishment of a molecular link between PTGS in plants<sup>56,57</sup>, QUELLING in *Neurospora crassa*<sup>58</sup> and RNA INTERFERENCE in *Caenorhabditis elegans*<sup>59</sup>.

#### Screens for gametophytic mutants

The plant life cycle alternates between a diploid and a haploid phase. The diploid, SPOROPHYTIC phase is the dominant one in flowering plants, whereas the haploid phase is restricted to the few cells of the male and female gametophytes: the embryo sac and the pollen grain, respectively. A genetic analysis of gametophytic mutants is challenging for several reasons: the gametophytes are restricted to a small number of cells; direct scoring for a phenotype is a difficult undertaking; and male and female gametophyte development occurs in sporophytic tissue (anthers and ovules, respectively) rendering it inaccessible for direct inspection.

Until recently, very few mutants that affect the gametophytic phase of the plant life cycle had been identified; most of the known mutants were found fortuitously. Mutants that disrupt female gametogenesis are expected to result in semisterility because, in a heterozygous plant, half of the ovules bear a wild-type and the other half a mutant female gametophyte, the latter not giving rise to seeds. Because chemical mutagenesis also drastically reduces fertility, the

**a Insertional mutagenesis of *Arabidopsis* plants using the *Ac/Ds* system****b Screening for reduced seed set/semisterility****c Screening for distorted Kan<sup>R</sup> segregation****d**

**Figure 5 | Identifying mutants that affect the female gametophyte.** **a** | An insertional mutagen (*Ds*; see BOX 1) disrupts a female gametophytic factor (FGF) and introduces a dominant marker that confers resistance to the drug kanamycin ( $\text{Kan}^R$ ). **b** | Screening for semisterility (low seed count) in a first step allows identification of potential gametophytic mutants that affect the female (and possibly the male) sex. **c** | Because reduced fertility can also be caused by reasons other than gametophytic lethality (for example, inappropriate environmental conditions, partially penetrant mutation of a sporophytically required gene involved in ovule development or reciprocal chromosomal translocations), a second screening step is necessary. Distorted segregation ratio of the dominant  $\text{Kan}^R$  marker indicates gametophytic lethality. For an idealized mutation, which affects exclusively one sex with full penetrance, a 1:1 segregation ratio of sensitive to resistant seedlings is expected. The indirectly identified mutants are analysed in detail. **d** | Left, wild-type female gametophyte with normal cellularization (arrow); right, *hadad* mutant gametophyte arrested at the two-nuclear stage before the formation of a central vacuole (arrow)<sup>27</sup>. The proximo-distal axis of the ovule from the CHALAZAL (Ch) to the MICROPYLAR (Mi) pole is indicated. Panels **b** and **d** provided by Norbert Huck, University of Zürich, and Jean-Philippe Vielle-Calzada, CNVETSAV Irapuato, Mexico, respectively.

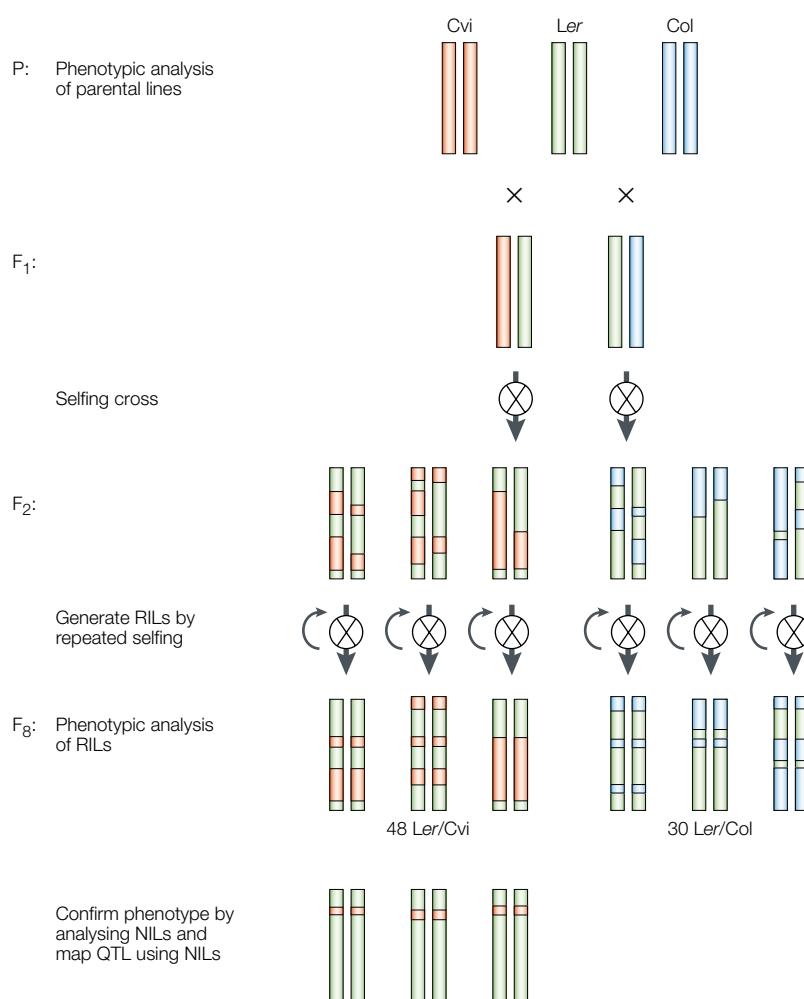
semsterile phenotype is not suitable as the basis for a classical genetic screen; it can be used efficiently, however, if very few lesions are introduced, for example by using insertional mutagens (FIG. 5a). By contrast, male gametophytic mutants are expected to be fertile owing to the large excess of wild-type pollen compared with the number of ovules that are available for fertilization. Laborious microscopic inspection did allow the isolation of mutants in which half of the pollen showed a phenotype<sup>60–62</sup>, although these screens are restricted to a subset of phenotypic classes that show visible defects in mature pollen.

The fact that a mutation affecting the development or function of a gametophyte is transmitted to the progeny at a reduced frequency offers an alternative to microscopic inspection. Reduced transmission through one or both sexes leads to a distorted, non-Mendelian segregation ratio of the mutation (and linked loci), such that segregation ratio distortion can be used as a reliable indicator of a gametophytic defect. Although strategies that use multiply marked chromosomes to follow segregation ratios have been successful<sup>63</sup>, such screens can be facilitated by using a marked insertional mutagen. The progeny of a hemizygous plant that contains an inserted dominant drug resistance gene are expected to segregate 3:1 for resistance. Any deviation of this ratio indicates that the drug resistance gene is transmitted at a reduced frequency through one or both sexes (FIG. 5c). Mutageneses based on either T-DNA or transposon insertions have been successful in identifying male and/or female gametophytic mutants<sup>27,64–66</sup> (FIG. 5D; BOX 1).

It is striking that the phenotypic spectrum of female gametophytic mutants seems to depend on the insertional mutagen used (J. Moore and U.G., unpublished observations). The reason for this difference might lie in the widely used 'floral-dip' transformation method, in which T-DNA is integrated in the female gametophyte<sup>67,68</sup>. If the insertion disrupts a gene that is essential for a subsequent developmental step in female gametogenesis, the mutation is not transmitted to the next generation and will be lost. By contrast, transposition creates a chimeric  $F_1$  plant and does not immediately interfere with female gametogenesis. If the mutation is not lethal to all male and female gametophytes, it will be transmitted to the next generation so that the mutation can be recovered.

**Natural allelic variation**

The identification of gene function by studying induced mutants is limited by the genetic backgrounds that are analysed. *Arabidopsis* is a predominantly self-fertilizing species and the strains generally used for forward, as well as reverse, genetic approaches are Landsberg *erecta* (*Ler*), Columbia (*Col*), C24 and *Ws*, originally collected from the wild by Friedrich Laibach and colleagues, the pioneers of *Arabidopsis* research<sup>69</sup>. These accessions represent practically homozygous inbred lines. The mutant phenotypes, and therefore the genes, that can be identified depend on the wild-type genetic background that is used for mutagenesis. It is



**Figure 6 | Natural variation as a source of new genes.** Schematic illustration of the procedure used by Swarup *et al.*<sup>74</sup> to identify quantitative trait loci (QTL) that affect the *Arabidopsis* circadian clock. Two parallel bars symbolize the diploid genotype of an individual plant. The fraction that originates from the different wild-type accessions from which recombinant inbred lines (RIL) and near-isogenic lines (NIL) are derived are colour coded. By taking advantage of previously generated RILs and NILs, the circadian period length of parental wild-type strains (Cvi, Cape Verde Islands; Ler, Landsberg erecta; Col, Columbia), which have similar rhythmic periods, were compared with the period length of RILs between the three wild-type accessions. The RILs have extensive variation in rhythmic period length. To confirm and map the QTL, NILs containing small chromosomal pieces of one parent (Cvi) introgressed into another (Ler) are tested for the circadian period length phenotype. By using this strategy, Swarup *et al.* identified four putative QTL that control the period of the circadian clock.

**EPISTATIC**  
When one gene masks the expression of another. If mutant *a* gives phenotype A and mutant *b* gives phenotype B, and if the double mutant *ab* gives phenotype A and not B, then gene *a* is epistatic to gene *b*.

**RECOMBINANT INBRED LINE**  
Homozygous plant line obtained after crossing two parental accessions and subsequent inbreeding over several generations.

unlikely or impossible to detect mutant phenotypes of genes for which the laboratory strain carries a functional null allele (either mutated or silenced). Because of EPISTATIC interactions that might differ in different accessions, some phenotypes will be apparent only in certain genetic backgrounds.

This limitation can be overcome by using the variation found among and within naturally occurring populations of *Arabidopsis* as a source of genetic variation — an alternative to induced mutants. It has been known since the earliest days of *Arabidopsis* research that plants collected from different geographical regions showed considerable genetic variation<sup>69,70</sup>.

However, exploring this variation was limited because the multigenic variation between accessions often leads to quantitative (continuous) traits, in contrast to the qualitative (discrete) variation of commonly studied mutants. Only recently, the study of variation among *Arabidopsis* accessions has been renewed by the application of methods for quantitative genetics that were developed and used extensively in crop plants. *Arabidopsis* is becoming a model system for questions based on quantitative genetics, such as population structure, plasticity and evolution, because it allows the extension of these genetic analyses to the molecular level (reviewed in REFS 71,72).

Alonso-Blanco and Koornneef<sup>73</sup> propose three main areas in which natural variation will become increasingly important: first, the study of genes for which phenotypic functions cannot be detected by current approaches in standard laboratory strains; second, the identification of mutants that can only be identified by laborious or expensive assays; and third, the identification of new functional alleles that reveal important information about the molecular function and evolution of known genes.

The first successful studies in which natural variation was used with respect to these areas have been reported recently<sup>74,75</sup>. The circadian system relies on an endogenous biological clock that controls a wide range of rhythmic processes with periods close to 24 h, even under constant environmental conditions. In all model systems studied so far, forward genetic screens have been essential for identifying components of the circadian oscillator. However, the isolation of mutants requires the monitoring of candidates over several days at different time points, making such brute-force screens extremely labour intensive. Taking advantage of the natural variation in crosses between the most commonly used laboratory strains Col and Ler and an *Arabidopsis* accession from the Cape Verde Islands (Cvi), Swarup *et al.* scored changes in the circadian period of leaf movement<sup>74</sup>. Although the parental lines show similar rhythmic periods, the progeny of crosses between them revealed extensive variation for this trait. An analysis in RECOMBINANT INBRED LINES (RILs) between Ler/Cvi and Ler/Col identified four putative QUANTITATIVE TRAIT LOCI (QTL) that control the period of the circadian clock (FIG. 6). The positions of the QTL were mapped by using NEAR-ISOCENIC LINES (NILs) that contain a QTL in a small, defined region. In summary, the testing of a relatively small number of individuals (48 RILs from the Ler/Cvi population) led to the identification of four major QTL for the circadian period, which could be confirmed either in NILs or by the identification of similar QTL in the Ler/Col population. The power of this approach becomes most apparent in the further analysis of one NIL: the testing of a candidate mutation that lies in the identified region led to the identification of the FLOWERING LOCUS C (*FLC*) gene that underlies the QTL. For comparison, direct screening of 8,000 M<sub>2</sub> lines using a luciferase reporter gene-based assay led to the identification of at least 21

Box 3 | Reverse genetics resources in *Arabidopsis*

The complete inactivation of a gene is generally the most direct way to understand its function. Unlike in other model systems, such as yeast or mouse, no effective methods for targeted gene disruption exist in flowering plants. Transposons or T-DNA (BOX 1) are used as (almost) random mutagens to create loss-of-function mutations in plants. Because the sequence of the inserted element is known, the gene in which it inserted can easily be recovered using various cloning- or PCR-based strategies.

A growing number of insertion mutants are available to the *Arabidopsis* community and can be screened for an insertion in a gene of interest, either *in silico* by using BLAST, if the genomic sequence that flanks the insertion is known, or through a PCR-based screening approach. The [Nottingham \*Arabidopsis\* Stock Centre](#) (NASC, see link), the [Salk Institute](#) (see link), the [Flanking Sequence Tags \(FST\) Project](#) at Versailles (see link) and the [Torre Mesa Research Institute](#) (TMRI, see link) maintain insertion databases that can be searched for known insertions in a gene of interest. The [Knockout Facility](#) at the University of Wisconsin, Madison (see link) generated large populations of T-DNA insertion lines and offers a user-fee service to obtain 'knockout' mutants on the basis of a systematic screen of DNA pools from independent T-DNA lines.

If a gene of interest has a known loss-of-function phenotype, the [Arabidopsis TILLING Project](#) (see link) is useful for further characterization of the gene.

TILLING is a large-scale reverse genetic strategy that uses a high-throughput method based on an endonuclease that preferentially cleaves mismatches in heteroduplexes between wild-type and mutant DNAs. Subsequent analysis of cleavage products on a sequencing gel allows for the rapid identification of induced point mutations<sup>70</sup>. In contrast to insertional mutagenesis, TILLING can yield a traditional allelic series of point mutations. TILLING is not the method of choice in cases in which a 'knockout' phenotype of an unknown gene is the primary interest, but the alleles of different phenotypic strength obtained by this method are extremely useful for the detailed functional characterization of a gene.

independent timing of CAB expression mutants (probably also representing a handful of loci), of which one was further characterized (*toc1*)<sup>47</sup>.

A second study used natural *Arabidopsis* strains to identify alleles that control variation in the response of plants to flowering cues. Certain plant species require a 3–8-week cold treatment for the induction of flowering (vernalization), which is a principal factor in determining flowering time in some *Arabidopsis* accessions. Although many genes are known to regulate flowering time (reviewed in REF. 76), the vernalization requirement of different *Arabidopsis* accessions segregates as a single genetic trait that maps to the *FRIGIDA* (*FRI*) locus<sup>77</sup>. Therefore, the *FRIGIDA* gene has a discrete, large-effect allelic variation. To analyse the molecular basis of this allelic variation, Johanson *et al.*<sup>75</sup> cloned *FRI* by map-based cloning procedures. The correlation of the molecular nature of the *FRI* allele in different accessions with the flowering time phenotype and the requirement for vernalization yielded important insights into the evolution of the variation in flowering time in *Arabidopsis*. Rapid cycling accessions, such as the laboratory strains Col and Ler, carry recessive *FRI* alleles. Sequence comparison of a dominant *FRI* allele from a late-flowering ECOTYPE with the early-flowering Ler and Col revealed that the latter carry loss-of-function alleles. In Ler, a 16-bp deletion changes the reading frame and leads to early termination. In Col, a 376-bp deletion combined

with a 31-bp insertion removes the putative translation start. These findings indicate that the *FRI* allele from late-flowering ecotypes might be the ancestral form of the gene, with early flowering evolving at least twice from late-flowering ecotypes through loss of *FRI* activity<sup>75</sup>.

#### Reverse genetics in *Arabidopsis*

At present, the *Arabidopsis* researcher also has great tools for doing reverse genetics, the genetic analysis that proceeds from genotype to phenotype using several gene manipulation techniques. Different consortia have generated collections of insertion mutants, which can either be screened for a knockout in a gene of interest by PCR<sup>78</sup> or by comparing sequences that flank the insertion with the sequence of a gene of interest (BOX 3). Systematic screens for gene disruption and also for overexpression are under way in the public and private sectors. However, reverse genetic screens depend on the researcher's judgement on the candidacy of a gene by sequence alone. So, reverse genetic screens are biased and less likely to lead to new insights than unbiased forward screens.

Recently, an additional, extremely powerful tool has become available: high-throughput screening for induced point mutations. This allows the identification of induced lesions in a particular gene of interest (targeted, induced lesions in genomes, TILLING)<sup>79</sup>. Unlike insertional mutagenic approaches, which often provide knockout mutations, EMS-mutagenesis-based TILLING yields point mutations that provide an allelic mutant series; for instance, it might identify sublethal alleles, which are especially valuable for analysing the phenotype of essential genes. TILLING has the potential to identify conditional (for example, temperature-sensitive) alleles in a relatively simple way. Reverse genetics approaches provide a powerful tool to study genes of unknown function (BOX 3) and therefore complement the study of genes identified by forward genetic approaches.

#### Conclusions and outlook

Classical genetic screens have been a powerful means to dissect plant function. Indeed, *Arabidopsis* has become the 'trendsetter' in plant biology as was predicted by Laibach in 1965 (REF. 80). The completion of the *Arabidopsis* genome sequence has brought us into an era in which genetic screens will be even more important in assessing the function of *Arabidopsis'* ~25,000 genes. The ability to map and clone QTL has opened a new avenue for the geneticist — the exploitation of natural variation. This approach bears a tremendous potential to further our understanding of plant growth and development, and its evolution. Genetic screens are bound to become more sophisticated through the design of tailor-made genetic backgrounds that allow ever more complex processes to be addressed. However, the conceptual framework for genetic screens is still the same as that laid out by the pioneers of *Arabidopsis* genetics and will remain the basis for future genetic approaches.

**QUANTITATIVE TRAIT LOCUS**  
A genetic locus identified through the statistical analysis of complex traits (such as plant height or body weight). These traits are typically affected by more than one gene, and also by the environment.

**NEAR-ISOGENIC LINE**  
Homozygous plant lines obtained after crossing two parental accessions, then repeatedly backcrossing the progeny to one of the parents. Near-isogenic lines differ from the parental line only in a small genomic region.

**ECOTYPE**  
A subdivision of a species that survives as a distinct population through environmental selection and reproductive isolation.

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