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EpiRILs: lessons from Arabidopsis

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Abstract

In recent times, epigenetic marks have emerged as important players involved in the regulation of gene expression and transposable element silencing in many organisms. In plants, many epigenetic changes, mainly at the level of DNA methylation, are transgenerational stable and contribute to formation of epialleles, affecting developmental and agronomical traits. In this scenario, it becomes critical to differentiate the genetic from the epigenetic contribution to plant phenotypes. In Arabidopsis, epigenetic Recombinant Inbred Lines (epiRILs), obtained by an initial cross of isogenic parents with different DNA methylation profiles, provide a powerful tool to investigate the role and significance of epigenetic alteration in identical or almost identical genetic backgrounds. Such populations have greatly increased our knowledge in mechanisms involved in epialleles formation and stability, as well as in the consequences of DNA methylation changes in genomic stability, transposable elements activation and phenotypic traits.

Introduction

While it is known that DNA is the support of heredity, it is more and more recognised that heritable phenotypic variation can be also caused by epigenetic changes, and not only by change in the DNA sequence. Methylation of cytosines (DNA methylation) is an epigenetic mark conserved across many species and plays an important role in regulating gene expression (X. J. He, Chen, & Zhu, 2011). Widespread perturbation in DNA methylation has been shown to lead to heritable phenotypic changes in plants (X. J. He et al., 2011; Seymour & Becker, 2017). Moreover, in plants changes in DNA methylation can be transmitted through generations because, contrary to what occurs in mammals, there is no clear evidence of global DNA methylation resetting at each generation (Heard & Martienssen, 2014). DNA methylation in plants occurs at cytosines and can been observed in all three contexts CG, CHG and CHH. DNA methylation in genes is almost only observed in the CG context, and seems to be associated with gene activation. On the other hand, dense DNA methylation at Transposable Elements (TEs) is observed in all three contexts and is associated with transcriptional repression (Gehring & Henikoff, 2007).

To date only a handful of natural epialleles (see definitions) have been described in eukaryotes (Table 1). These epialleles are characterised by a gain or loss of DNA methylation, often associated with a change in gene expression, as well as strong phenotypes. Such changes of DNA methylation at epialleles are usually observed in repeated sequences or TEs that are either in close proximity or inside genes. While these epialleles are stable over a generation, some are metastable, which means that some level of instability as well as gradual reversions have been observed. This is in striking contrast to

genetic alleles (see definitions), as changes in the DNA sequence are more stable than changes in DNA methylation for several orders of magnitude (Becker et al., 2011).

The small number of natural epialleles described so far could be explained by the fact they were either identified thanks to a strong phenotypes (Bender & Fink, 1995; Cubas, Vincent, & Coen, 1999; Manning et al., 2006; Martin et al., 2009; Melquist, Luff, & Bender, 1999; K. Miura et al., 2009; L. Zhang et al., 2012; X. Zhang, Sun, Cao, & Song, 2015), to an allelic incompatibility between accessions (Agorio et al., 2017; Blevins, Wang, Pflieger, Pontvianne, & Pikaard, 2017; Durand, Bouche, Perez Strand, Loudet, & Camilleri, 2012) or by chance (Silveira et al., 2013). We can suppose that many epialleles have not been discovered yet as they might be associated with mild phenotypes, or phenotypes only visible under certain circumstances (e.g environmental stress). Moreover, while identifying alleles underlying a certain phenotype is nowadays straight-forward, identifying DNA methylation changes at an unknown position associated with a phenotype is still challenging and requires more sophisticated analysis. In the context of this chapter, we will use a broad definition of epialleles: any stably transmitted change in methylated profiles, with or without phenotypic consequences.

[Insert Table 1 here]

Perturbing DNA methylation, by mutating genes involved in DNA methylation deposition or maintenance, is a way to increase the chance of detecting epialleles. Hence epialleles have been detected in *Arabidopsis thaliana* mutants characterised with a global loss of DNA methylation. Some of these epialleles are characterised by DNA hypomethylation, such as *fwa*, associated with a late flowering phenotype (Kakutani, 1997; Kakutani, Jeddeloh, Flowers, Munakata, & Richards, 1996; Lippman et al., 2004; Ronemus, Galbiati, Ticknor, Chen, & Dellaporta, 1996; Soppe et al., 2000) and *sqn*, associated with an increased

expression (Catoni et al., 2017; Habu et al., 2006). Others are characterised by DNA hypermethylation, such as *sup*, associated with an excess of stamens (Jacobsen & Meyerowitz, 1997; Jacobsen, Sakai, Finnegan, Cao, & Meyerowitz, 2000), *ag*, associated with an absence of carpels (Jacobsen et al., 2000) and *bns*, associated with a dwarf phenotype (Saze & Kakutani, 2007). These epialleles are stably maintained after removal of the inducible mutation, with a certain degree of metastability, as also observed for natural epialleles. Except for QQS (Silveira et al., 2013), until now, none of these induced epialleles have been naturally observed in *Arabidopsis thaliana*.

In order to identify alleles with milder or quantitative phenotypes (in contrast to strong qualitative phenotypes), recombinant inbred lines (RILs, see definitions) are commonly used (Mackay, 2001). These populations are used to identify loci at which the segregation of parental alleles are associated with phenotypic changes. Such an approach could also allow the detection of epialleles associated with mild or quantitative phenotypes. However, as this will be described in more detail in this chapter, alleles as well as epialleles are segregating in RIL populations, making it difficult to separate epigenetic from genetic impact on phenotypes (Johannes, Colot, & Jansen, 2008). In order to specifically identify epialleles associated with phenotypic changes, epigenetic RIL (epiRILs, see definitions) have been generated in *Arabidopsis thaliana* (Johannes et al., 2009; Reinders et al., 2009). In short these populations have been created in order to maximise DNA methylation changes, while reducing (if not completely removing) DNA sequence differences.

In this chapter we will be discussing the many aspects in which epiRIL populations have been of a great use and how the acquired knowledge could be translated in crops in the future.

Definitions

Allele: Genetic variants of a gene. Different alleles can result in different phenotypic traits.

Epiallele: Epigenetic variants of a gene. The genetic sequence of the epialleles is identical, but the level of DNA methylation, or other epigenetic marks, are different. Epialleles can result in differences in gene expression, which can potentially lead to differences in phenotypic traits.

RIL (Recombinant Inbred Lines): Set of homozygous lines that incorporate a combination of genomic regions derived from the cross of two parent lines. Each RIL is developed by self-pollination and single seed descent propagation of a segregating F2 plant obtained from the initial cross. Inbreeding continues for at least six/eight generations, determining the fixation in homozygous form of most of alleles and epialleles. RILs are often used for mapping QTLs.

EpiRIL (Epigenetic Recombinant Inbred Lines): Similarly to RILs, epiRILs are a set of fixed homozygous lines, descending from a F2 population. However, contrary to RILs, the parents used to generate epiRIL population have identical (or almost identical) genomic sequence but different DNA methylation profiles. EpiRILs are thus maximising epialleles segregation, while reducing (if not removing completely) allelic segregation. EpiRILs can be used for mapping epiQTLs.

QTL (Quantitative Trait Locus): A QTL is a locus of the genome at which genetic variation correlates with variation of a quantitative trait.

EpiQTL (Epigenetic Quantitative Trait Locus): An epiQTL is a locus of the genome at which variation in DNA methylation correlates with variation of a quantitative trait.

Additive alleles: Different alleles of a gene that combine in a way that the phenotype or expression level of the heterozygous is equal to the sum of each allele.

Dominant alleles: The dominant allele dictates the phenotype or expression level of the heterozygous, when paired with a recessive allele.

Transgressive transcripts: In the context of a hybrid, locus expression level that is not explained neither as additive, nor than as dominant allelic effect.

Definition and description of the epiRIL populations

The study of epiallele stability and phenotypic consequences can be performed by taking advantage of natural variation in *Arabidopsis thaliana*. DNA methylation at the level of genes has been shown to be highly polymorphic between *A. thaliana* accessions, making possible to follow epialleles segregation and their stability in F2 populations (Vaughn et al., 2007). Natural accessions not only differ in their levels of DNA methylation, but also in their genetic sequences, and genetic polymorphism can be used to identify the parent of origin for genomic regions in F1 and F2 populations (Greaves et al., 2012; Shen et al., 2012; Vaughn et al., 2007; X. Zhang, Shiu, Cal, & Borevitz, 2008).

However, the presence of genetic and epigenetic variation across *A. thaliana* natural accessions often impairs proper quantification of the epigenetic contribution to phenotypic differences. Indeed, several studies in plants (but also in mammals) reported many examples of DNA methylation variations associated to either local (*cis*) or distant (*trans*) changes in DNA sequence (Eichten et al., 2011; Gibbs et al., 2010; Hellman & Chess, 2010; D. Zhang et al., 2010). On the other hand, mutation rate of methylated cytosines is higher than

non methylated cytosines (Xia, Han, & Zhao, 2012), suggesting that DNA methylation and DNA sequence polymorphisms can be linked and also influence each others.

Therefore, a classification of epialleles has been proposed depending on their link with DNA sequence polymorphism (Richards, 2006): (i) obligatory epialleles, for which a *cis* or *trans* genetic polymorphism influences the DNA methylation status; (ii) facilitated epialleles, which can be linked to or caused by a genetic polymorphism, but that are not fully dependent on it; and (iii) pure epialleles, that are not affected by any genetic changes.

Two epiRIL populations have been independently created in *Arabidopsis thaliana* (Figure 1), to maximise DNA methylation variation and minimise (if not abolishing) DNA sequence polymorphisms, in order to discriminate epialleles that are not influenced by DNA sequence polymorphisms (Johannes et al., 2008). These epiRILs have been generated by crossing an epigenetic mutant, *met1-3* (Reinders et al., 2009) or *ddm1-2* (Johannes et al., 2009), with its corresponding wild-type (Columbia-0 accession). The two parents thus have the same genome, except for the mutated gene, but they have very contrasting DNA methylation profiles. Each epiRIL within the population essentially contains a mosaic epigenome derived from either wild-type and *ddm1-2* or wild-type and *met1-3.*

Although both *met1-3* and *ddm1-2* mutants are hypomethylated, their effects on genome wide DNA methylation are different, and these differences are conserved in the epigenetic perturbations segregating in two epiRILs populations. The DNA methyltransferase MET1 maintains CG methylation in *Arabidopsis thaliana* and the *met1-3* null mutant is characterised by a virtual complete erasure of CG methylation and indirect loss of plant-specific non-CG methylation (Saze, Mittelsten Scheid, & Paszkowski, 2003). On the other hand, *DDM1* encodes an ATPase chromatin remodeler primarily involved in allowing DNA

methyltransferases to access heterochromatin (Zemach et al., 2013). *ddm1-2* mutation mainly affects DNA methylation in all cytosine contexts (CG, CHG and CHH) at heterochromatic TEs (Kakutani, Jeddeloh, & Richards, 1995; Lippman et al., 2004; Vongs, Kakutani, Martienssen, & Richards, 1993). Consequently, the epialleles generated in *met1*-derived epiRILs are equally distributed in euchromatic and heterochromatic areas, including gene bodies that are exclusively CG methylated (Bewick et al., 2016; Catoni et al., 2017), while epialleles in *ddm1*-derived epiRILs are mostly involving TE loci (Cortijo et al., 2014). The *met1-3* mutant used to create the *met1*-epiRIL population also shows very severe phenotypic defects, including reduced fertility (Mathieu, Reinders, Caikovski, Smathajitt, & Paszkowski, 2007). Hence, a high level of mortality (29%) has also been observed while propagating 100 individuals of the *met1*-epiRIL population over generations (Reinders et al., 2009). On the contrary *ddm1*-derived epiRILs have been generated starting from the *ddm1-2* mutant, which displays only minor developmental defects. This strategy allowed the production of a large population of 505 different *ddm1*-derived epiRILs, with no evidence of selection against deleterious phenotypes (Colome-Tatche et al., 2012).

The crossing scheme of the two populations also differs. In both cases, the mutant (*met1-3* or *ddm1-2*) has been crossed with a wild-type plant and only F2 plants segregating the wild-type allele have been used to generate the epiRIL populations. The *met1*-epiRILs originate directly from the F2 individuals resulting from this cross, while the *ddm1*-epiRILs descend from a second back cross of the F1 with the wild-type. Thus, DNA methylation changes segregate with a 1:1 ratio in the *met1*-epiRILs and with a 1:3 (mut/WT) ratio in the *ddm1*-epiRILs.

[Insert Figure 1 here]

DNA methylation transgenerational stability and its phenotypic consequences

- Understanding of the stability of DNA methylation perturbations

Contrary to mammals, in plants there is no evidence of a consistent global resetting of DNA methylation during development, making the transmission of epialleles over generations more probable. Indeed, it has been shown that the loss of DNA methylation induced by the *ddm1-2* mutation can be stably inherited over many generations once the DDM1 wild-type allele is re-introduced (Kakutani, Munakata, Richards, & Hirochika, 1999). The analysis of the transmission of *ddm1-2* and *met1-3* induced hypomethylation at six TEs, after a cross with wild-type, showed that the hypomethylation is transmitted at some loci and reversed to a wild-type methylation state at other loci (Lippman et al., 2003). Methylated regions have been divided into two categories: (i) those that can form two distinct epialleles that are maintained over generations once in a WT background, and (ii) those that revert to the WT epigenetic state (remethylatable). EpiRILs are a great tool to study the mechanisms and consequences of DNA hypomethylation stability or reversion over generations. Indeed, the analysis of the DNA hypomethylation stability at multiple loci in three *ddm1*-epiRILs and three *met1*-epiRILs confirmed the presence of stable and remethylatable *ddm1*- and *met1*-induced hypomethylation (Reinders et al., 2009; Teixeira et al., 2009). Transgressive DNA methylation patterns have also been observed in these populations. Using bisulfite sequencing it was shown that remethylation to a level similar to wild-type was observed occurring at many loci in all cytosine contexts. This remethylation requires sRNA and factors involved in RNA-directed DNA methylation and is progressive over generations in the *ddm1*-epiRILs (Teixeira et al., 2009), while remethylation has been observed directly occurring in

the F1 in the case of *met1*-induced hypomethylation (Rigal et al., 2016). Further analysis of *cis* factors influencing remethylation in the *met1*-epiRILs as well as in the F2, containing the wild-type allele of MET1, originating from a backcross between *met1-3* and wild-type, showed that remethylation is associated with repetitiveness and relative scarcity of CpGs. In contrast, stable epialleles are associated with low copy number and high CpG content (Catoni et al., 2017). The link between these *cis* factors and the level of epigenetic stability was confirmed in rice (Catoni et al., 2017), and also observed generally associated to the susceptibility of transgenes to be epigenetically silenced (Sidorenko et al., 2017). This observation shows how epiRILs in *Arabidopsis thaliana* could be of great help to identify general rules associated to epiallele stability in different plant species or even for synthetic or heterologous DNA sequences (like transgenes).

- From epialleles to epigenomic recombination maps

The identification of epialleles in epiRILs has been used advantageously to identify the parental origin of genomic regions along the genome, exclusively using DNA methylation information (Colome-Tatche et al., 2012; Reinders et al., 2009). Parental origin was identified for three *met1*-epiRILs using whole-genome methylation analysis (Reinders et al., 2009). Genome-wide DNA methylation data for 123 *ddm1*-epiRILs were also used in order to construct a recombination map derived from 126 epialleles covering 81.9% of the total genome (Colome-Tatche et al., 2012). The genetic length of this map is comparable to those obtained from classical *Arabidopsis* crosses, suggesting that the hypomethylated loci segregating in the *ddm1*-epiRILs do not affect the global meiotic recombination rates. However, it has been seen on a local scale that recombination rates are reduced within

repeat-rich pericentromeric regions and increased in chromosome arms (Colome-Tatche et al., 2012). This remodelling of recombination hotspots, without changing the global rate, was also independently observed using *met1*-epiRILs (Mirouze et al., 2012). A later study shown that this remodelling of local recombination requires genes involved in the redistribution of interfering crossovers (Yelina et al., 2015).

Interestingly, the creation of epigenomic recombination maps using epialleles has also been done using mutation accumulation (MA) lines in *Arabidopsis thaliana* (Hofmeister, Lee, Rohr, Hall, & Schmitz, 2017). MA lines are self-pollinated single-seed descent lines originating from a single founder, such that the lines are nearly genetically identical. MA lines display DNA methylation variation, and more than half of the differentially methylated regions identified in MA lines were stably transmitted in the progeny of a cross between two of them (Hofmeister et al., 2017). The creation of epigenomic recombination maps using stable DNA methylation variation is thus not restricted to epiRILs and will be of great interest to identify epialleles underlying phenotypic variation.

- Epialleles and phenotypic consequences

Knowing that a proportion of DNA methylation perturbations are transmitted through generations in *met1* and *ddm1*-epiRILs, one important question is to define if these can have phenotypic consequences. The two epiRIL populations have been extensively phenotyped for qualitative as well as quantitative traits such as flowering time, biomass or response to biotic and abiotic stresses. Two types of phenotypic variation have been observed. A first type of recessive variation has been observed sporadically occurring in only one epiRIL line and thus arose specifically during the creation of that line (Figure 2). These specific

252 phenotypic changes are unlikely to be transmitted from the parents used in the creation of
253 the epiRIL populations, and it was shown that TE mobilisation impairing gene functions were
254 the cause of such specific phenotypes in the *met1*-epiRIL population (Mirouze et al., 2009).
255 The second type of phenotypic change is affecting a significant proportion of the epiRIL lines
256 and is thus potentially inherited from the parents. We will discuss more in detail this second
257 type of phenotypic change, as they are more likely to be caused by epialleles segregating in
258 the epiRIL populations.
259 [Insert Figure 2 here]
260 One strong phenotype observed in the two epiRIL populations is delayed flowering time,
261 which has been shown to be associated with the hypomethylated epiallele at the *FWA* locus
262 (Johannes et al., 2009; Reinders et al., 2009). However, continuous variation for flowering
263 time is still observed in the *ddm1*-epiRIL population after removing individuals for which late
264 flowering is caused by this *fwa* epiallele (Johannes et al., 2009). This suggests that DNA
265 methylation changes at other loci are also involved in the segregation of this trait in the
266 *ddm1*-epiRIL population.
267 A large proportion of the *met1*-epiRIL population is also characterised with retarded growth
268 (85% of *met1*-epiRILs) as well as delayed germination under elevated salinity (60% of *met1*-
269 epiRILs). Moreover, 34% and 4% of *met1*-epiRILs showed respectively increased resistance
270 or susceptibility to the biotrophic bacterial pathogen *Pseudomonas syringae* pv. tomato
271 (Pst) (Reinders et al., 2009).
272 Given the high number of lines in the *ddm1*-epiRIL population (505 lines), many quantitative
273 traits have been measured in this population and their heritability estimated (i.e. the degree
274 of variation in the phenotypic trait in the population due to genetic, and here epigenetic,
275 variation between individuals). A continuous variation and high heritability have been

observed for several traits such as flowering time, plant height, primary root length, fruit number, total biomass and others (Cortijo et al., 2014; Johannes et al., 2009; Roux et al., 2011). Many traits such as flowering time, plant height, fruit size, dry biomass and rosette diameter have also been measured in common garden experiments, alongside natural accessions of *Arabidopsis thaliana* (Roux et al., 2011). It was found that phenotypic variation in the *ddm1*-epiRIL population displays a level of trait heritability similar to the natural Arabidopsis accessions grown in parallel. Phenotypic plasticity, which is the ability of one genotype to produce multiple phenotypes in response to the environment, has also been measured for flowering time, plant height, fruit number, total biomass and root:shoot ratio in response to drought and nutrient addition (Zhang et al., 2012). A high heritability was observed for these traits in the absence and presence of environmental perturbations, but also for their plasticity (Y. Y. Zhang, Fischer, Colot, & Bossdorf, 2013). Theoretical predictions indicate that these heritability values are consistent with a small number of parentally derived quantitative trait loci (QTL, see definitions). These results suggest that phenotypic variability in the *ddm1*-epiRILs can be caused by the segregation of epialleles, or by DNA sequence polymorphisms caused by mobilisation of transposable elements, reactivated by DNA hypomethylation.

In order to identify the loci underlying heritable phenotypic variability in the *ddm1*-epiRIL population, and to define their genetic or epigenetic origin, epigenetic quantitative trait loci (epiQTL, see definitions) have been mapped in *ddm1*-epiRILs for flowering time and primary root length (Cortijo et al., 2014). This was done taking advantage of a genetic map generated using differentially methylated regions in 123 *ddm1*-epiRILs, and covering 81.9% of the total genome (Colome-Tatche et al., 2012). Several epiQTLs were detected on chromosomes 1, 4 and 5 for flowering time, and on chromosomes 1, 2 and 4 for primary

root length (Figure 3). These QTLs could be associated to epigenetic polymorphisms, but also caused by TE mobilisation. In order to discriminate between these two possibilities, association between DNA methylation status and primary root length was confirmed for the markers at the peak of the three epiQTLs in an independent F3 population. Moreover, new TE mobilisations detected at these epiQTLs in the epiRIL population are not present in this F3 population. These results strongly suggest that changes in DNA methylation are causing the epiQTLs detected for primary root length (Cortijo et al., 2014).

The next step will be to identify the epialleles underlying these epiQTLs. However, as for mapping alleles underlying QTLs, this operation is challenging and will require more time and work. A first step would be to generate a fine mapping population in order to reduce the size of QTLs and thus the number of potential epialleles (Loudet, Gaudon, Trubuil, & Daniel-Vedele, 2005). Once potential epialleles will be detected, manipulating their DNA methylation status will be required in order to confirm the link between DNA methylation and phenotypic variability at this locus. Targeted DNA methylation is still challenging but could be now achieved using a deactivated Cas9 fused with a DNA methyltransferase (Vojta et al., 2016), by VIGS (Bond & Baulcombe, 2015) or by using RNA hairpins to trigger RdDM (Mette, Aufsatz, van der Winden, Matzke, & Matzke, 2000).

However, the complete characterization of epialleles responsible for the identified epiQTL associated to traits of interest is not necessarily a requirement in order to use this knowledge to improve plants. Methods such as marker-assisted selection could be used to introgress the desired trait in the cultivar of interest, taking advantage of markers associated to the identified epiQTL (Kumar et al., 2017). The DNA methylation status of these markers, rather than the DNA sequence polymorphisms, would have to be used during the selection process. Assays based on DNA digestion with enzymes sensitive to DNA

methylation, as for example McrBC (Teixeira et al., 2009), associated to qPCR, would provide a cheap and high throughput approach to perform such selection based on the markers epigenetic status.

[Insert Figure 3 here]

Using epiRILs to understand TE mobilisation

Transposable elements (TEs) are a heterogeneous group of mobile DNA elements integrated in the genome of virtually all organisms, with the ability to move from their original position to a new genomic location. TEs can be classified in two main classes based on their transposition strategy: (i) Class I TEs (or retrotransposons), which transpose with a copy-and-paste mechanism through reverse transcription of a RNA intermediate and (ii) Class II TEs, transposing with a cut-and-paste mechanism mediated by a transposase (Wicker et al., 2007). Although initially considered as selfish genes and assimilated to "junk DNA" (Doolittle & Sapienza, 1980), the importance of the contribution of TEs to gene and genome structure and evolution is currently recognised across the entire tree of life (Hurst & Werren, 2001; Rebollo, Romanish, & Mager, 2012), including plants (Lisch, 2013). Consequently, transcriptional silencing of TEs ensures genetic stability, and is controlled in plants by a network of self-reinforcing epigenetic pathways, marking TEs with repressive marks at the level of DNA (cytosine methylation) and chromatin (histone repressive marks). Therefore, epigenetics mutants often show release of TE expression, and have been used to reveal and study real time TE mobilization (Ito et al., 2011; A. Miura et al., 2001; Tsukahara et al., 2009). In this context, epiRILs represent a valuable alternative to homozygous *met1*, *ddm1* and other epigenetic mutants in studying TE mobilization for several reasons. First, epiRILs are in the wild-type genetic background and are therefore genetically and phenotypically

more stable compared to the mutant from which they derived (Reinders et al., 2009). Moreover, the epiallele segregation and homozygous fixation that occurred through many inbred generations contributed to “dilute” the epialleles with deleterious effects, reducing the amount of developmental defects that are normally displayed in the homozygous mutant. For example, the Arabidopsis *met1-3* mutation is semi-lethal with transgenerational decrease of fitness, and homozygous mutant plants can be maintained viable for a maximum of four generations (Mathieu et al., 2007). Although not as severe as for *met1-3* mutants, *ddm1-1* and *ddm1-2* homozygous mutants accumulate strong phenotypic defects through generations, (Kakutani et al., 1996). Stochastic bursts of several TEs independently occur in different *ddm1* inbred lines, and are contributing to at least some of the developmental phenotypes observed in *ddm1* (A. Miura et al., 2001; Tsukahara et al., 2009). By contrast, *met1* and *ddm1*-derived epiRILs have been maintained for more than eight generations without noticing a significant decrease in fertility (Johannes et al., 2009; Reinders et al., 2009), providing a much more reliable platform to study transposition events. Indeed, the mobilization of the Class II DNA transposon CACTA1 (Reinders et al., 2009) and the Class I retrotransposon EVADE (EVD) (Mirouze et al., 2009) were reported in *met1*-derived epiRILs, while not detected in the *met1-3* mutant. Similarly, many transposons have been found active in *ddm1*-epiRILs, indicating that *ddm1-2* mutation is necessary to release TE silencing, and that TEs can remain active after re-introduction of the DDM1 wilt-type allele (Cortijo et al., 2014; Gilly et al., 2014). In *ddm1*-epiRILs the fraction of the demethylated genome was initially diluted through one *ddm1* backcross of the F1 with the wild-type, reducing in average to 25% the fraction of hypomethylated genome inherited from the *ddm1-2* mutant parent, and contributing to stabilize epiRILs phenotypes at late generations.

Therefore, both *met1* and *ddm1* derived epiRIL populations demonstrated a longer transgenerational viability and stability compared to the mutant parents from which they are derived. The advantage of this condition is that the plethora of epiallelic effects and multiple TE activation observed in the homozygous mutants can be isolated in independent epiRILs, making it possible to study the activation and *de novo* silencing of independent TEs in real time experiments. For example, the transgenerational dynamic evolution of EVD mobilization was studied in inbred epiRILs (Mari-Ordonez et al., 2013). The EVD burst and its *de novo* silencing was reconstructed in a *met1*-epiRIL, observing that efficient silencing is associated to a change in small RNA composition, and consistently occurs approximately at the 14th generation after EVD activation, when its copy number in the genome reaches a threshold of 40 copies (Mari-Ordonez et al., 2013).

Although the first events of real time transposition were discovered in maize more than 50 years ago (Mc Clintock, 1950), the impact of TE mobilization on genome stability and the biology of complex organisms is still poorly investigated, and essentially extrapolated from comparative genomics and phylogenetic studies. This limitation is the direct consequence of the rarity of TE mobilization events so far observed in nature, likely due to the epigenetic silencing normally associated to repeated DNA sequences.

The most evident effect of TE mobilization is the recessive mutation of genes with a new TE insertion occurring in their coding region, in many cases producing a visible phenotype. Nonetheless, phylogenetic studies produced evidence of several TE-induced non-destructive effects on gene expression responsible for agricultural important traits in crops (Lisch, 2013). It is however unclear if these non-destructive effects derived from positively selected exceptional aberrant transposition events or are the result of transposition strategies of different TE families. In this scenario, epiRILs offer the opportunity to identify and

characterize new active TEs, and to study the impact of their real time mobilization across generations in a limited number of plant lines. Therefore, the study of epiRILs may contribute to elucidate the role of TE on genetic and biology in higher plants, and more generally in eukaryotic multicellular organisms.

Heterosis

Heterosis, or hybrid vigour, is a phenomenon describing the improved phenotype of a hybrid offspring compared to the average of both parents, first recorded by Charles Darwin in 1876 (Darwin, 1876). In agriculture, heterosis has been adopted as a routine strategy for plant breeding, leading to improved biomass, yield or resistance to biotic and abiotic stimuli in hybrids (Baranwal, Mikkilineni, Zehr, Tyagi, & Kapoor, 2012). Despite such an extensive use in agriculture, the underlying mechanisms of heterosis are still poorly understood. Traditionally, it is generally accepted that heterosis directly correlates with the level of genetic distance between the two parents (Birchler, Yao, Chudalayandi, Vaiman, & Veitia, 2010). However, more recent experiments performed in Arabidopsis have shown that hybrids generated from accessions with very similar genome can also display a high level of hybrid vigour (Groszmann, Greaves, Fujimoto, Peacock, & Dennis, 2013; Schneeberger et al., 2011), suggesting that epigenetic differences could also contribute to heterosis (Figure 4).

[Insert Figure 4 here]

Indeed, changes in small RNA level and DNA methylation have been associated to hybrid vigour in both interspecific (i.e. between species) or intraspecific (i.e. between accessions) hybrids systems studied in Arabidopsis (Greaves et al., 2012; Groszmann et al., 2011; Shen et al., 2012) and other plant species, including rice (Chodavarapu et al., 2012; G. He, He, & Deng, 2013), maize (Barber et al., 2012; G. He, Chen, et al., 2013), wheat (Kenan-Eichler et

al., 2011) and tomato (Shivaprasad, Dunn, Santos, Bassett, & Baulcombe, 2012). However, the coexistence of genetic and epigenetic differences in hybrids makes it intrinsically difficult to quantify the epigenetic contribution to heterosis.

In contrast, epiRILs are isogenic to wild-type but differ at localized hypomethylated chromosomal areas. Interestingly, some lines from both *met1*-derived and *ddm1*-derived epiRIL populations displayed increased biomass or higher resistance to a pathogen if compared to wild-type Columbia-0 accession (Johannes et al., 2009; Reinders et al., 2009), similar to what is observed in heterotic hybrids. These results suggest that epigenetic variation by itself might be involved in the generation of hybrid vigour.

In a recent work, heterosis for growth-related traits was investigated in epigenetic hybrids generated by pollinating *met1*-derived epiRIL plants with pollen from their isogenic wild-type line (Col-0) (Dapp et al., 2015). In the case of one *met1*-derived epiRIL (epi31), a consistent and reproducible increase in rosette size was observed in F1 plants compared to both parental lines. Remarkably, epi31 displayed a clear parent-of-origin effect on hybrid vigour, as also observed in certain crosses between Arabidopsis accessions (Barth, Busimi, Friedrich Utz, & Melchinger, 2003; Meyer, Torjek, Becher, & Altmann, 2004). Although the authors could not associate any change in gene expression with the hybrid vigour observed, several additive, dominant and transgressive (see definitions) transcripts have been identify in the F1 hybrids (Dapp et al., 2015), supporting the existence of multiple scenarios for DNA methylation-mediated gene regulation in epi-hybrids.

More recently, the contribution of differences in parental methylation to heterosis was quantified measuring six different traits in a larger panel of over 500 *A. thaliana* epi-hybrids obtained starting from *ddm1*-derived epiRILs (Lauss et al., 2018). Several positive and negative heterotic effects were documented, and specific differentially methylated regions

444 in parental genomes were associated with heterotic phenotypes observed in nineteen epi-
445 hybrids (Lauss et al., 2018).
446 In conclusion, there is growing evidence supporting the epigenetic contribution to heterosis.
447 In this context, epiRILs may be the optimal tool to isolate and characterize epigenetic
448 determinants of hybrid vigour, for example by mapping epiQTLs associated to different
449 favourable traits. In addition, altering the epigenetic landscape of parents can potentially
450 increase the heterotic effect of hybrids, and could be used as a tool to increase plant
451 productivity.
452
453 **Challenges with crops**
454 The investigation of the epigenetic landscape in Arabidopsis epiRILs critically contributed to
455 reveal general plant epigenetic proprieties and mechanisms. Such findings include the
456 mapping of epiQTLs (Cortijo et al., 2014), the discovery of genetic proprieties that predict
457 epialleles, common in Arabidopsis and rice (Catoni et al., 2017), and a model for origin and
458 evolutionary consequences of gene body DNA methylation in Angiosperms (Bewick et al.,
459 2016). However, despite a general conservation of most epigenetic factors and proprieties
460 across plants, epiRILs are so far only available for *Arabidopsis thaliana*. Creating epiRILs in
461 crops could improve our understanding of the source of epiallelic creation and also help
462 detecting epialleles with potential agronomic advantages.
463 The introduction in crops of a level of epigenetic variation similar to that observed in
464 Arabidopsis epiRILs might be of great interest for agriculture. Especially when considering
465 that crops have larger genomes containing a much higher number of transposons and
466 repetitive DNA, suggesting an elevated potential for the generation of epialleles.
467 Consistently, rice, maize and tomato mutants in components of epigenetic regulation

display strong developmental phenotypes and partial or complete infertility (Gouil & Baulcombe, 2016; Hu et al., 2014; Li et al., 2014). Remarkably, developmental phenotypes described in crop epigenetic mutants do not correlate with extensive genome hypomethylation as observed in Arabidopsis (Mathieu et al., 2007), suggesting that in most plants small perturbations of the methylome have stronger deleterious phenotypic effects than in Arabidopsis.

Taking this into account, the generation of crop epiRILs may be impaired by the inability of producing viable hypomethylted mutants required for the initial cross. However, alternative strategies should be considered to induce stable epiallele formation without affecting plant viability (Figure 5).

[Insert Figure 5 here]

One possibility to reduce genome methylation is the use of hypomethylated partial loss-of-function epigenetic mutants with mitigated deleterious developmental phenotypes. In Arabidopsis, while the null *met1-3* allele causes complete loss of CpG methylation and is semi-lethal (Mathieu et al., 2007), the partially functional MET1 protein produced in the *met1-1* allele can retain CpG methylation in approximately one quarter of the genome, causing only minor developmental defects and allowing transgenerational conservation of the *met1-1* mutation in the homozygous form (Kankel et al., 2003). In addition, mobilization of TEs has also been observed in the *met1-1* mutant background (Griffiths, Catoni, Iwasaki, & Paszkowski, 2018) as well as the formation of epialleles that are stably maintained for several generations after transgenic complementation with a wild-type MET1 allele (Catoni et al., 2017). This suggests that the use of partial loss-of-function mutants might replace null alleles in epiRIL construction, if a viable knock-out mutant cannot be obtained. However, the production of partial-loss of function mutants for a chosen gene may be difficult to achieve

492 in plants, and is normally associated to fortuitous screening starting from random
493 mutagenized populations. Nonetheless, DNA editing strategies, such as CRISPR/ CAS9 (Cong
494 et al., 2013) and TALEN (Miller et al., 2011) have been successfully extended to plants,
495 allowing an unprecedented high level of accuracy in targeting chromosomal sequences to
496 induce mutations (Malzahn, Lowder, & Qi, 2017). Using these approaches, the effect of well
497 know partial loss-of-function mutations observed in Arabidopsis might be more easily
498 obtained in the species of interest by targeting a similar mutation in the corresponding
499 homologous genes.
500 Alternatively, passive DNA hypomethylation has been proposed to occur during
501 gametogenesis in heterozygous *met1* mutant. The haploid male and female gametophytes
502 undergo two and three post-meiotic divisions, respectively. Therefore, genomic DNA is
503 duplicated in gametophytes with the *met1* mutant allele, in absence of the MET1
504 methylation maintenance system, leading to the passive reduction to 50% and 75% of the
505 genome methylation respectively in male and female gametes (Saze et al., 2003). This
506 hypothesis was confirmed by later studies, observing also a genome-wide demethylation
507 and the formation of stable epialleles in heterozygous inbred *met1* mutant lines, similar to
508 what was observed in epiRILs (Catoni et al., 2017; Stroud, Greenberg, Feng, Bernatavichute,
509 & Jacobsen, 2013). Therefore, genome-wide hypomethylation in crop plants may be simply
510 achieved by inbreeding the usually more fertile heterozygous *met1* mutant, without the
511 necessity of a viable homozygous mutant allele.
512 One alternative to the generation of epigenetic mutants is the use of drugs interfering with
513 epigenetic pathways. Inhibitors of DNA methylases, such as 5-Azacytidine and Zebularine,
514 have been successfully used to induce DNA demethylation in plants (Griffin, Niederhuth, &
515 Schmitz, 2016; Pecinka & Liu, 2014), including crops (Sano, Kamada, Youssefian, Katsumi, &

Wabiko, 1990; Santos et al., 2002; Zhu et al., 2018). Although most of hypomethylation and transcriptional changes induced by these drugs are only transient (Baubec, Pecinka, Rozhon, & Mittelsten Scheid, 2009), transgenerational effects have been observed in rice treated with 5-Azacytidine (Sano et al., 1990). Recently, simultaneous application of Zebularine and the RNA polymerase II inhibitor α-amanitin on Arabidopsis wild-type seedlings was sufficient to mobilize the heat-responsive Class I retrotransposon ONSEN, demonstrating that drug application can efficiently release transposon transcriptional silencing (Thieme et al., 2017). Finally, another very valuable alternative in order to reduce DNA methylation in plant is the heterologous expression of enzymes promoting DNA hypomethylation. For example, the human Ten-eleven translocation (TET) methylcytosine dioxygenases are an enzyme family catalysing the conversion of 5mC in 5-hydroxymethylcytosine (5hmC), and are involved in active DNA demethylation in embryonic stem cells (Tahiliani et al., 2009). The transgenic expression of TET3 catalytic subunit in Arabidopsis was enough to decrease DNA methylation at ribosomal repeats (Hollwey, Watson, & Meyer, 2016). In addition, the transgenic expression of the same TET3 gene in Tomato induced hypomethylation and ectopic expression of the CEN1.1 gene in leaves, promoting vegetative growth (Hollwey, Out, Watson, Heidmann, & Meyer, 2017). In a more recent work, ectopic overexpression of a different TET gene in Arabidopsis induced widespread DNA demethylation and phenotypic variations, mimicking the effects of *met1* mutation (Ji et al., 2018). In addition, a Cas9-based targeted demethylation system using the TET1 catalytic subunit was recently generated and was shown to be able to target demethylation and activate gene expression when directed to known switchable epialleles in Arabidopis (Gallego-Bartolomé et al., 2018).

The combination of these approaches could thus potentially be used in order to promote global or specific changes in DNA methylation profiles and be the first step to create epiRILs in crops.

Conclusion

Arabidopsis epiRIL populations have allowed major advances in understanding the genetic determinant controlling DNA methylation stability as well as mechanisms involved in the transgenerational transmission of epigenetic information. Several studies used epiRILs to highlight the phenotypic consequences of epiallele segregation and the epigenetic contribution to quantitative traits. While epiRILs have been initially created with the intention of minimising DNA polymorphisms, the TE reactivation induced by the global loss of DNA methylation has been used advantageously in order to better understand how TE mobilisation is controlled, and to study the transgenerational effect of TE activation. EpiRILs have also helped to better understand the importance of DNA methylation on heterosis, commonly used in crops to improve yield.

The next step to extend the epigenetic potential to improve agricultural traits will be the creation of epiRILs in crops. This step is challenged by the amount of developmental defects associated to genome wide hypomethylation observed in epigenetic mutants. Nonetheless, the better understanding of the epigenetic contribution to phenotypes, and the use of more sophisticated genome editing stategies might be critical to successfully obtain crop epiRILs in the near future.

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Figure legends:

Table 1: Non-exhaustive list of known epialleles in plants.

Figure 1: Allelic and epiallelic segregation in RIL and epiRIL populations.

RIL populations (left) are usually created by crossing two distinct Arabidopsis accessions that are different in their genomes (depicted with different chromosome colours) and epigenomes (depicted as full or empty dots beside chromosomes). Alleles and epialleles are thus segregating in F2 population derived by this cross, and fixed in homozygous form by self-pollination and single seed-descend. By contrast, epiRILs (right) are created by crossing parents that have identical (or almost identical) genomic sequence but different DNA methylation profiles. This is obtained in Arabidopsis by mutation of *MET1* or *DDM1* genes (represented by a red horizontal line on chromosome sequence), coding for factors involved in DNA methylation maintenance. During the generation of epiRILs, only F2 plants with a MET1 or DDM1 wild-type allele are carried out, to avoid new events of genome wide hypomethylation. EpiRILs are thus maximising epialleles segregation, while reducing (if not removing completely) allelic segregation.

Figure 2: Origin of phenotypic changes observed in epiRILs.

Phenotypic changes occurring in epiRILs are of two types. The first type (left) is sporadic and recessive and occurring specifically in one line, probably caused by TE mobilisation or other genetic mutation. These phenotypes are unlikely to be transmitted from the parents used in the creation of the epiRIL populations. The second type of phenotypic changes (right) appears on a significant proportion of epiRIL lines. These traits are potentially inherited from the parents and likely caused by epialleles segregating in the epiRIL populations.

Figure 3: Principle of epiQTL mapping in epiRILs for root length, followed by epiallele identification and validation.

In order to identify epiQTLs for a quantitative trait, every line of the population is phenotyped (top left) and epigenotyped (top right). EpiQTLs are then identified by measuring the co-segregation of phenotype and epigenotype. Several QTLs were identified on chromosomes 1, 2 and 4 for root length in the *ddm1*-derived epiRILs (middle). The next step is to identify epialleles underlying epiQTLs and to validate them by changing their DNA methylation level (bottom).

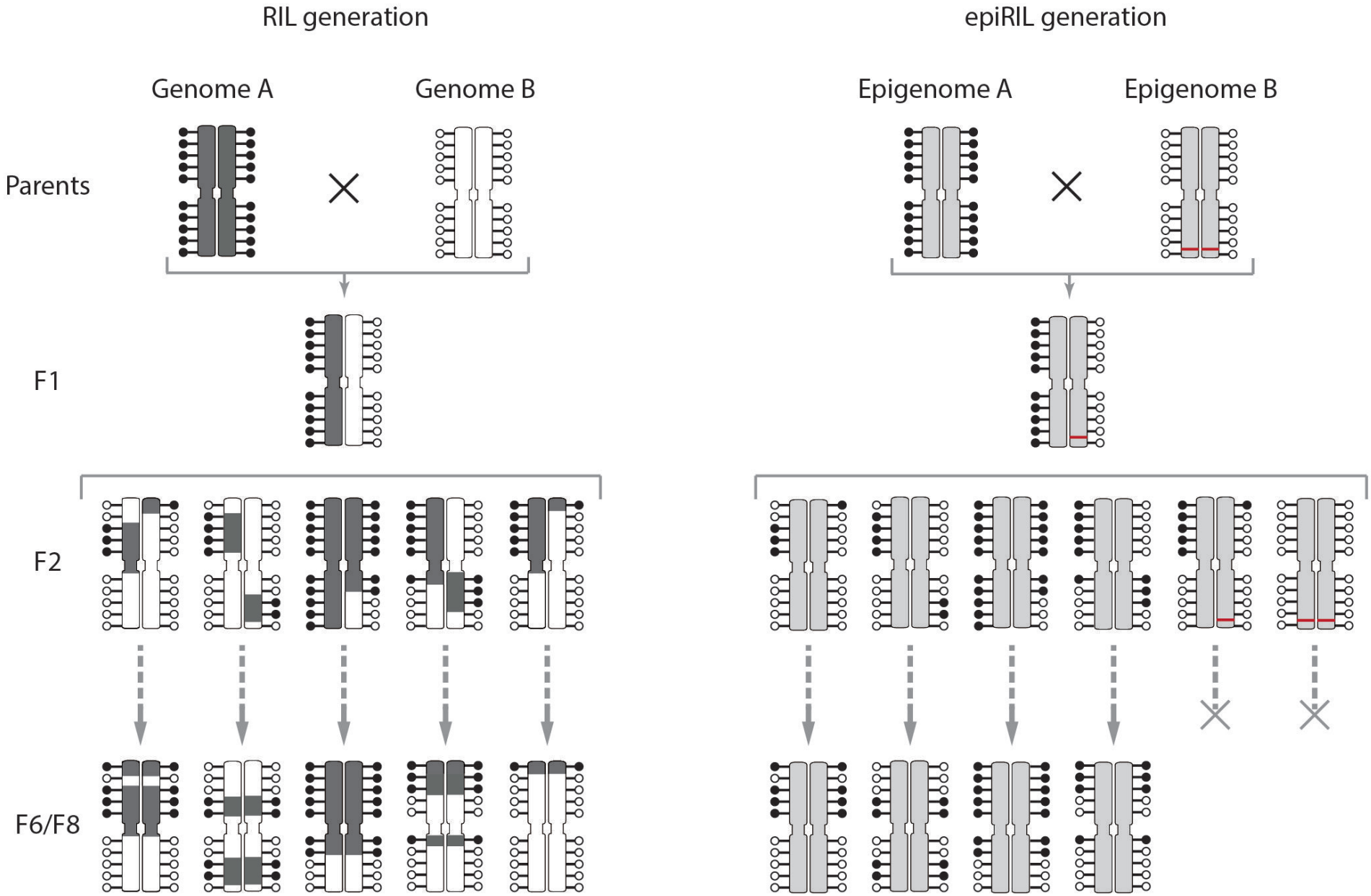
Figure 4: Comparison of epi-hybrid and intraspecies hybrid in *Arabidopsis thaliana*.

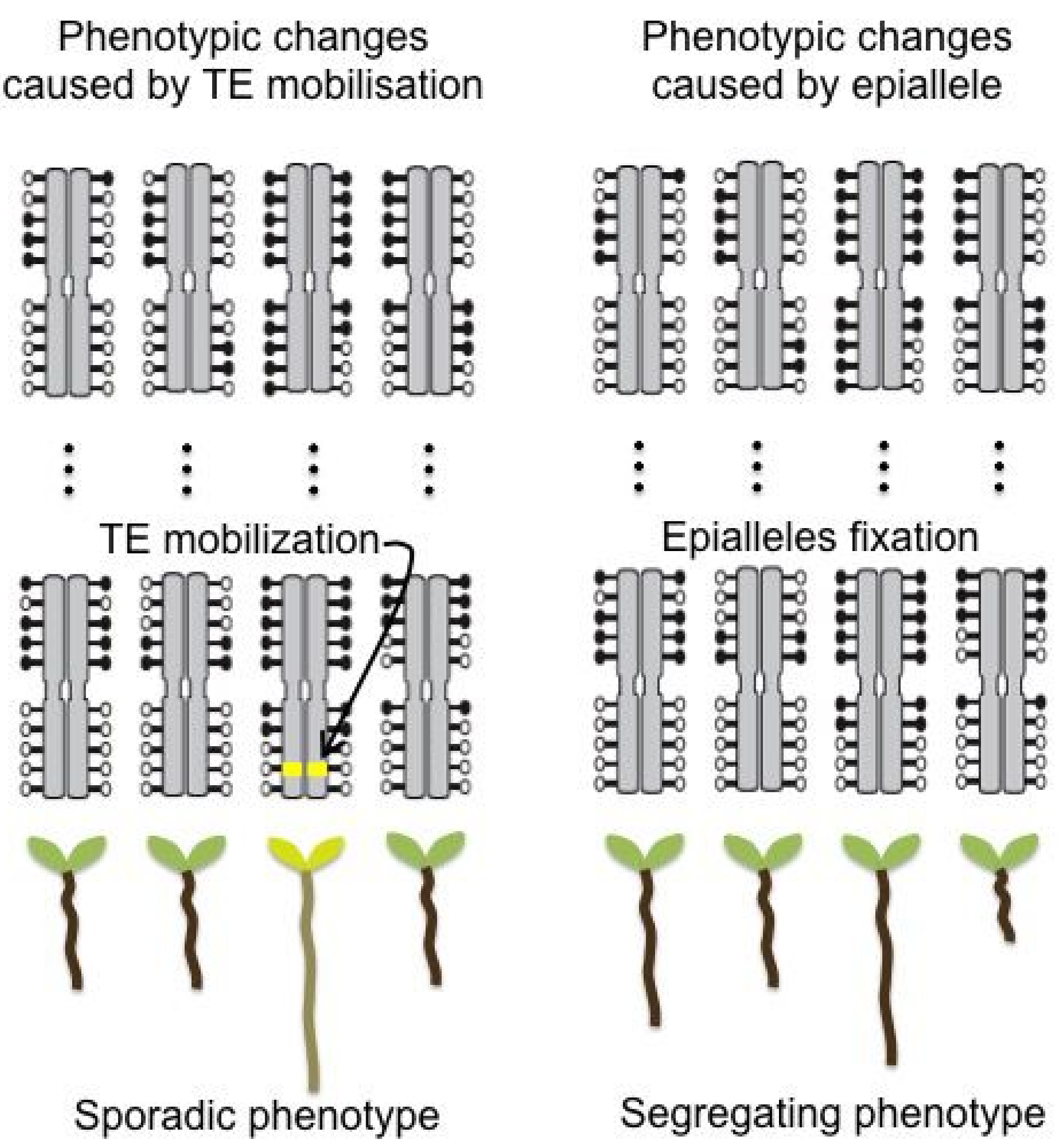
Examples on enhanced vigour in an epi-hybrid, compared with its two parents, epi31 and wild-type Col-0 (top), and in an intraspecies hybrid compared to its two parent accessions, Col-0 and C24 (bottom). In both cased, the epi-hybrid and the intraspecies hybrid are bigger than their parents, indicating a heterotic effect.

Figure 5: Different approaches to induce global DNA demethylation in order to create epiRIL populations.

In wild-type, DNA maintenance mechanisms ensure conservation of epigenetic marks (i.e. DNA methylation, represented as black dots). In *met1* or *ddm1* knock-out mutants, DNA methylation is strongly impaired and normally associated to strong developmental phenotype. Alternative strategies to reduce DNA methylation limiting the impact on plant fitness include the use of partial loss-of-function mutations with partial de-methylation; the self-propagation of heterozygous knock-out mutants, resulting in gametophyte

hypomethylation; the application of drugs interfering with methyltransferase activity; and the ectopic overexpression of TET methylcytosine dioxygenases.


RIL generation
Genome A
Genome B
Parents
F1
F2
F6/F8
epiRIL generation
Epigenome A
Epigenome B

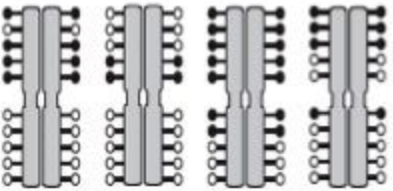

Phenotypic changes caused by TE mobilisation
Phenotypic changes caused by epiallele
TE mobilization
Epialleles fixation
Sporadic phenotype
Segregating phenotype

Phenotype

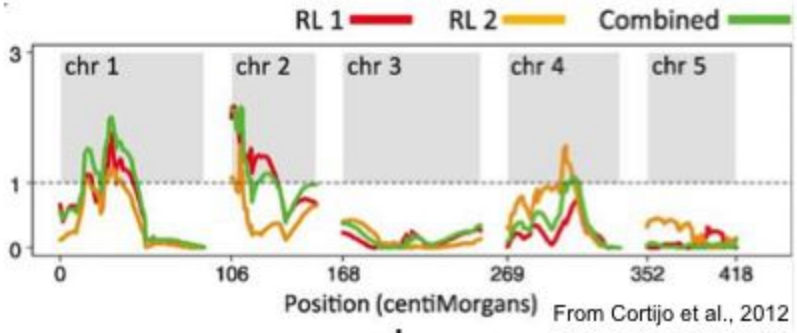
Line 1 Line 2 Line 3 Line 4 ...

Epigenotype

Line 1 Line 2 Line 3 Line 4 ...



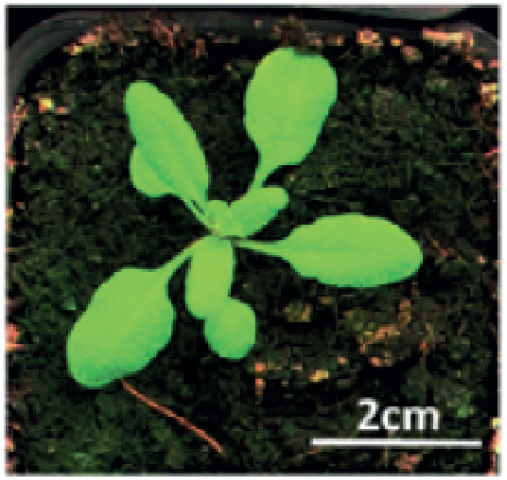
epiQTL mapping



From Cortijo et al., 2012

Epiallele identification and validation

WT (Col-0)

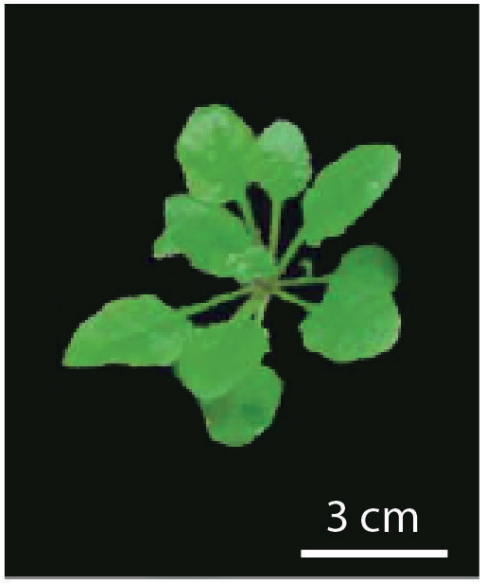


epi31 x WT

epi31 (Col-0)

From Dapp et al. 2015

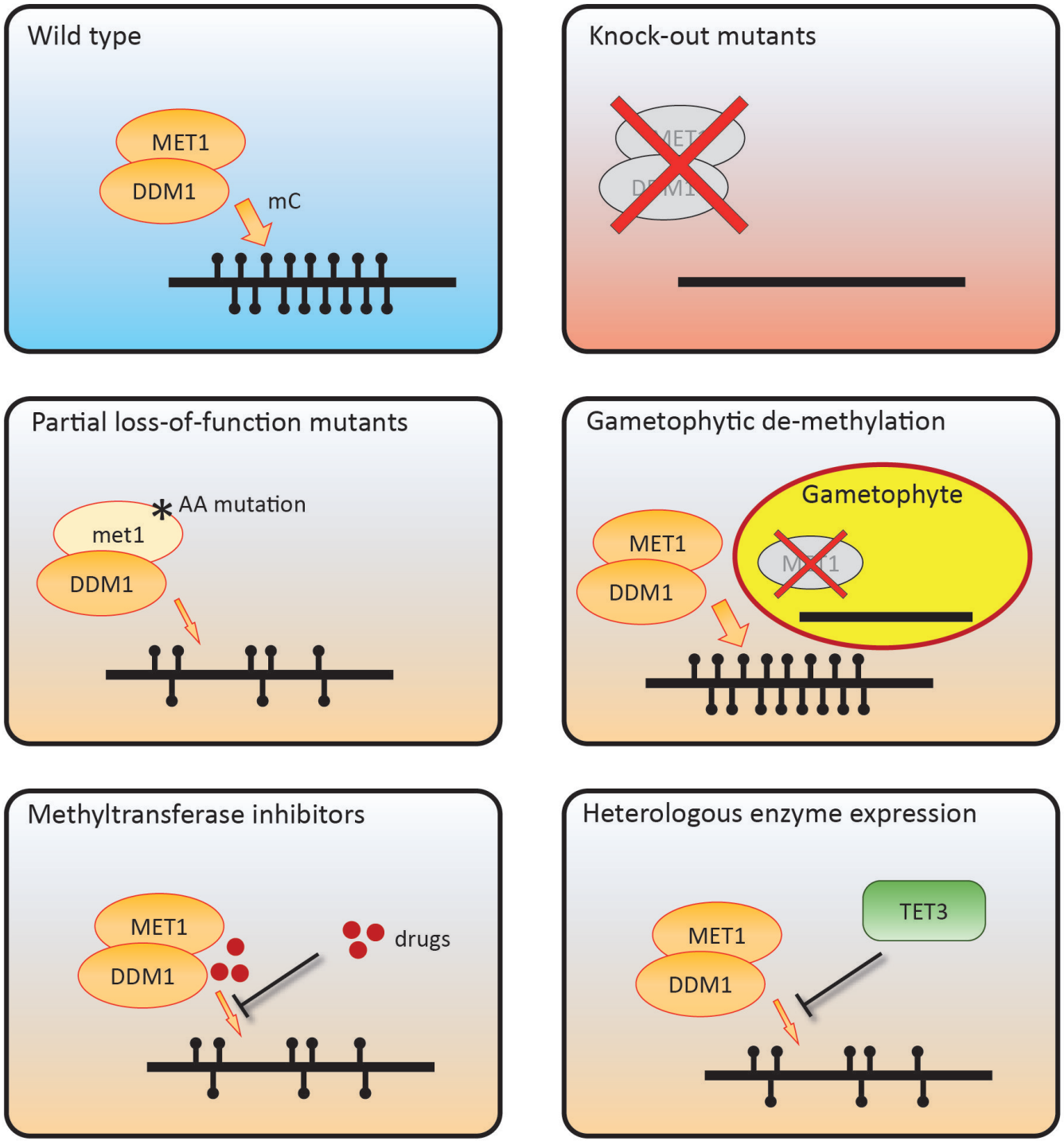
Col-0



Col-0 x C24

C24

From Chen 2010


Wild type
MET1
DDM1
mC
Knock-out mutants
Partial loss-of-function mutants
AA mutation
met1
DDM1
Gametophytic de-methylation
Gametophyte
MET1
DDM1
Methyltransferase inhibitors
MET1
DDM1
drugs
Heterologous enzyme expression
TET3
MET1
DDM1