Chapter 2
Plant Epigenetic Mechanisms in Response to Biotic Stress

Michael R. Roberts and Ana López Sánchez

Abstract The environment changes faster than the ability of genetic mutation and recombination to generate natural genetic diversity. In this context, epigenetic regulation of gene expression has the potential to provide organisms with an alternative mechanism for phenotypic variation by controlling the extent of plasticity that can be achieved in response to environmental changes. There is now substantial evidence suggesting roles for epigenetic regulation of several different aspects of the plant response to biotic stress. At the basic level of gene expression, posttranscriptional gene silencing mediated by small RNAs and chromatin remodelling controlling transcriptional gene silencing are essential for the induced resistance responses activated during pest and pathogen attack. Beyond this, there is also evidence that histone modifications and DNA methylation are associated with immune memory, or defence priming, such as systemic acquired resistance (SAR). In addition, recent evidence indicates that epigenetic modifications can also generate longer-term defence priming responses that can be inherited across generations. In this chapter, we will discuss the roles of epigenetics in these different modes of biotic stress resistance, and suggest ways in which we may in the future be able to exploit epigenetic systems for crop protection.

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R. Alvarez-Venegas et al. (eds.), *Epigenetics in Plants of Agronomic Importance: Fundamentals and Applications*, https://doi.org/10.1007/978-3-030-14760-0_2

2.1 Introduction

Like all living organisms, plants need to adapt to environmental changes in order to persist. Until the last decades, neo-Darwinian evolutionary theories assigned the origin of phenotypic variability to a set of characteristics determined by the genetic information. In the case of facing a change in the environment, different individuals within a population will have differential survival, depending of their characteristics, that will determine the genetic, and therefore phenotypic features of the new generations (Pigllucci 1996). Surprisingly, some recent observations lead different experts to claim for an implementation of this now classical new Darwinian perspective (Rando and Verstrepen 2007). For example, mutation rates are usually slower than environmental changes, and the phenotypic plasticity observed in natural populations wider than genetic variability. Therefore, an extra source of phenotypic plasticity is expected (Grativol et al. 2012). On the other hand, some environmentally induced adapted states seem to be relatively stable or even inherited for few generations without involving a change in the genetic information. This extra layer of relatively stable phenotypic plasticity has also been called epigenetic buffering (understood as 'something' beyond genetics). Epigenetic buffering could have a special importance in the case of plants, which because of their sessile nature, face threats to their survival and fitness from biotic stresses (O'Dea et al. 2016).

Against a pathogen attack, plants counter with a broad range of defence mechanisms. Plants possess some constitutive barriers to protect themselves against potential pathogens that are usually effective against a variety of microbes (Malinovsky et al. 2014). Along their evolutionary arms race for survival, pathogens have developed several strategies to overcome those defences and produce infections. In response to those, plants are able to actively induce defences when they identify a microbe or herbivore as a threat (Jones and Dangl 2006). This is associated with a reprogramming of gene expression. In some cases, once they have suffered a stress that induced their defences, plants are able to remember this first stress encounter. Then, in the case of recurrent stresses, the induced responses are faster and stronger (usually more effective). This is the concept of priming of defence responses, which involves a different control of gene expression and it is inevitably associated with a memory of the stress (Prime-A-Plant Group et al. 2006). Waddington in 1942 coined the term epigenetics to describe the study of phenomena in which the phenotypes observed in nature cannot be explained just by the understanding of their genotypes (Waddington 2012). He was studying development. At that time, the scientific community understood the genome as packages of information encoding specific features, but the control of that information (gene expression control) was an unknown. Of course, they could not imagine that part of the information stored in the genome works, in fact, by controlling gene expression (regulatory regions, transcriptional factors, etc.) and it is therefore genetic. Along the last decades, epigenetics has been redefined as phenotypic changes that can be transmitted through mitotic or even meiotic divisions in the absence of changes in the DNA sequence. Thus, epigenetics is associated with the control of gene

expression and certain memory. Importantly, some of the initially considered epigenetic mechanisms have been found to be encoded in the genome (miRNAs, chromatin modellers, histone variants, etc.). However, at all stages of induced immunity, the considered epigenetic mechanisms have been demonstrated to be of a key importance.

In this chapter, we will first introduce the different epigenetic mechanisms. We will focus on their role in controlling gene expression at the transcriptional and posttranscriptional level, contextualizing them by the use of some examples of their involvement in plant response to pest and diseases. Then, we will dedicate a section to assess the role of epigenetics in the memory of the stress and priming of defence responses. We will present and discuss publications supporting the role of epigenetic mechanisms in priming at different timescales, from short to long periods of time or even *trans*-generationally. Finally, we will summarize and discuss the potential application of epigenetics in the development of alternative programs for plant protection. We believe, this integrated view of epigenetics in plant defence and priming could be inspiring for a new generation of plant scientists aiming to understand plant defence mechanisms, as well as to develop alternative, hopefully more effective and sustainable, crop protection strategies.

2.2 Epigenetic Mechanisms Involved in Plant Defence

As originally defined, epigenetics allows plants to show different phenotypes with the same genotype. The underlying question for years was how? Nowadays, we know that the majority of what are considered epigenetic mechanisms are centred on the control of gene expression. From this perspective, the so-called epigenetic buffering, which is used to explain the extra source of phenotypic plasticity, does not involve a change in the information itself, but the different observed phenotypes are a consequence of modifying the speed and intensity at which genes are expressed (Grativol et al. 2012). Although epigenetics has traditionally been associated with repression of gene expression (reason why they are called ‘silencing’ mechanisms), we now also know of mechanisms considered as epigenetic that are able to promote or facilitate gene expression (Eamens et al. 2008; Matzke and Matzke 2000). This gene expression control can be imposed at either the transcriptional or posttranscriptional level. For this reason, epigenetic mechanisms are typically classified in two groups: those controlling gene expression at transcriptional level, known as ‘transcriptional gene silencing mechanisms’ (TGS), and those controlling gene expression at posttranscriptional level, ‘posttranscriptional gene silencing mechanisms’ (PTGS).

2.2.1 *Posttranscriptional Control (PTGS): The Role of Small RNAs*

In general terms, the main part of posttranscriptional epigenetic control is associated with the action of small RNAs (sRNA). Despite the fact that sRNAs pathways play important roles in developmental processes, they seem to have evolved originally from a defence mechanism against viruses and transposable elements to later start silencing endogenous genes (Borges and Martienssen 2015; Matzke and Matzke 2000). The first reports of epigenetic mechanisms in plant defence were examples of posttranscriptional gene silencing. Actually, reports of plant defence and priming processes as part of the cross-protection observed against viruses in the 1920s could be considered the very first recognized examples of epigenetic posttranscriptional gene silencing (Ross 1961; Waterhouse et al. 2001). They described how infections with relatively avirulent virus strains can induce protection against related virulent viruses. Unfortunately, at that time, the mechanisms were far from being discovered, and remained unknown for decades. It was not until the late 1980s that the first examples of posttranscriptional gene silencing mechanisms emerged, during experiments by plant biotechnologists trying to alter the colour of petunia flowers (Eamens et al. 2008). They observed how the expression of a transgene could trigger the silencing of the transgene and also homologous endogenous genes, by mechanisms involving small RNAs. Today we know both observations were related. It is generally accepted that all the epigenetic mechanisms involving RNA intermediates were originally part of the plant defence mechanisms against viruses. Virus transcription and replication is carried out inside the host cell, so the plants evolved the ability to detect exogenous nucleic acids as a potential threat (Waterhouse et al. 2001). Thus, plants first developed a system to defend themselves against viruses (exogenous RNAs). Then, the system was adapted to an endogenous control for gene expression and genome stability (controlling the movement of transposable elements—TEs—that are similar to viruses). That is the reason why advances in sRNA-mediated silencing mechanisms and plant defence have been feeding from each other during decades.

Derived from this original antiviral function, all small RNAs possess common features in their biogenesis. For example, most of the small RNAs required a longer double-stranded RNA (dsRNA) precursor molecule. This could be due to the fact that the 90% of plant viruses depend on a dsRNA molecule for their replication (Waterhouse et al. 2001). The dsRNAs are processed by DICER-LIKE proteins (DCLs) to generate fragments of 21–24 nt length. These molecules are then 2′-*O*-methylated by the protein HUA ENHANCER 1 (HEN1) at the 3′ end, which is a specific aspect of plant sRNAs (Yang et al. 2006). Finally, mature sRNAs are loaded into ARGONAUTE (AGO) proteins which can interact with other proteins to form the RNA-induced silencing complexes (RISCs; Fang and Qi 2016). In posttranscriptional gene silencing mechanisms, RISC complexes find the target mRNA by base pairing and affect its stability through mRNA cleavage (degrading the target mRNA), or repress its translation.

2.2.1.1 Silencing of Exogenous RNAs. The Special Case of Viruses

Due to its evolutionary origin, the majority of antiviral defences are triggered by exogenous RNAs (viral RNAs). Viral infection activates epigenetic mechanisms in order to destroy or silence the invading viral genome (Waterhouse et al. 2001). In Fig. 2.1 we represent a summary of the different silencing mechanisms against viruses as discovered in *Arabidopsis* (adapted from Ruiz-Ferrer and Voinnet 2009). These biological roles of the silencing components were mainly deciphered by analysing plant defective mutants (reviewed in Katiyar-Agarwal and Jin 2010; Seo et al. 2013). For RNA viruses (the majority of plant viruses), the viral genome is replicated by a viral replicase to generate a dsRNA molecule (Fig. 2.1a). This dsRNA would be processed by plant DCL proteins to trigger the production of sRNAs that would lead to viral silencing by degrading the dsRNA replication intermediates, the viral transcripts or impeding their translation. Viral transcripts (single-stranded RNAs—ssRNAs) can also trigger silencing by being copied to a dsRNA molecule by plant RNA-dependent RNA polymerases (RDR proteins), feeding the system. Those RDR proteins are also involved in the silencing of the viruses which have ssRNA genomes. In the case of the DNA viruses (Geminivirus—family Geminiviridae—for instance), the viral transcripts are copied to dsRNA by the plant RDR2 protein which in this case would mediate the production of sRNAs similar to the heterochromatic sRNAs, triggering the silencing of the virus at transcriptional level (Fig. 2.1b, and further discussed in Sect. 2.2; Raja et al. 2008). Moreover, viruses spread through the plant using the vasculature (Hipper et al. 2013). In this respect, a striking property of the sRNAs is their cell-to-cell mobility, increasing the efficiency of the silencing mechanism. Once the silencing is locally triggered in the

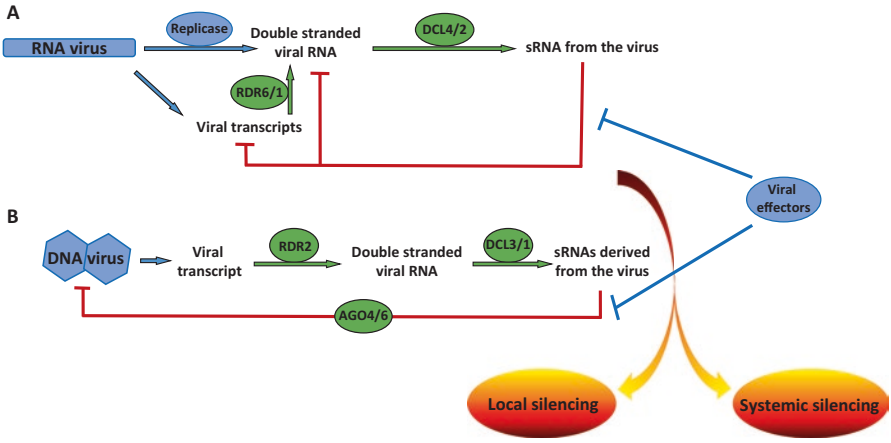

Fig. 2.1 Epigenetic mechanisms controlling viral infection. Adapted from Ruiz-Ferrer and Voinnet (2009). Epigenetic mechanisms against RNA (**a**) and DNA (**b**) virus. Pathogen components are highlighted in blue. Plant proteins are represented in green. Plant defence as a process is represented with red and orange colours

infected tissue, the sRNAs can travel through the plant xylem and phloem system and provide systemic resistance by silencing targets in distal parts of the plant (Chitwood and Timmermans 2010; Kalantidis et al. 2008). This systemic silencing has been broadly used by biotechnologists for decades, but more importantly, it has positioned the sRNAs as good candidates to be the mobile signal involved in some systemic resistance processes (Voinnet 2005).

2.2.1.2 Endogenous RNAs: From an Antiviral Defence to the Control of Endogenous Sequences

From these early mechanisms designed as antiviral defences, plants (and other organisms) evolved the capacity to use the silencing machinery in controlling other sequences (Waterhouse et al. 2001). This evolution leads to the sRNA-mediated regulation of endogenous genes and the suppression of transposable element (TE) movement. Nowadays there is an increasing number of different sRNAs recognized (Borges and Martienssen 2015). Several classifications have been proposed. On the basis of their biogenesis, we can consider two major classes: small interfering RNAs (siRNAs) and micro RNAs (miRNAs). Maybe the better-known of the two are the miRNAs, which are typically 20–22 nt length, transcribed by RNA polymerase II (Pol II) and processed by DCL1. Among the endogenous siRNAs there are many different subclasses. On the one hand, there are the hairpin-derived siRNAs (hp-siRNAs) and natural antisense siRNAs (natsiRNAs), both 21–24 nt length. On the other hand, the 'secondary siRNAs', including *trans*-acting siRNAs (tasiRNAs), phased siRNAs (phasiRNAs), epigenetically activated siRNAs (easiRNAs) and the long siRNAs (lsiRNAs). The lsiRNAs represent a class of endogenous siRNAs identified specifically in plant–pathogen interactions with a length of 30–40 nt. Finally, plants also produce heterochromatic siRNAs (hetsiRNAs), which from a classical view of the pathway are 24 nt length and generated by the plant-specific RNA-dependent DNA polymerase IV (Pol IV). hetsiRNAs are involved in transcriptional gene silencing, so they will be addressed later (see Sect. 2.2). Both miRNA and non-heterochromatic siRNAs bind the RISCs complexes and find the target mRNA (in this case a plant gene transcript), by base pairing. In the same way that the viral sRNAs are eliminated, the control of the target gene is achieved by mRNA cleavage (degrading the target mRNA) or repression of its translation.

The majority of the sRNA classes have been demonstrated to play a role in the control of plant defence at almost all stages. In general terms, once the pathogen is recognized by the plant, the induced defence process includes some conserved elements (Tsuda et al. 2008) such as the production of reactive oxygen species (ROS), which on specific occasions can lead to a hypersensitive response (HR) and apoptosis, an intracellular cascade mediated by MAP kinase proteins (MAPK) and hormonal signalling which generally involves a transcriptional reprogramming. Salicylic and jasmonic acid (SA and JA, respectively) are considered the two main hormonal pathways involved in plant defence. Plants adjust their immune system

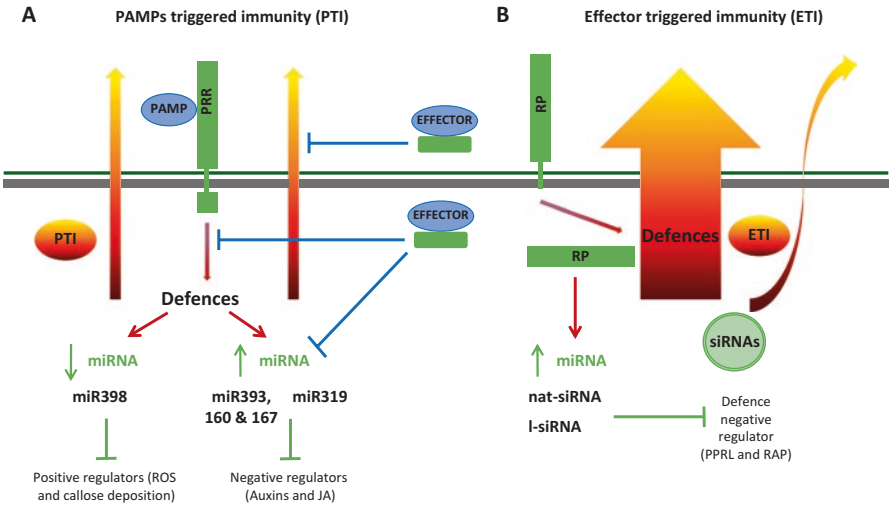

Fig. 2.2 Posttranscriptional epigenetic mechanisms involved in plant defence. The diagram represents some of the identified small RNAs controlling defence responses at the level of PAMP triggered immunity (**a**) and effectors triggered immunity (**b**). Pathogen components are highlighted in blue. Plant elements are represented in green. Plant defence as a process is represented with red and orange colours

depending on the lifestyle of the attacker they encounter. Generally, defence against attack by biotrophic pathogens, which feed from living cells, is mediated by the SA pathway. Conversely, necrotrophic pathogen infections, which kill the tissues to feed from them, or herbivores are resisted by the JA/ethylene (ET) pathway (Pieterse et al. 2012). An effective focus of the resources in defence is achieved by the prioritization of one of the pathways at the expense of the other, once the pathogen is recognized (Glazebrook 2005). As a consequence, it is common to find opposite phenotypes for different lifestyle pathogens when one of the pathways is active (Vos et al. 2015). In addition to SA, JA and ET, other hormones such as auxins, abscisic acid (ABA), cytokinins, gibberellins and brassinosteroids play a secondary, but nevertheless important, role in plant defence (Pieterse et al. 2012). These are general defence mechanisms triggered by the plant independently of the recognition of the pathogen.

It is generally accepted that there are two main routes by which the plant can activate these various defences, depending on the recognition of the pathogen. These are 'PAMP triggered immunity (PTI)' and 'effector-triggered immunity (ETI)'. In Fig. 2.2, we contextualize some of the most important sRNA examples controlling PTGS described to date in both branches of plant defence.

sRNAs in PAMP Triggered Immunity (PTI)

The first branch in plant defence is triggered when the plant recognizes general microbial- or pathogen-associated molecular patterns (PAMPs), such as flagellin (common to different bacteria) or chitin (common to many fungi) by the use of transmembrane pattern recognition receptors (PRRs). This recognition leads into the PAMP triggered immunity (PTI) by the activation of a defence signalling cascade (Fig. 2.2a). miRNAs modulate different stages of the plant defences but they have a special contribution in PTI (Voinnet 2008). As a reflection, *dcl1* mutants (strongly impeded in the production of miRNAs) are effectively infected by the usually avirulent *hrcC* strain of *Pseudomonas syringae* DC3000 (Navarro et al. 2008). One of the first observations in this respect was a remarkable change in miRNAs populations during infection, and in response to exogenous PAMP applications. During PTI some miRNA species are repressed (represented as ↓ miRNAs in Fig. 2.2a). This is the case of miRNAs controlling positive elements in the defence response, like the miRNA398 (Fig. 2.2a; Jagadeeswaran et al. 2009). The repression of miR398 releases its targets (superoxide dismutases CSD1 and CSD2), and as a consequence, enhances callose deposition, reinforcing the cell wall and impeding the pathogen infection (Li et al. 2010). In contrast, other miRNAs are induced during PTI (↑RNA), which are usually the ones that control negative regulators. Maybe the one that could be considered the most relevant example until date is the case of the miR393. Transcription of miRNA393 is induced during PTI (by both flagellin treatments and *Pseudomonas syringae—Pst*—infections) and it is accompanied by a repression of its targets, which in turn causes repression of the auxin signalling pathway (Fig. 2.2a). Repression of the auxin pathway in this way seems to lead in resistance against *Pst* by hormonal crosstalk (Navarro et al. 2006). Apparently, by this hormonal crosstalk, the plant would prioritize the expense of the resources in defence over growth by repressing auxin pathway during a defence response. In fact, there are other miRNAs involved in the repression of the auxin pathway during PTI, like miR160 and miR167 (Fig. 2.2a; Zhang et al. 2011). The fine-tuning of the defence responses through a hormonal control is not exclusive of the auxin pathway. Actually, as introduced before, it is accepted in the field that plants tailor their defence responses in accordance with the attacker's lifestyle by the negative crosstalk of SA and JA pathways, the two main hormonal pathways involved in immune responses. This crosstalk is also under the control of miRNAs. This is the case of the miR319 which is induced in response to a/virulent hemibiotrophic bacteria (*Pst* DC3000, *Pst* DC3000 *hrcC,* and *Pst* DC3000 *avrRpt2*). miR319 represses JA pathway components, and its induction during defence responses against biotrophic pathogens could therefore have the objective of prioritizing SA-related defences at the expense of JA responses (Fig. 2.2a; Zhang et al. 2011). This kind of immunity has been demonstrated to stop the colonization of many different pathogens. However, some plant pathogens evolved to somehow interfere with these PTI mechanisms by the use of specific molecules called as effectors.

Host-adapted pathogens use effectors to suppress PTI and thus successfully colonize their host. There are few identified cases of pathogens manipulating the plant

silencing mechanisms as part of the immune system. One of the most relevant cases of pathogen effectors suppressing silencing comes once again from viruses. As effectors, viruses produce viral suppressors of RNA silencing (VSRs) to counteract their silencing (Fig. 2.1). Those are the best-studied examples of suppressors of silencing and are able to interfere with silencing at different stages (Fig. 2.1; Csorba et al. 2015). However, there are also a few identified cases of other pathogens manipulating the plant silencing mechanisms. This is the case of the miR393, and probably miR159, which are repressed by AvrPto effectors of *Pst* (Navarro et al. 2008; Zhang et al. 2011). One of the most interesting examples of this co-evolution comes from the discovering siRNAs produced by the pathogen *Botrytis cinerea.* The fungus produces siRNAs as effectors in order to hijack the plant silencing machinery, facilitating its infection (Weiberg et al. 2013). Nonetheless, along their shared evolutionary path, some plants have also acquired the ability to detect pathogen effectors, triggering the second branch of plant immunity.

sRNAs in Effector-Triggered Immunity (ETI)

Plant recognition of the presence of effectors triggers the second branch of plant defence, which is known as effector-triggered immunity (ETI). Effector detection is carried out by what are sometimes referred to as R proteins (from 'resistance proteins'). R proteins, also called guard proteins, can be intra- or extracellular and detect the presence of effectors either directly (e.g. by direct protein–protein interaction) or indirectly, via the outcome of the effector's interference with PTI. During ETI, induced defences are typically much stronger than PTI (Fig. 2.2b). One remarkable example of epigenetic control of ETI concerns the small interfering RNAs *nat-siRNAATGB2* and *AtlsiRNA-1* (Fig. 2.2). They were both found to take part in the ETI response against the strain *avrRpt2 of Pseudomonas syringae* pv *tomato*. In an avirulent interaction, the plant R protein RPS2 is able to detect the action of the effector avrRpt2 degrading the plant defence protein RIN4, and triggers an ETI response. As part of that system *nat-siRNAATGB2* and *AtlsiRNA-1* are induced and contribute to the releasing of the defences by the inhibition of *PPRL* (Katiyar-Agarwal et al. 2006) and *AtRAP*, both negative regulators of RPS2-related defences (Katiyar-Agarwal et al. 2007). Those are just some examples of the roles of silencing mechanisms along the co-evolutionary history between plants and their pathogens. Strikingly, some recent discoveries show how, as a last counteracting measure, some plant sRNAs can be transferred by extracellular vesicles to the pathogen in order to trigger silencing of virulence factors as part of ETI response (Fig. 2.2, Cai et al. 2018). This demonstrates once again the important role of the small RNAs in the plant–pathogen arm race.

2.2.1.3 The State of the Field, from *Arabidopsis* to Other Species

Unfortunately, even though the first studies started many years ago using tobacco as a model species, in the rise of epigenetics as a hot field during the last 20 past years, the majority of the research has been done in *Arabidopsis* plants. Infections of *Pst* in *Arabidopsis* have been consolidated as a model pathosystem. However, as translational science strategies are rapidly building on this fundamental knowledge, nowadays there is evidence coming from many different pathosystems, including crop species. For instance, the role of the silencing machinery against viruses has been investigated in rice against the rice stripe virus (Jiang et al. 2012). In *Brassica*, there has been identified a miRNA (bra-miR1885) which appears to be specifically targeted by viral effectors from TuMV virus. In addition, TuMV infections (but not TMV or CMV) induce bra-miR1885 levels which represses a defence-related protein, facilitating virus infection (He et al. 2008). It has also been reported that miR393 is conserved across different plant species such as rice and cucumber (Bian et al. 2012; Xu et al. 2017), and there is even evidence in soybean pointing to a conserved role of its function in PTI (soybean—*Phytophthora sojae* infections; Wong et al. 2014). Another notable example is the tomato miR482 family, which represses components of the plant basal defences and is down-regulated during bacterial and viral infections (Shivaprasad et al. 2012). Last decade there has also been an increment in the range of pathogen interactions analysed, including bacteria (Alizadeh et al. 2018; Zhao et al. 2013), fungi (Ellendorff et al. 2009; Li et al. 2014, 2016; Shen et al. 2014), oomycetes (Li et al. 2012), viruses (Li et al. 2012), cyst nematodes (Hewezi et al. 2008; Zhao et al. 2015) and other herbivores. Importantly, the majority of the crop studies started with genomic and transcriptomic analysis, with a strong in silico component (Guo et al. 2011; He et al. 2014; Jeyaraj et al. 2017; Kapoor et al. 2008; Lu et al. 2007; Pandey et al. 2008; Pérez-Quintero et al. 2012; Qiu et al. 2009; Radwan et al. 2011; Warren and Covert 2004; Xin et al. 2010; Yin et al. 2012). Much more work needs to be done in vivo to be able to include these epigenetic mechanisms as agronomical tools for the development of crop protection strategies.

2.3 Transcriptional Control (TGS): Chromatin Remodelling

Epigenetics can also modify gene expression at the transcriptional level. The DNA is compacted in the nucleus by association with proteins in what we know as chromatin (Kornberg 1974). The basic units of chromatin are called nucleosomes and are formed by an octamer of histone proteins (two of each H2A, H2B, H3, and H4; Van Holde et al. 1974) and approximately 146 bp of DNA, or 1.7 turns, wrapping the histone octamer. The DNA between nucleosomes is called linker DNA and for higher levels of compaction can be associated with histone H1 (Fig. 2.3a). Depending on the physicochemical interaction of the DNA and histone proteins in the nucleosome, the chromatin can have different levels of compaction (Fig. 2.3b). This

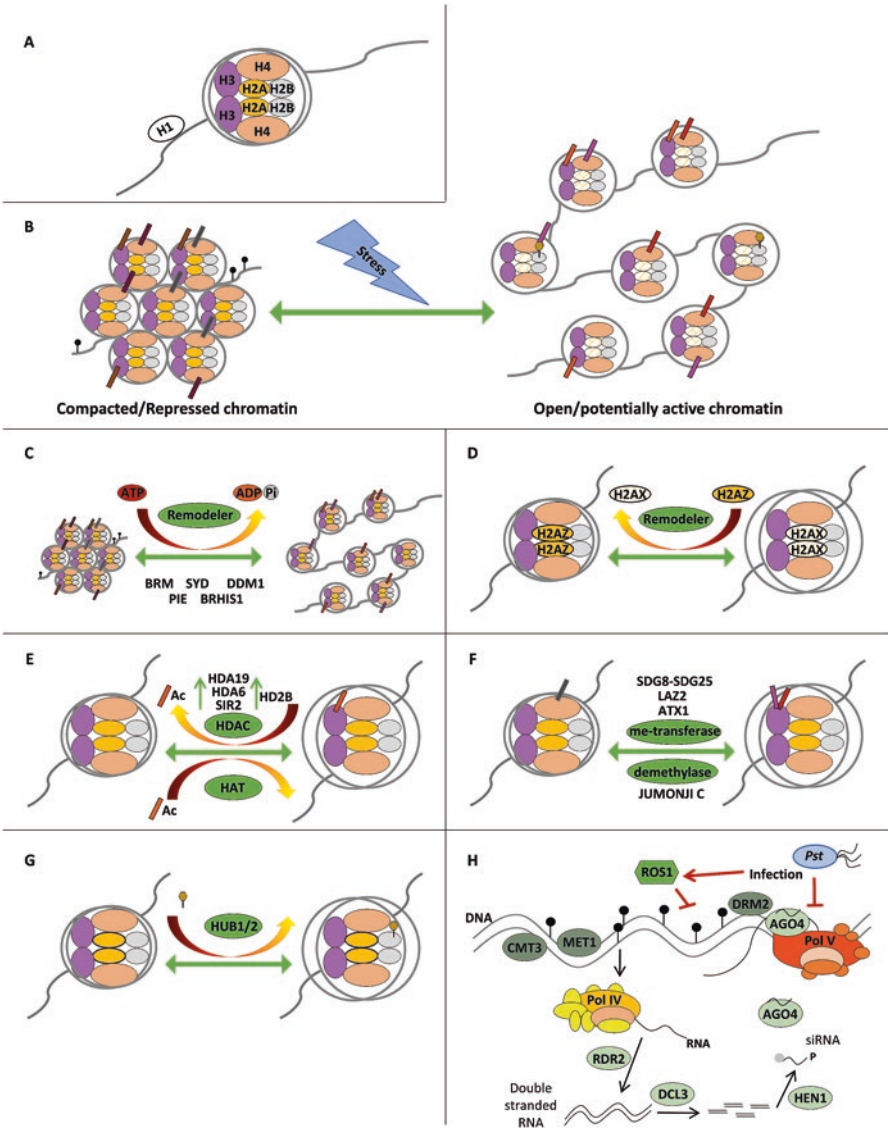

Fig. 2.3 Transcriptional epigenetic mechanisms involved in plant defence. (**a**) Representation of a nucleosome unit. The DNA wraps around an octamer of histone proteins (2× -H2A, H2B, H3 and H4-). H1 is located in the linker DNA (between nucleosomes). (**b**) The main part of the epigenetic mechanisms controlling defence at transcriptional level influences the chromatin compaction. In response to pathogen attack, plants activate epigenetic mechanisms to open the chromatin at the level of genes involved in defence responses, facilitating their expression and/or compacting chromatin regions containing defence repressors. DNA methylation, histone acetylation, histone methylation as a negative mark (for instance, H3K9me2), histone methylation as a positive mark (for instance, H3K4me3 or H3K36me), histone ubiquitination. (**c**) Some of the chromatin remodellers involved in plant defence. (**d**) Deposition of histone variants in plant defence processes. Fig. 2.3 (continued) Specifically, the case of the H2AZ is represented in the diagram. (**e**) Some histone acetylation examples in response to pathogen attack. (**f**) Histone methylation examples in response to pathogen attack. (**g**) Histone ubiquitination associated with plant defence. (**h**) DNA methylation changes mediated by the RdDM pathway and ROS1 in response to *Pst* infections

compaction is essential as, for the main part of the functions of DNA such as gene expression and replication, the DNA should be accessible to large protein complexes. However, the entire genome does not fit into the nucleus in the uncompacted state (Li et al. 2007). Thus, during interphase, the transcribed regions are uncompacted constituting what is known as open chromatin, while other regions are preserved (silenced) in very compacted chromatin regions. At the same time, the compaction of the DNA at some regions should be maintained, as this preserves the genetic information from damage and the jumping of TEs. In response to an environmental stimulus, the chromatin has been proposed to interpret the signal and facilitate the gene reprogramming (Fig. 2.3b; Badeaux and Shi 2013). As the chromatin compaction has been directly related to the transcriptional control of gene expression, all mechanisms modifying chromatin compaction are considered epigenetic mechanisms controlling transcriptional gene silencing (Fransz and de Jong 2011). Among such mechanisms, the most important are: chromatin remodellers, deposition of histone variants, histone posttranslational modifications and DNA methylation. Similar to the control of PTGS, chromatin compaction has been demonstrated to play a crucial role in the fine-tuning of defence responses. Importantly, the chromatin state has been proposed to be the mechanism underlying priming processes and memory of the stress, which will be addressed in Sect. 2.3.

2.3.1 ATP-Dependent Chromatin Remodellers

The ATP-dependent chromatin remodellers are multiprotein complexes that are able to disrupt the interaction between the DNA and histone proteins using energy provided by the hydrolysis of ATP molecules (Fig. 2.3c; Han et al. 2015). They are conserved cross-kingdom in eukaryotes. In plants, the ATPase function resides in proteins from the Snf2 superfamily. Members of the Snf2 superfamily involved in plant defence identified to date are: BRAHAMA (BRM), SPLAYED (SYD), DECREASE IN DNA METHYLATION 1 (DDM1), PHOTOPERIOD-INDEPENDENT EARLY FLOWERING 1 (PIE1), BIT-RESPONSIVE HISTONE-INTERACTING SNF2 ATPASE 1 (BRHIS1) and CHROMATIN-REMODELLING FACTOR 5 (CHR5). BRM is maybe the most canonical and well-studied chromatin remodeller in plants, and it has been widely studied in responses against abiotic stresses, where it controls abscisic acid (ABA)-related genes (Han et al. 2012; Peirats-Llobet et al. 2016). However, some defence-related genes have been observed as misregulated in the *brm101* mutant, pointing to a defence role of BRM, probably by crosstalk between ABA and SA hormonal pathways (Bezhani et al. 2007; Ramirez-Prado et al. 2018). SYD has been reported to play a role in the activation of JA/ET related defences. On the one hand, SYD seems to bind some promoter regions for JA/ET-related genes, probably promoting the opening of the chromatin. On the other hand, mutants defective in SYD are consistently more susceptible to the necrotrophic pathogen *B. cinerea* and unable to properly induce appropriate target genes (Walley et al. 2008). In addition, some SYD mutants are

more resistant to biotrophic pathogens such as *Hyaloperonospora arabidopsidis* (*Hpa*) and *Pseudomonas syringae* pv. *maculicola* (*P.s.m.*; Johnson et al. 2015). DDM1 plays a critical role in the maintenance of the DNA and histone H3 methylation pattern. In this case, DDM1-mediated chromatin opening does not recruit gene activators, but it seems to allow the recruitment of methyltransferases to its target regions, inducing gene silencing (Gendrel et al. 2002; Jeddeloh et al. 1998; Zemach et al. 2013). It therefore links the direct opening of the chromatin with changes in the histone protein modifications and DNA methylation. DDM1 has been related with the control of the SA-related defences and the silenced basal state of *RPP5*, a six-defence gene cluster (Yi and Richards 2007, 2009). Accordingly, the *ddm1* mutant plants are more resistant to *Hpa* (López Sánchez et al. 2016). Recently it has been reported how the *SNC1* gene from the *RPP5* cluster is also regulated by another ATP-dependent chromatin remodeller, CHR5. In this case, CHR5 acts as a positive regulator (classical chromatin remodeller function) opening the chromatin at the level of *SNC1* gene. The mutant *chr5* shows an inability to open the chromatin at *SNC1* level, exhibits increased nucleosome deposition along the whole genome, and hyper-susceptibility to virulent and avirulent strains of *Pst* (Zou et al. 2017). Finally, the chromatin remodellers PIE and BRHIS1, like DDM1, act as intermediaries for other chromatin modifications such as the deposition of histone variants and histone monoubiquitination, respectively (discussed later).

2.3.2 Deposition of Histone Variants: Histone Replacement

One of the mechanisms involved in chromatin remodelling is the replacement of the canonical histone by specific histone variants. Due to different physicochemical properties, the interaction of the histone variants with the DNA in the nucleosome can modify chromatin compaction and thus, gene expression at transcriptional level (Coleman-Derr and Zilberman 2012). That is the reason why the deposition of different histone variants has been proposed as a mechanism mediating responses to environmental changes (Talbert and Henikoff 2014). One of the most important examples for this mechanism is the case of the H2A.Z (Fig. 2.3d). First, March-Diaz et al. (2008) described how mutants defective in the H2A.Z coding genes, as well as the SWR1 chromatin remodelling complex, which facilitates its deposition (including the above-mentioned PIE protein), constitutively express SA-related genes and are more resistant against biotrophic pathogens. The authors suggested that the deposition of H2A.Z has a role in controlling the silencing of SA-related genes in a basal state (March-Díaz et al. 2008). Recent works have confirmed the role of this histone variant in repressing SA-related genes, but they report hyper-susceptibility for both biotrophic and necrotrophic pathogens (Berriri et al. 2016). However, the deposition of the histone variant H2A.Z seems to be key for the fine-tuning of the defence responses.

2.3.3 Modification of the Histone Proteins

Histone proteins can be posttranscriptionally modified at different residues, having an important impact in the chromatin state (Kouzarides 2007). More than 60 positions have been detected in the tail of the core histone proteins that can potentially carry posttranscriptional modifications (PTMs) of a variable nature. It is becoming clear that PTM of histone proteins imparts a dynamic and complicated regulation of gene expression, particularly in plant defence processes. This regulation is a consequence not just of the appearance of one PTM, as they can have an individually positive or negative contribution for chromatin compaction, but the consensus of several interacting PTMs, in what is called 'the histone code'. The PTMs that have been demonstrated to play a key role in plant defence are related with acetylation, methylation and ubiquitination of different residues (Fig. 2.3e–g). There have been very recent and comprehensive reviews in this field (Chen et al. 2017; Ding and Wang 2015; Ramirez-Prado et al. 2018). The majority of cases have been described once again in *Arabidopsis*, so here we will just outline some of the most notable examples to offer a general view.

2.3.3.1 Acetylation

Generally, the acetylation of residues in histone H3 and H4 proteins is associated with a relaxation of the chromatin (openness). As DNA is negatively charged, the addition of acetyl groups (also negative), loosen the nucleosome association, facilitating gene expression. Histone acetylation is carried out by histone acetyltransferases and the deacetylation by histone deacetylases (HATs and HDACs, respectively, Fig. 2.3e; Berger 2007). In *Arabidopsis*, two HDACs in particular, HDA19 and HDA6, have been linked with plant defence. Both of them are induced by necrotrophs and/or JA-related signals (for example, wounding; Zhou et al. 2005), suggesting some overlapping functions. *hdc19* mutants show increased susceptibility to necrotrophic pathogens and an inability to induce JA-related genes. This phenotype does not seem to be related with direct changes in the PTMs at the level of the JA-related genes, but by the hormonal crosstalk with the SA hormonal pathway (Choi et al. 2012; Koornneef et al. 2008). HDA19 seems to play a key role in the maintenance of the silent basal state of the SA-related genes. At the basal state, HDA19 seems to inhibit the acetylation of histone proteins at the *PATHOGENESIS-RELATED1* and *2* (*PR1* and *PR2*) defence gene loci (genes considered marker genes for the SA hormonal pathway). In fact, *hda19* mutants show enhanced expression of those genes and hyper-resistance against some biotrophic pathogens (even when originally there were contradictory results at this respect; Choi et al. 2012; Kim et al. 2008). Other histone deacetylases involved in plant defence belong to the SIR2 protein family. In *Arabidopsis*, *AtSRT2* controls the basal repression of the SA-related defences (Wang et al. 2010). Thus, histone deacetylations seems to cause a general repression at the level of different SA-associated defence genes.

However, some recent studies have described how during PTI responses, the phosphorylation cascade induced by MAPKs proteins can lead to the specific activation of the HDAC, HD2B. HD2B would deacetylate genic regions, inhibiting their expression and therefore contributing to the gene expression reprogramming during defence processes (Latrasse et al. 2017). Lastly, nitric oxide has been proposed as a repressor of the histone deacetylation during plant defence processes, mediating the hyperacetylation and contributing to the induction of defence-related genes (Mengel et al. 2017; Ramirez-Prado et al. 2018).

2.3.3.2 Methylation

As for acetylation, methylation of different residues of the histone proteins has been proven to play a crucial role in plant defence (De-La-Peña et al. 2012; Ramirez-Prado et al. 2018). It mainly occurs in lysine and arginine residues of histones H3 and H4. For each residue, from one to three methyl groups can be added (Bannister and Kouzarides 2005; Kouzarides 2007). These forms of histone methylation can differentially impact on the chromatin structure. Unlike the case of acetylation that usually involves chromatin relaxation, methylation as a PTM of histone proteins has been linked with both inhibition and priming of gene expression (Fig. 2.3f). The enzymes involved in histone de/methylation are very specific. The most notable examples related to plant defence come from the analysis of methyltransferases. This is the case, for instance, of *ARABIDOPSIS* HOMOLOG OF TRITHORAX (ATX1), which trimethylates the lysine 4 of histone 3 (H3K4me3) and positively regulates the expression of *WRKY70*, a transcriptional factor involved in SA hormonal pathway and postulated to be crucial to the SA-JA hormonal crosstalk (Alvarez-Venegas et al. 2007). LAZARUS2 (LAZ2) is another histone methyltransferase involved in trimethylation of lysine 36 of histone 3 (H3K36me3), which is required to activate an R gene involved in ETI responses against *Pst* (Palma et al. 2010). Recently, roles for demethylases in defence have also been reported. This is the case of Jumonji C demethylases. For example, the H3K9 demethylase JMJ27 is induced during bacterial infection, required for the resistance to the pathogen and the correct expression of defence-related genes (Dutta et al. 2017). A peculiar case is the demethylation of lysine 9 of histone 3 (H3K9me2). H3K9me2 has been traditionally considered as a repressive chromatin mark of TEs. Surprisingly, it has been reported that the levels of H3K9me2 at the defence gene *RPP7* can affect the selection of the polyadenylation site, and thus, the production of a different transcript (Tsuchiya and Eulgem 2013). As pointed out previously, the chromatin environment is determined not by just individual marks, but the appearance of different ones at the same time. Therefore, it is easy to imagine that there is co-regulation of the different PTM pathways. This was evident in the study of the methyltransferases SDG8 and SDG25. Mutants in those proteins are altered in plant defence against necrotrophs, biotrophs, and show differences in methylation of several residues of the histone proteins at the level of some defence-related genes (Berr et al. 2010; Lee

et al. 2016). They also display an altered pattern in H2B ubiquitination (Lee et al. 2016), which will be the subject of the next section.

2.3.3.3 Ubiquitination

Ubiquitination of proteins typically refers to the addition of the 76-residue peptide known as ubiquitin by the action of three consecutives enzymes, E1, E2 and E3 (Weake and Workman 2008). In general, those enzymes can add one or more residues of ubiquitin to the target proteins. Many different proteins can be ubiquitinated, but in the specific case of histone proteins, ubiquitination has only been found in the form of a single residue in H2A or H2B, and usually acts as a positive mark for gene expression marking open chromatin (Fig. 2.3g). As with the other PTMs, ubiquitination is reversible. *Arabidopsis* HUB1 and HUB2, the two RING E3 enzymes, have been reported to be required for plant defence against necrotrophs (Dhawan et al. 2009; Hu et al. 2014) and to play a certain role against biotrophic pathogens (Zou et al. 2014), probably through modifications of the cuticle (Ménard et al. 2014).

2.3.4 DNA Methylation

DNA methylation usually refers to the addition of a methyl group at the fifth carbon of the cytosine residues of the DNA. In addition to cytosine methylation, adenine methylation has also been reported in many different organisms, being a key factor for protecting prokaryotic DNA. However, adenine methylation has so far received little attention in plants (Liang et al. 2018). Cytosine DNA methylation in plants can be found in every sequence context and in different extents, depending on the species (Niederhuth et al. 2016; Takuno et al. 2016). Attending to the nature of the context, both symmetrical and asymmetrical contexts can be considered (Cokus et al. 2008). Symmetrical contexts are CG and CHG (H refers to A, T or C), where both strands of the DNA are methylated. Asymmetrical context is CHH. The maintenance of the DNA methylation pattern is carried out by the METHYLTRANSFERASE1 (MET1) in CG context and CHROMOMETHYLASE2 and 3 (CMT2 and CMT3) in CHG contexts. The maintenance of CHH methylation is mainly performed by an RNA-directed DNA methylation pathway (RdDM), and requires the constant production of siRNAs (Fig. 2.3h; Law and Jacobsen 2010). Probably as a consequence of its origin as defence systems against exogenous nucleic acids, the establishment of a de novo pattern in DNA methylation is primarily controlled by siRNAs and has some homologies with the PTGS mechanisms. The current view of the process in *Arabidopsis* includes an initiation phase (not included in the figure for simplicity reasons) in which it is likely that over-accumulated RNAs are copied to a double-stranded molecule by RDR6, and then

processed by DCL2 and DCL4 into 21–22 nt siRNAs. Probably these siRNAs trigger PTGS by binding to AGO1 or AGO2 proteins. However, in subsequent stages, they are loaded into AGO6, which directs the plant-specific DNA-DEPENDENT RNA POLYMERASE V (Pol V) and the DNA methyltransferase, DOMAINS REARRANGED METHYLTRANSFERASE 2 (DRM2), to the target regions in the genome to induce low levels of DNA methylation (Nuthikattu et al. 2013). After this initiation phase, the second branch of the RdDM pathway is activated (Fig. 2.3h). In this, the other plant-specific DNA-DEPENDENT RNA POLYMERASE IV (Pol IV) is involved in the production of RNA molecules from the targets that after being processed by RDR2, DCL3 and HEN1 will be loaded onto AGO4. The base pairing between the siRNA with Pol V-produced RNA transcripts enables the recruitment of DRM2 for establishment of DNA methylation (Matzke and Mosher 2014). DRM2-dependent CHH methylation requires the constant production of siRNAs, and ongoing activity by the Pol IV-RDR2-dependent RdDM pathway. Both the overall level of genome-wide DNA methylation and the pattern of methylation are controlled by a balance between DNA methylation and demethylation processes. Removal of DNA methylation can happen passively during replication, or actively by the action of DNA glycosylase/lyases, of which four have been identified to date in *Arabidopsis* (Zhu 2009). Among these, REPRESSOR OF SILENCING 1 (ROS1) is predominantly responsible for DNA demethylation in vegetative tissues. The primary functions of DNA methylation are controlling genome stability and gene expression. In general, the epigenetics community tend to associate DNA methylation at the level of promoter regions with repression of gene expression, while the consequences of methylation within gene bodies remain uncertain (Bewick and Schmitz 2017).

The first characterized roles for DNA methylation in plant defence came again from the defence against viruses, with several examples showing DNA methylation of the viral genome for the Geminivirus family (Blevins et al. 2006; Raja et al. 2008). The majority of plant viruses possess RNA genomes, but the Geminiviridae family genome is a single-stranded DNA. The silencing of the viral genome is carried out by the TGS mechanisms of the cell as a defence mechanism against exogenous nucleic acids. Therefore, Geminivirus genomes are silenced by part of the RdDM pathway (Fig. 2.1b). A beautiful example of plant–DNA virus interaction is seen between tomato and the TOMATO YELLOW LEAF CURL CHINA VIRUS. During their co-evolution, tomato plants first developed the ability of defend themselves from viral infection by methylating the viral DNA. Some virulent strains of the virus carry what is known as the betasatellite encoding βC1, which is a repressor of silencing used as an effector (Yang et al. 2011). However, resistant strains of tomato plants have developed the ability to polyubiquitinate βC1 to mediate its degradation via the proteasome. Nowadays, a range of different viral effectors acting as repressors of transcriptional silencing are known (Rodríguez-Negrete et al. 2013; Wang et al. 2014). Although the TGS assigned to the defence against Geminiviruses was thought to act at the level of DNA methylation of the viral genome, recent studies found that the role of the RdDM pathway in silencing the

viral genome is performed by triggering H3K9 methylation (Jackel et al. 2016). This reflects once again the crosstalk between different TGS mechanisms.

As in the case of the PTGS mechanisms, plants took advantage of the TGS defence system to control different endogenous sequences. In fact, the activation of antiviral defences has direct consequences in endogenous sequences (Castillo-González et al. 2015; Coursey et al. 2018), as the most important role of DNA methylation is controlling TE repression (considered invasive DNAs). The changes in chromatin caused by TE silencing can also modify the expression of some plant genes, and in our case of interest, defence-related genes. There has been increasing evidence for a role of the DNA methylation and demethylation machineries in controlling plant defence. On the one hand, plants trigger changes in DNA methylation during pathogen attack (Dowen et al. 2012; Pavet et al. 2006; Yu et al. 2013). In general terms, an active DNA demethylation process in response to infections of *Pst* and the application of PAMPs such as flagellin has been observed. This demethylation seems to be a consequence of the repression of some RdDM components and the active removal of methyl-cytosines by the protein ROS1 (Fig. 2.3h; Dowen et al. 2012; Yu et al. 2013). On the other hand, *Arabidopsis* mutants impeded in DNA methylation (such as *met1*, *drd1*, *cmt3* and mutants defective in Pol V) have been reported to show increased resistance to biotrophic pathogens like *Pst* and *Hpa* (Dowen et al. 2012; López et al. 2011; Yu et al. 2013). Correspondingly, with the SA-JA hormonal crosstalk, those same mutants show hyper-susceptibility against necrotrophic pathogens like *Plectosphaerella cucumerina* and *B. cinerea* (López et al. 2011; López Sánchez et al. 2016). Also in accordance, mutants in ROS1 protein, which cannot actively demethylate the DNA, show the opposite phenotype (hyper-susceptibility against biotrophs and enhanced resistance against necrotrophs). The exact mechanisms by which DNA methylation controls plant defences are not known and even when transcriptomic analysis of the mutants during infection point to changes in several genes, only in very limited cases, *cis*-regulation has been demonstrated (Le et al. 2014; Yu et al. 2013). In just a few specific cases, differentially methylated regions have been directly associated with the transcriptional control defence-related genes, for instance, being TEs located at the promoter regions of defence genes (Yu et al. 2013). Alternative *trans*-regulatory mechanisms have been proposed, which will need further research in the future. Importantly, even when mutants defective in DNA methylation are more resistant against biotrophic pathogens and they show a better induction of the SA-related defence genes, those mutants do not display constitutive expression of those genes (López et al. 2011; López Sánchez et al. 2016). These and other evidences involving chromatin states and the requirement of DNA methylation machinery in transgenerational phenomena have been crucial in determining their role in priming of induced defences and memory of stress, which will be assessed below.

2.3.5 *The State of the Field, from* Arabidopsis *to Other Species*

In line with the case of the PTGS mechanisms, there are not many studies carried out in non-model organisms. Here, we will introduce some of the works reported until now. The BRHIS1 chromatin remodeller from rice is a nice example. It has been demonstrated to repress defence-related genes and maintain them in the basal state. As part of the plant response to fungal pathogens/priming agents, plants repress *BRHIS1*, which would in turn favour the induction of the defence genes (Li et al. 2015). A few more examples have been reported of posttranslational modification of histone proteins. Also in rice, there has been nice work in the rice interaction with the pathogen *Magnaporthe oryzae*. The over-expression of the histone deacetylase HDT701 confers to the rice plants hyper-susceptibility to *M. oryzae* and *Xanthomonas oryzae* pv. *oryzae* (*Xoo*). Thus, HDT701 has been proposed as defence repressor, probably targeted by effectors, as its induction has been detected during infections (Ding et al. 2012a). There is also an elegant work in rice studying the Jumonji C histone demethylases. During *Xoo* infection, rice plants have been reported to induce the expression of 15 JmjC proteins (Hou et al. 2015). Among these, JMJ704 and JMJ705 have been demonstrated to play a key role for plant defence. Accordingly, a *jmj704* mutant is more susceptible, and *JMJ705* over-expression lines more resistant, to *Xoo* (Hou et al. 2015; Li et al. 2013). Both seem to play a role in repressing defence genes during basal conditions and releasing their expression during defence. On the one hand, JMJ704 seems to be important in maintaining low levels of the positive mark H3K4me2/3 in the defence genes during basal conditions (Hou et al. 2015). On the other hand, JMJ705 seems to induce the removal of negative marks such as H3K27me2/3 during infections (Li et al. 2013). Apart from rice, following a similar strategy to the genome-wide analysis of sRNAs, a whole-genome analysis during rust infection in common bean reported global changes in histone methylation and acetylation (Ayyappan et al. 2015). In addition, and built on the basis of the work in *Arabidopsis*, the histone ubiquitination pathway has also been studied in other species such as tomato (Zhang et al. 2015). In this study, the tomato homologs of the HUB1/2 proteins: SIHUB1 and SIHUB2 were identified. The authors demonstrated that SIHUB1/2 are required for defence against *B. cinerea* and seem to play a role in the crosstalk of the SA-JA hormonal pathways, probably by a combination of the cuticle properties and the priming of defence genes (Zhang et al. 2015). Lastly, consistent with the role of DNA methylation in response to biotrophic pathogens, treatments with 5-azadeoxycytidine in rice seedlings have been reported to show resistance to *Xoo*, a phenotype which correlated with the demethylation of specific regions, including a defence gene *Xa21G* (Akimoto et al. 2007). Again, even though the role of the different TGS mechanisms has been proved to be crucial for plant defence, more work is needed in order to apply the fundamental knowledge generated in models to crop protection programs.

2.4 Epigenetics Is Involved in the Memory of the Stress: Priming

In addition to the roles of small RNAs and chromatin remodelling in the regulation of immediate defence responses and defence-related gene expression, epigenetic processes also play key roles in longer-term defence priming. Priming refers to the immunological memory that can develop following stress exposure, such that responses to future stresses are more effective. The best-studied example of priming in relation to biotic stress is systemic acquired resistance (SAR). As well as transient up-regulation of defence genes in systemic leaves (which have not suffered the infection), SAR typically includes a priming element, such that defence responses are stronger and more rapidly induced in response to a secondary infection for up to several weeks following an initial pathogen infection (Klessig et al. 2018). More recently, evidence has accumulated that under some circumstances, longer-lasting priming memory can be established, which is then inherited by one or more future generations.

2.4.1 Short-Term Priming Memory

2.4.1.1 Changes in Defence Response Signalling Components

Several mechanisms have been proposed that could potentially generate the memory of stress that is required for priming. Short-term memory could be generated relatively easily by changes in the quantity or activity of signalling components required for the regulation of ETI and PTI (Conrath et al. 2015). For example, systemic leaves of *Arabidopsis* undergoing SAR display elevated levels of unphosphorylated (therefore inactive) mitogen-activated protein kinases MPK3 and MPK6. When inoculated with *P. syringae*, these primed leaves exhibit higher levels of MPK3/6 activity than non-primed leaves, due to their faster activation, as they are already synthetized (Beckers et al. 2009). The accumulation of transcription factors necessary for defence gene expression may be another similar mechanism for priming. Van der Ent et al. (2009) identified panels of *Arabidopsis* transcription factors that were up-regulated upon priming by either induced systemic resistance (ISR) triggered by *Pseudomonas fluorescens* or by root drenching with β-aminobutyric acid (BABA), a well-known chemical inducer of priming. They suggested that the different groups of transcription factors responsive to each of these priming treatments not only provide a mechanism for priming, but can also act as markers for different priming responses. The up-regulation of pattern recognition receptors (PRRs) involved in recognition of biotic attackers has also been suggested as a mechanism for priming (Tateda et al. 2014).

2.4.1.2 Chromatin Remodelling

Aside from the production of additional signalling molecules, the other main area that has received attention is epigenetic mechanisms for encoding stress memories.

As well as being essential for immediate, short-term transcriptional responses, chromatin remodelling via histone and DNA modifications also has the potential for conferring stable patterns of gene expression. Indeed, during development, epigenetic mechanisms are central to changes in gene expression associated with cell-type specialization (Heard and Martienssen 2014). As described above, regulation of defence gene expression involves various histone and DNA modifications which ultimately make genes more accessible to the transcriptional machinery. After a transient burst of stress-induced transcription, the chromatin of a defence-related gene may revert to the basal state, in which case it would be expected to show the same response characteristics to any subsequent experience of stress. Alternatively, if the reversion was only partial, then it might be possible that subsequent access of the transcriptional machinery would be less restricted. This would represent a primed state, in which chromatin modifications, brought about by previous transcriptional activation, leave a memory imprint. Primed genes might therefore be expected to reside on more open chromatin associated with altered levels of key histone and DNA modifications. To date, evidence from several different biotic and abiotic stress response systems has identified a range of histone marks and DNA methylation patterns, but in particular, reduced nucleosome occupancy and increased H3K4me3 are emerging as especially common features of primed stress-related genes.

Histone Modifications

Several authors have identified H3K4 hypermethylation associated with transcriptional memory/priming, most notably the trimethylation state. Key work linking histone modifications and defence priming came from Jaskiewicz et al. (2011), who demonstrated that local inoculation with *P. syringae* or treatment with benzothiadiazole (BTH) led to increases in H3K4me2 and H3K4me3, along with increased acetylation at a number of histone H3 and H4 lysine positions in the promoters of several WRKY genes. Histone H3K4me2/3 methylation is generally associated with a permissive transcriptional chromatin state (Berger 2007). Importantly, the increase in H3K4 methylation following BTH treatment was not associated with any immediate change in gene expression, but the affected WRKY genes exhibited augmented expression in response to a secondary stimulus. Furthermore, H3K4me3 hypermethylation was not observed in the SAR-deficient *npr1-1* mutant, whereas constitutively primed *cpr1* and *sni1* mutants showed constitutive high levels of H3K4me3 in the WRKY gene promoters. Similarly, mutants defective RNA polymerase V, which is required for initiation of DNA methylation in the RdDM pathway, also demonstrate constitutive priming of SA-dependent defence genes, and exhibit enhanced H3K4me3 at defence genes (López et al. 2011). Priming responses

following application of BABA in common bean were also linked with elevated H3K4me3 states at defence gene promoters (Martínez-Aguilar et al. 2016). Importantly, BABA treatment resulted in enhanced H3K4 trimethylation without any change in gene expression. The subsequent transcriptional activation of genes with this modification was primed, being higher in BABA-treated plants than in control plants, in response to bacterial infection (Martínez-Aguilar et al. 2016).

Other histone modifications may act alongside H3K4 methylation to establish priming. A role for histone acetylation in priming of PTI was identified by Singh et al. (2014a), who showed that priming for bacterial disease resistance was eliminated by mutation of *histone acetyltransferase1* (*HAC1*). Interestingly, the *Arabidopsis FLD* gene, which encodes a protein homologous to a human lysine-specific demethylase and also associated with histone deacetylase complexes, was identified in a genetic screen for mutants defective in the ability to express SAR (Singh et al. 2013). Mutants in *FLD* display wild-type levels of basal resistance to *Pst*, but do not exhibit priming of *PR1*, *WRKY6* and *WRKY29* gene expression following secondary inoculation of systemic leaves (Singh et al. 2013, 2014b). H3K4me2 appears to be the major substrate for FLD (Liu et al. 2007), but H3K4me2 methylation was decreased overall rather than increased in the promoters of *WRKY6* and *WRKY29* following challenge inoculation of an *fld* mutant (Singh et al. 2014b). Although the data do not identify a clear role for FLD in regulating histone modifications during priming of these genes, the authors suggested it may function as a negative regulator of an alternative histone demethylase that represses H3K4me2 methylation, and therefore priming, of defence genes.

As well as posttranslational modifications of histones, nucleosomes are also regulated by inclusion of variant histone proteins. In particular, H2A.Z has been linked with plant–pathogen resistance responses, although its precise function remains unclear. As noted in Sect. 2.2, March-Díaz et al. (2008) found that mutants defective in the SWR1 chromatin remodelling complex, which is responsible for substitution of canonical H2A with H2A.Z, are resistant to *P. syringae* and over-express a suite of SAR-regulated genes. More recently, different roles were identified for genes in the SWR1 complex and H2A.Z in SA and JA-mediated basal and effector-triggered immunity, indicating a complex interaction between H2A.Z substitution and immune regulation (Berriri et al. 2016). Overall, while loss of H2A.Z increases basal immunity, it reduces inducible responses. Whether it plays any role in priming of defence genes following induced resistance remains to be tested.

Nucleosome Occupancy

A second, related, common feature that has been identified in the promoters of primed genes is a more open chromatin configuration. Chromatin assembly factor CAF-1 is required for assembly of nucleosomes on newly replicated DNA, and mutants in CAF-1 subunits display pleiotropic developmental phenotypes (Ramirez-Parra and Gutierrez 2007). One of these phenotypes is constitutive priming of many

defence genes. Under normal growth conditions, the *fas2-4* mutant (a null allele for one of the CAF-1 subunits) exhibited constitutive expression of SAR-responsive genes. When grown under sterile conditions, these genes were no longer constitutively expressed, but displayed primed expression in response to SA treatment (Mozgová et al. 2015). CAF-1 therefore appears to be involved in repression of the primed state in wild-type plants. In the CAF-1 mutant, chromatin assays identified reduced nucleosome occupancy but increased abundance of H3K4me3 around transcription start sites (TSS) of several SAR genes, and similar chromatin states were observed in the same genes when priming was induced by either SA or BABA treatment (Mozgová et al. 2015). Interestingly, similar profiles were also detected in the promoters of genes exhibiting priming memory following drought stress. In this work, promoters of primed genes possessed stalled RNA polymerase II (PolII) and H3K4me3 hypermethylation (Ding et al. 2012b). Finally, genome-wide profiling of nucleosome positioning in response to SA treatment also identified reduced nucleosome occupancy at the TSS of SA-responsive genes (especially those regulated by *NPR1*), while SA-repressed genes showed nucleosomal enrichment (Singh et al. 2015).

DNA Methylation

Changes in DNA methylation are another obvious candidate mechanism for long-term stress memory. As discussed above, DNA hypomethylation has been detected following infection of *Arabidopsis* with *P. syringae* in several studies (Dowen et al. 2012; Pavet et al. 2006; Yu et al. 2013). This may play a functional role in immediate induced resistance responses, since hypomethylated loci centred around transposable elements are enriched in defence genes (Dowen et al. 2012). Yu et al. (2013) also identified demethylation of TEs near defence genes in response to treatment with the PTI-eliciting FLG22 peptide. This response was dependent on the DNA glycosylase *ROS1*. These stress responsive changes in methylation are consistent with the phenotypes of hypomethylated DNA methylation mutants, which are typically more resistant to infection (Dowen et al. 2012; Le et al. 2014; López et al. 2011; López Sánchez et al. 2016; Luna et al. 2012; Luna and Ton 2012). Whether mutants that suffer from extensive genome-wide hypomethylation genuinely reflect what happens following infection is open to question, but plants with more restricted regions of hypomethylation, such as in the so-called epiRIL (epigenetic recombinant inbred) lines generated by back-crossing hypomethylated mutants to wild-type *Arabidopsis*, can also exhibit altered responses to defence hormones and variations in resistance to pathogens (Latzel et al. 2012). Importantly, increased resistance in some DNA methylation mutants has been attributed to priming rather than constitutive SA-responsive gene expression (López et al. 2011; López Sánchez et al. 2016). Since DNA methylation is readily inherited during mitotic cell divisions, it provides a potential mechanism for long-term priming memory that can extend even into cells and tissues not present during the initiating stress.

2.4.2 Long-Term Priming Memory

Responses such as SAR are well documented to persist over several weeks, but in recent years, examples of defence priming that persist for much longer periods of plant development have also emerged. For example, seed treatments with elicitors including JA, chitosan and BABA resulted in defence priming that persists for many weeks (Haas et al. 2018; Strapasson et al. 2014; Worrall et al. 2012), while seedling root drenches with BABA prime long-lasting disease resistance in *Arabidopsis* and tomato (Luna et al. 2014; Wilkinson et al. 2018). Not only that, priming can extend from one generation to the next. One early report hinting at what is now referred to as transgenerational acquired resistance (TAR) in the case of SA-dependent disease resistance, or transgenerational immune priming (TGIP) more generally, suggested that tobacco mosaic virus (TMV) infection enhanced resistance in progeny of tobacco (*Nicotiana tabacum*; Roberts 1983). Other studies found that *Brassica spp*. suffering biotic stress produced seeds containing higher concentrations of glucosinolates compared with non-infested control plants (Lammerink et al. 1984; Shattuck 1993). Another series of papers demonstrated that insect herbivory on wild radish (*Raphanus raphanistrum*) enhanced resistance in seedlings of progeny plants (Agrawal 2001, 2002; Agrawal et al. 1999). The increase in resistance observed in these studies was transient and no mechanism was identified. Although intriguing in the context of the potential ecological benefits of TGIP, the examples described above could also be explained by simple maternal effects—the provisioning of seeds with altered resources from the mother plant in response to stress. While widely recognized as ecologically important, maternal effects are distinct from *bone fide* transgenerational inheritance, which enables the offspring generation to express phenotypes independently of any non-genetic contribution from the parental plants. Such transgenerational inheritance is most likely to be epigenetically encoded.

2.4.2.1 Transgenerational Immune Priming

Several examples have emerged over recent years which provide much stronger evidence for true transgenerational inheritance of biotic stress priming. As well as following pest and pathogen attack, priming can be induced by various chemical agents. One of the best studied among these is β-aminobutyric acid (BABA; Cohen et al. 2016). As well as within-generation priming, it was recently found that the progeny of plants primed either by BABA treatment or infection with avirulent *P. syringae* bacteria exhibited TAR. Offspring of treated plants were more resistant to infection by both *Pst* and *Hpa* because of primed SA-dependent gene expression (Slaughter et al. 2012). Intriguingly, offspring of BABA-primed parents were more responsive to BABA treatment than offspring of control plants, suggesting a 'primed-to-be-primed' phenotype. Similarly, treatment of barley (*Hordeum vulgare*) with the commercial resistance-inducing agent acibenzolar-*S*-methyl, or with saccharin, resulted in TAR against leaf blotch disease caused by the fungal pathogen, *Rhynchosporium commune* (Walters and Paterson 2012).

Luna et al. (2012) also reported TAR in *Arabidopsis* following repeated inoculations with virulent *P. syringae*. A key aspect of this work was that the authors were not only able to identify priming in the immediate offspring generation, but it could also be detected in the grandchildren of the infected plants. This demonstrates that priming memory can be inherited over at least one stress-free generation and eliminates the possibility that maternal effects alone are responsible for the increased resistance. Subsequent work from the same group now shows that priming can be detected, albeit weakly, even after two stress-free generations (Stassen et al. 2018), indicating that the phenomenon must be epigenetically regulated. In evolutionary terms, the gradual loss of priming after the initial stress episode would be expected in order to avoid excessive costs of priming and could readily be achieved through reversible epigenetic changes. In parallel with this work on transgenerational disease resistance, similar responses to herbivory were reported. terHorst and Lau (2012) found that both herbivore resistance and reproductive fitness were affected by parental exposure to insect herbivory in a field experiment with *Lotus wrangelianus*. Moreover, herbivory also resulted in JA-dependent transgenerational priming of defence against insects in both *Arabidopsis* and tomato (Rasmann et al. 2012).

2.4.2.2 Mechanisms for TGIP

The most likely mechanisms for true transgenerational priming effects are epigenetic. Similar to within-generation priming responses such as SAR, histone modifications could be detected in the promoters of the SA-regulated genes, *PR1*, *WRKY6* and *WRKY53* in plants derived from *P. syringae*-infected parents (Luna et al. 2012). Such histone modifications may well contribute to primed defence gene expression in TAR, but there is still wide debate over the roles that histone modifications might play in epigenetic inheritance between generations (Heard and Martienssen 2014). Much better understood is the ability of DNA methylation to be meiotically inherited, and DNA methylation has therefore been suggested as the more likely mechanism for encoding transgenerational epigenetic memory (Quadrana and Colot 2016). It has become increasingly clear over recent years that modifications to the DNA methylome can be maintained through the plant's lifespan and into subsequent generation(s), and can generate heritable phenotypic changes (Bossdorf et al. 2010; Johannes et al. 2009; Mathieu et al. 2007; Verhoeven et al. 2010). Accordingly, several studies have found that mutants affected in various regulatory mechanisms controlling DNA methylation show altered biotic stress resistance, and/or fail to establish transgenerational priming (López Sánchez et al. 2016; Luna et al. 2012; Luna and Ton 2012; Rasmann et al. 2012). These two modes of epigenetic regulation are not mutually exclusive, since histone modifications and DNA methylation are somewhat inter-dependent (Heard and Martienssen 2014).

The strongest current candidate for generating methylation-dependent stress memory is RNA-dependent DNA methylation (RdDM). As noted above, several studies have shown that mutations in genes involved in the RdDM pathway can have a direct impact on defence responses (López et al. 2011; Le et al. 2014; López

Sánchez et al. 2016; Luna and Ton 2012). RdDM-mediated methylation is initiated by the generation of 21–24 nt siRNAs by DICER-like proteins, and transgenerational priming responses to both biotic and abiotic stress have been found to require the production of siRNAs. Furthermore, offspring priming phenotypes triggered by different abiotic stresses in *Arabidopsis* required *DCL2* and/or *DCL3*, which are responsible for the production of 21/22 and 24 nt siRNAs, respectively (Boyko et al. 2010). Similarly, for biotic stress, the *dcl3–1* mutant failed to establish TAR against biotrophic pathogens (Luna and Ton 2012) and a *dcl2/dcl3/dcl4* triple mutant failed to establish transgenerational priming against herbivores (Rasmann et al. 2012). siRNAs are able to move systemically throughout the plant (Dunoyer et al. 2010; Lewsey et al. 2016; Molnar et al. 2010), and can therefore be viewed as candidates for long range priming signals both within and between generations, since reprogramming of methylation in germ line cells by siRNAs would allow inheritance by offspring tissues. Interestingly, sRNA populations have been reported to be significantly influenced by parental/grandparental environmental stress in both *Brassica rapa* and the dandelion, *Taraxacum officinale* (Bilichak et al. 2015; Morgado et al. 2017). Since pathogen infection triggers genome-wide methylation changes associated with reactivation of transposon sequences, generation of mobile siRNAs and subsequent RdDM presents a feasible mechanism for maintaining stress memories.

Active DNA demethylation also appears to be required for TAR. The DNA glycosylase ROS1 plays a major role in global demethylation, and was found to be essential for transgenerational memory in offspring of *Pst*-infected *Arabidopsis* (López Sánchez et al. 2016). Interestingly, the same authors found that ROS1 was not required for within-generation SAR. The nature of the changes imposed by ROS1-dependent demethylation and RdDM in response to biotic stress and the mechanisms by which they impact on defence phenotypes remain to be elucidated. Although methylome profiling of different generations of plants expressing TAR identified differentially methylated sites that correlate with ancestral stress experiences (Stassen et al. 2018), the analysis of these sites does not yet provide a clear insight into the mechanism of transgenerational priming.

2.4.2.3 Re-Setting Epigenetic Priming Memory

Epigenetically mediated transgenerational defence priming provides a novel system by which plants can display phenotypic plasticity in response to environmental stress, providing enhanced evolutionary fitness when parental environments are good predictors of offspring environments. To have evolved, the benefits of transgenerational phenotypic plasticity must outweigh any costs. Because plasticity is epigenetically mediated, it is readily reversible, such that when stress is not present, defence reverts to basal levels, thus minimizing costs. Costs would also be minimized when the signals initiating priming are good predictors of future stress. In other words, long-lasting priming would be expected only under strong, consistent stress, and should decay in the absence of stress. These predictions appear to hold

true in the limited instances where these ideas have been tested. Singh et al. (2014a) found that repeated, but not single, mild stress exposures provided short-lived priming of biotic stress resistance but was not sufficient for long-term priming. TAR induced by *P. syringae* infection of *Arabidopsis* was maintained at high levels when successive generations of plants were inoculated, but was gradually lost when only a single generation suffered disease (Stassen et al. 2018). The costs of transgenerational priming remain relatively poorly explored, but one clear cost is seen in the antagonism between the major SA- and JA-dependent defence pathways. TAR against biotrophic pathogens was associated with transgenerational increased susceptibility to necrotrophic pathogens (Luna et al. 2012), while transgenerational priming of herbivore resistance caused increased susceptibility to *P. syringae* infection (Singh et al. 2017). The storage of epigenetic marks reflecting stress memories of previous generations could therefore have deleterious impacts when offspring experience different stresses than their parents (Crisp et al. 2016; Iwasaki and Paszkowski 2014). Avoidance of such maladaptive memories requires a system for re-setting of epigenetic stress memories that can balance the forces imposing them. As an example to keep in mind, the *Arabidopsis FLC* locus, involved in vernalization, is epigenetically silenced in response to low temperature. The low temperature memory is re-set each generation, meaning that it is the experience of individual plants, not their ancestors, that determines flowering time. Mutation of the *ELF3* gene, a histone H3K27me3 demethylase, causes a failure of the ability to re-set the low temperature signal, meaning that offspring flower early regardless of environmental conditions (Crevillén et al. 2014). Other re-setting systems have also been identified. Two chromatin regulators, *DECREASE IN DNA METHYLATION1* (*DDM1*) and *MORPHEUS' MOLECULE1* (*MOM1*), prevent the transmission of memories of heat stress exposure from parents to their progeny. Genome-wide transcriptional signatures induced by stress were found in the subsequent generation in *ddm1/mom1* double mutants, but not wild-type plants (Iwasaki and Paszkowski 2014). More recently, MOM1 has also been identified as an epigenetic regulator of the expression of pattern recognition receptor (PRR) and nucleotide-binding leucine-rich repeat (NLR) genes in *Arabidopsis*. PRRs and NLRs act as receptors for activation of PTI and ETI, respectively, and therefore changes in their expression could potentially be a mechanism for defence priming. *MOM1* indirectly regulates PRR/NLR gene expression via an RdDM-dependent pathway for transposon silencing. Mutants deficient in *MOM1* displayed higher expression of PRRs/NLRs and were more resistant to bacterial infection (Cambiagno et al. 2018), consistent with the idea that *MOM1* might function antagonistically with defence priming. Such mechanisms of chromatin re-setting could prevent or constrain transgenerational priming of defence to optimize trade-offs between the costs and benefits of priming.

2.5 Potential Application of Epigenetics

World population is growing at a speed that places food security at risk at a global level (FAO 2009a). In this context, increasing crop yields by reducing losses caused by pests and diseases would appear to be essential. At the same time, the use of pesticides, which have had a major influence on protecting yields in the past, has been demonstrated to considerably contribute to soil degradation, ecosystem disturbance, climate change and even to have a negative impact on human health. Given this scenario, global entities are taking action to promote the development and implementation of alternative crop protection technologies and strategies (European Academies Science Advisory Council and Deutsche Akademie der Naturforscher Leopoldina 2014). Although when its impacts were first recognized, epigenetics represented an inconvenience in biotechnology, for example, because of unintended gene silencing effects (Napoli et al. 1990; van der Krol et al. 1990), today, the field of epigenetics presents a clear example of the importance of fundamental research in future applications (Connor 2002). On the one hand, advances in the understanding of epigenetic mechanisms have already brought novel tools for biotechnologists to analyse, control and tailor gene expression. On the other hand, the possibility to produce new stable (even heritable) phenotypes in the absence of genetic changes opens doors to new approaches avoiding transgenesis, which has important implications in view of the current regulatory framework around genetically modified organisms (GMOs) and their public perception. Moreover, boosting the plants' natural immune system has been suggested as the safest approach to improve crop yields while minimizing environmental impacts (Rapicavoli 2015; Dewen et al. 2017; Kothari and Patel 2004; Quintana-Rodriguez et al. 2018).

Bearing in mind that the origin of the epigenetic machinery seems to be linked to ancestral defence mechanisms, we believe the application of epigenetics to crop protection could provide significant breakthroughs in the development of future integrated pest management programs (Stenberg 2017). Here, we discuss some possible applications to translate the new knowledge of epigenetics to first, keep broadening our understanding of natural phenomena, and second, as a tool to a new generation of plant biotechnologists and breeders in the field of crop protection.

2.5.1 As a Tool in Research

Advances in the understanding of epigenetic mechanisms have significant potential to impact the future research of plant–pathogen interactions. Up to now, such understanding has enabled the development of various techniques to characterize and even manipulate the epigenome. Nowadays, we can detect small RNAs by northern blotting, hybridization with probes for detection and subtraction, PCR/qPCR, etc. (Boccara et al. 2017; Li and Zamore 2018; Ro and Yan 2010; Urbanek et al. 2015). Using the new advances in bioinformatics we can also sequence and map sRNAs in

different genomes, compare them in different species or even design specific systems to knock them down. Chromatin changes can be analysed by different techniques such as chromatin immunoprecipitation (ChIP; Furey 2012), DNase I treatments (Cockerill 2011), FAIRE (Simon et al. 2012) and chromatin conformation capture (3C; Dekker et al. 2002). There have also been enormous advances in the analysis of DNA methylation as a result of the development of bisulphite sequencing, which enables the detection of changes in DNA methylation at the level of individual cytosine residues across the whole genome. Other techniques, like ChIP analysis using antibodies against methyl-cytosines (MeDIP-ChIP), allow for the detection of regional differences in DNA methylation (Cortijo et al. 2014b). MeDIP-ChIP has lower resolution than bisulphite sequencing, but it can be used as an affordable method to detect regional differences in the whole genome, or, coupled to PCR, for the detection of differential methylation in discrete regions, which can be helpful in species for which the full genome cannot be sequenced. Another important set of tools is based on the differential activity of restriction enzymes in methylated and non-methylated DNA. This property of the endonucleases can be used for the detection of the whole-genome methylation level by southern blotting, in which no or minimum information about the DNA sequence is required. It can also be used in the design of chop PCR markers, which allow us to detect changes in DNA methylation at specific positions quickly and economically.

Currently, we can not only detect, but also modify the levels of DNA methylation. For example, following the identification of the proteins of the DNA methylation machinery, mutant plants are available with different and relatively stable patterns of DNA methylation that can be used for research and applied purposes. There are also several chemical reagents known that alter the epigenome. For example, 5-azacytidine (5-azaC), zebularine and sulfamethazine reduce the general levels of DNA methylation (Jones 1985; Zhang et al. 2012; Zhou et al. 2002). More targeted manipulation of the epigenome—epigenome editing—is now becoming possible as a result of new advances in molecular biology. By fusing proteins with DNA methylation/demethylation activities to sequence-specific DNA binding proteins such as zinc finger nucleases or the CRISPR/dCas 9 system, it is possible to target methylation/demethylation to specific target sites (Gallego-Bartolomé et al. 2018). Hence, our knowledge of epigenetics made it possible to develop these techniques, and we are now in a position to use them to speed up progress in understanding mechanisms of plant defence.

Now, we can also understand phenotypes that were baffling for decades, as they involved changes in gene expression in the absence of any change in DNA sequence. The use of the aforementioned techniques is accelerating the discovery of the epigenetic mechanisms controlling plant defence. It has opened the door to the identification of new natural epialleles, as well as the understanding of part of the observed natural variation found between species and ecotypes (Cubas et al. 1999; Niederhuth et al. 2016; Richards 2011; Turck and Coupland 2014; Vaughn et al. 2007; Zhai et al. 2008). It has also contributed to unravelling the evolution of the plant immune system. Since the plant immune system has been demonstrated to be controlled by epigenetic mechanisms at almost all levels, the study of the epigenotype will be

essential for the future understanding of plant defences and the translation of this new knowledge into crop protection strategies.

2.5.2 As a Tool in Biotechnology

Crop losses due to pests and diseases must be reduced to a minimum to ensure food security and sustainability in the coming decades. World population is expected to grow up to 9.7 billion by 2050. Thus, improving crop yields is a priority action for the world's public entities in order to produce more food on less land, avoiding the over-exploitation of natural ecosystems and the decline in biodiversity (FAO 2009b). At the same time, global climate change is introducing abiotic stress variables that promote outbreaks of different plant pathogens. To date, the use of pesticides has been the most successful agronomic strategy to protect crops from infections. Unfortunately, pests tend to easily acquire resistance to chemical pesticides. Moreover, the use of pesticides contributes to the disruption of natural ecosystems, degrading soils and contaminating water sources. It has also been demonstrated to be potentially toxic or carcinogenic, at the very least for workers who manipulate the products (Aktar et al. 2009). This provides additional impetus in the search for alternative strategies (European Commission 2009). Moreover, while biotechnologists focused their efforts on genetic engineering of plants to introduce new traits, there has been a strong public pressure against transgenesis and other forms of genetically modified organisms, especially in Europe (Collinge et al. 2010). With this in mind, some experts in the field agree that plant vaccination and priming (or plant immunization) is one of the most promising approaches in pursuit of designing safer and more sustainable crop protection programs (Kothari and Patel 2004; Rapicavoli 2015; Dewen et al. 2017; Quintana-Rodriguez et al. 2018).

As this chapter has described, the plant immune system and priming processes are epigenetically controlled at different levels. Given that it facilitates phenotypic changes while avoiding transgenesis, epigenetics appears to be an attractive tool/target for breeders and agronomical biotechnologists hoping for a new and more sustainable green revolution.

2.5.2.1 PDR: Pathogen-Derived Resistance

The first examples of the exploitation of epigenetics for plant protection were seen in the form of pathogen-derived resistance (PDR) or 'plant vaccination'. It had been known for some time that infections with some viruses triggered cross-protection against similar viruses (Hamilton 1980). The scientific basis for this observation was unknown for a long time, but resistance was assumed to be triggered by the production of viral components. Thus, PDR was proposed as a biotechnology strategy for plants in which pathogen-specific components could be altered and over-expressed in a non-functional form, or at the wrong developmental stage, to

somehow compete with the functional proteins produced by the virus (Sanford and Johnston 1985). Following the suggestion of this concept, there were a few successful cases of protein-mediated resistance (based on viral coat protein expression) that were probably unrelated to epigenetic mechanisms (Abel et al. 1986; Powell et al. 1990). In all cases, the mechanism involved the production of transgenic plants over-expressing genes (or modified versions of them) from the target pathogen. Surprisingly, in many subsequent cases, this approach was discovered to not be always associated with the levels of proteins of the transgene, but rather, RNA expression. In fact, instead of protein-mediated resistance, RNA-mediated resistance appeared to provide near complete immunity against, at least, the target virus. The basis of such RNA-mediated resistance remained obscure until it was linked with the co-suppression phenomenon observed in transgenic petunia plants (Lindbo et al. 1993). It then became clear that in most viral PDR cases, protection was due to the activation of gene silencing mechanisms against the pathogenic virus. With the discovery of the epigenetic mechanisms, PDR mediated by RNA expression has been improved (Prins 2003), optimizing the silencing of the pathogen. RNA silencing-mediated PDR is also now known as host-induced gene silencing (HIGS), which involves plant transformation with constructs expressing efficient siRNAs targeting different pathogen elements.

This strategy has been demonstrated to be effective not just against viruses in model organisms, but also other groups of pathogens and herbivores and in different crop species (Huang et al. 2016). Since its first uses in tobacco and *Arabidopsis*, PDR has been successfully applied to introduce virus resistance to crops such as papaya (against the papaya ringspot virus, PSRV; Gonsalves 1998; Jia et al. 2017), squash (against the Squash leaf curl virus; Taha et al. 2016), tomato (Schwind et al. 2009), cotton, rice, maize, corn, wheat and barley (Duan et al. 2012; Huang et al. 2016; Koch and Kogel 2014). Beyond its uses in plant-viral protection, it has been adapted to protect plants against many other different pest and diseases such as bacteria, fungi (Nowara et al. 2010; Nunes and Dean 2012), parasitic plants, nematodes or insects (Koch and Kogel 2014). Perhaps most remarkable is the work performed in plant–insect interactions, where HIGS-mediated resistance has been demonstrated following the ingestion of plant-produced siRNAs during insect feeding (Baum et al. 2007). Undoubtedly, the latest discoveries involving cross-kingdom sRNA trafficking will help us to better engineer PDR and/or HIGS, optimizing this kind of plant vaccination (Wang et al. 2016, 2017).

In some cases, PDR confers almost complete resistance. However, it also poses some problems. The first issue is that it involves transgenesis, which faces strong social opposition and is under the control of very restrictive legislation (Lucht 2015). On the other hand, alongside the benefits of resistance, there could be some deleterious consequences in the plant, due to the insertion and over-expression of the constructs. For example, the genomic insertions could disturb genic regions required for specific plant functions in response to environmental changes, which is difficult to predict or identify in field trials. A strong activation of silencing mechanisms could also have consequences for the natural roles of these pathways during plant development or responses. Finally, the expression of the transgene can be

modified by environmental changes, or even silenced due to its high expression (Martelli 2001; Zhao et al. 2014). Therefore, even though its high potential has been demonstrated for several decades, its success usually requires an important investment of time and optimization effort, which has restricted its successful commercial application to a relatively small number of specific cases.

2.5.2.2 Epigenetics Mediated Resistance

Since the role of DNA methylation in plant defence has only emerged relatively recently, commercial approaches that exploit epigenetics have not yet reached the market. However, the possibility to select or engineer plants at the epigenetic level to improve resistance without transgenesis makes this one of the most fashionable current strategies that is being actively pursued. In the same way that breeders have been searching for disease resistance genes in crop wild relatives and land races to develop introgression systems, we now know that a proportion of natural phenotypic variability is generated by the epigenome (Zhang et al. 2013). Given that some DNA methylation patterns appear to be stably inherited through meiosis, an attractive idea is the use of natural epigenetic alleles (epialleles) to provide traits for classical selective plant breeding (Gallusci et al. 2017; Hofmeister et al. 2017; Zhang and Hsieh 2013). In this respect, epialleles impacting on defence phenotypes from different backgrounds could be identified and introgressed (Ji et al. 2015).

There are a number of methods by which such epialleles can be identified. The first is through simple screening of populations of a single genotype for phenotypic variation. Repeated selection of an isogenic population of *Brassica napus* for individuals with elevated energy use efficiency (EUE) resulted in the isolation of 'epilines' with improved EUE and which gave increased yield (Hauben et al. 2009). The improvement in EUE appeared to come from altered DNA methylation profiles rather than genetic changes was inherited for eight generations and was stable over 3-year field trials. Later work used a similar strategy to isolate stable epilines with increased EUE and drought tolerance (Verkest et al. 2015). These lines showed increased expression of abiotic stress-related genes, and elevated H3K4me3 methylation at those genes. As well as simply screening for naturally occurring epigenetic variation, it is possible to create recombinant populations in which individual lines carry substantial portions of the genome with altered methylation patterns. Such lines are known as epigenetic recombinant inbred lines (epiRILs). EpiRILs can be generated from two varieties or ecotypes with similar genetic sequences but different epigenetic landscapes (Kawakatsu et al. 2016; Schmitz et al. 2013). The approach for isolating useful epialleles that provide novel traits assumes that novel methylation patterns carried by an epiRIL can be stably inherited and used for crop breeding. One method for generating novel epigenetic patterns is the generation of epiRILs by crossing mutants defective in DNA methylation machinery. Homozygous mutants develop a different epigenetic landscape (e.g. hyper- or hypomethylation), and following removal of the original genetic mutation by back-crossing, the resulting recombinants would carry a mosaic of different epigenetic marks at different

locations across the genome. This has already been applied successfully in *Arabidopsis* (Cortijo et al. 2014a; Johannes et al. 2009; Reinders et al. 2009), and several groups are currently working in epiRILs in crops like tomato, wheat and rice. Genome-wide epigenetic variability can also be induced by the application of DNA methylation inhibitors like sulfamethazine, 5-azacytidine (5-azaC) and zebularine (Jones 1985; Zhang et al. 2012; Zhou et al. 2002), or by introduction of novel DNA methylase/demethylase enzymes (Hollwey et al. 2017). An interesting alternative is to use natural instances of epigenome reprogramming. For example, plants regenerated from tissue culture often exhibit significant phenotypic variation, despite being clonal and therefore, in principle, identical at the DNA sequence level. This is known as somaclonal variation. Wibowo et al. (2018) recently found that Arabidopsis plants regenerated from root (but not leaf) cells were more susceptible to *Pst* and *Hpa* infection than non-regenerated controls. Genome-wide DNA methylation profiles also differed with the origin of regenerated plants and altered methylomes were inherited for at least three generations. Finally, where a priori information exists about desirable epigenetic traits, modifications at specific target loci can be achieved through the use of epigenome editing (Gallego-Bartolomé et al. 2018; Kungulovski and Jeltsch 2016) as described above. Despite the novelty of breeding with epialleles, it should not be forgotten that as for other forms of genetically encoded resistance, systems that result in constitutively elevated defences are likely to bring with them costs in yield in the absence of stress, due to the expense of allocating resources to defence. In nature, such costs are minimized by priming of defence, which could therefore be an attractive trait to target.

2.5.2.3 Epigenetic Priming

With the exception of the epigenome editing at specific sites, the approaches described above rely on natural or introduced random epigenetic variation as a source of natural variation from which to select desired traits, such as pest and disease resistance. However, the phenomenon of transgenerational immune priming described above, suggests that epigenetic resistance can be induced by exposure of plants to biotic stress. The primed state involves sensitization of defence responses following initial triggering of priming, but primed plants do not express higher levels of defence prior to attack by pests or pathogens, avoiding costs (van Hulten et al. 2006). Priming is therefore probably more similar conceptually to vaccination or immunization in vertebrates (Hilker et al. 2016; Mak et al. 2014) than PDR, as it involves a certain memory of the first stress-indicating signal. Priming falls within the concept of natural plant immunization, which has been suggested as one of the new targets for the next green revolution (Dewen et al. 2017; Quintana-Rodriguez et al. 2018).

The long-lasting nature of priming is one of its strengths for crop protection. Importantly, across shorter to longer periods, priming memory has been correlated with epigenetic changes (Jaskiewicz et al. 2011; López et al. 2011; Luna and Ton 2012). In fact, the majority of *Arabidopsis* mutants impeded in de novo DNA

methylation show resistance against biotrophic pathogens, but do not constitutively express defences. Rather, they show a constitutive priming phenotype. The lower cost of priming as a defence strategy is evident as such mutants do not usually show alterations in growth or development under control conditions. Thus, the already mentioned induced changes in the epigenetic landscape by the use of mutants, chemical compounds or epigenetic editing could be applied to trigger the epigenetic changes that induce the priming state. One successfully commercialized example is the use of seed treatments with elicitors of defence. Seed treatment with JA or BABA provides long-term priming of pest and disease resistance with either no or minimal costs in terms of growth and development (Paudel et al. 2014; Worrall et al. 2012). Obviously, the most natural priming induction would be to moderately stress plants in order to induce long-lasting priming, or even transgenerational priming. This approach does not involve the use of transgenesis, chemical treatments or any artificial systems, does not involve the constitutive induction of defences and is long-lasting. Priming should also be a sustainable approach, since it is not expected to artificially impact other species or the physicochemical properties of natural ecosystems. Moreover, it is expected to be more resilient to environmental changes as it is naturally responsive to them. Unfortunately, the phenotype induced by these methods is very variable and our poor understanding of the underlying mechanisms prevents the design of molecular markers to track resistance at the current time. Undoubtedly, more research is needed in this promising field to make it feasible and widely applicable. Advances in fundamental understanding, including the interactions between multiple stresses, durability of priming and the development of markers to trace the epigenetic changes are all needed to optimize its application in future crop protection programs.

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