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Chapter 5
Tomato Epigenetics: Deciphering the "Beyond" Genetic Information in a Vegetable Fleshy-Fruited Crop

Fabio T.S. Nogueira

Abstract The first natural plant mutant for which the molecular basis was determined to be an epimutation rather than a change in DNA sequence was a peloric variant of toadflax, *Linaria vulgaris*. Remarkably, the second example of a natural epimutant came from the vegetable fleshy-fruited crop tomato (*Solanum lycopersicum*). The discovery of the molecular basis for the *Colorless nonripening* (*Cnr*) epimutation was a landmark for plant epigenetics and, importantly, linked epigenetic mechanisms with an important agronomical trait. More recently, several studies on tomato have contributed to our better understanding of epigenetic mechanisms underlying important heritable crop traits, such as ripening and stress response. Epigenetic mechanisms have also been associated with transgressive segregation in hybrids generated from crosses between cultivated tomato and close wild relatives. Therefore, we can only envision that tomato will became a model for studying the epigenetic basis of economically important phenotypes, allowing for their more efficient exploitation in plant breeding.

Keywords Tomato • Small RNAs • DNA methylation • Epiallele

5.1 Introduction

Tomato (*Solanum lycopersicum*) is a major vegetable fleshy-fruited crop, accounting for 14 % of the world vegetable production. Over 100 million metric tons/year, a $1.6 billion market, were produced in 2010 (FAO 2013). Tomato is a rich source of micronutrients for human diet and its fruits can be used either for fresh consumption or for processing. It is also an important model species for research on fruit development and metabolite accumulation.

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Tomato belongs to the large and diverse *Solanaceae* family also called Nightshades, which includes more than three thousand species from several habitats. Among them, major crops arose from the "Old World" (Eggplant from Asia) and the "New World" (pepper, potato, tobacco, and tomato). The *Lycopersicon* clade contains the domesticated tomato and its 12 closest wild relatives (Peralta and Spooner 2005). Tomato originated in the Andean region of the Americas, and its domestication is thought to have taken place in Central America (Bai and Lindhout 2007). Domesticated tomato has been bred to improve productivity, fruit quality, and resistance to biotic and abiotic stresses, most of which are agronomically key traits for several crops. Modern cultivars are commercialized as hybrids with high performance in the field.

In spite of its importance as a crop and as a model plant for research, only recently the genome of domesticated tomato was sequenced (The Tomato Genome Consortium 2012). Tomato chromosomes contain pericentric heterochromatin and distal euchromatin, with repeats concentrated within and around centromeres, in chromomeres and telomeres (The Tomato Genome Consortium 2012). Interestingly, tomato has fewer high-copy, full-length long terminal repeat (LTR) retrotransposons when compared with *Arabidopsis thaliana* and *Sorghum bicolor* (The Arabidopsis Genome Initiative 2000; Paterson et al. 2009). This data supports previous findings that tomato genome is largely comprised of fast-evolving, low-copy DNA (Zamir and Tanksley 1988). This unique feature is likely to play an important role in tomato breeding.

A new step for understanding how the tomato genome "behaves" and evolves and its implication in tomato breeding and genetic control of agronomical traits is coming from next generation sequencing techniques. Such techniques allow the identification of not only genetic but also epigenetic "players". As an example of the latter, information from high throughput sequencing of tomato small RNA (sRNA) populations suggests that most sRNAs map preferentially to the euchromatin portion of its genome, which is contrasting to what is generally observed in Arabidopsis. Differential expression of tomato sRNAs was observed during fruit development and they apparently mapped to a number of gene promoters, including those of genes associated with cell-wall biogenesis (The Tomato Genome Consortium 2012). These sRNAs may function as "triggers" to generate epigenetic modifications that likely affect gene regulation and genome stability. Indeed, it is well established in model plants, such as Arabidopsis, that epigenetic modifications of the DNA and histones serve as heritable marks that can influence gene expression states. Therefore, deciphering the tomato epigenome and its function may help to identify candidate genes for tomato improvement, should epigenetic variants be discovered.

In this chapter I will first highlight the main findings on tomato epigenetics until today. I will then discuss how we may combine valuable information regarding epigenetic and genetic natural variation to help to improve the future of tomato breeding.

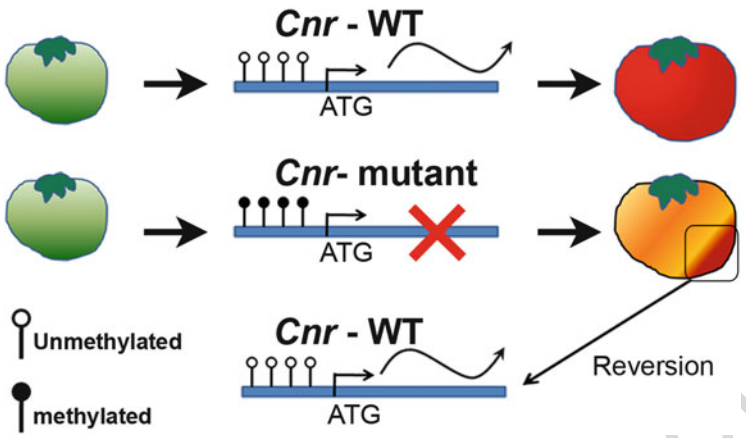

Fig. 5.1 Graphic representation showing how the natural epiallele *Cnr* prevents ripening, resulting in yellow fruits. Such epiallele is the result of changes in methylation status on CpG and CpHpG regions within the promoter and 5′-UTR of *SBP3*-like/*CNR* gene. Interestingly, some occasional revertant 'ripening' sectors that have a wild-type ripening phenotype are observed in mutant fruits

5.2 Epigenetic Studies on Tomato 71

5.2.1 DNA Methylation and Histone Modifications 72

Given that only a few spontaneous epimutations have been described in plants 73
(Cubas et al. 1999; Kalisz and Purugganan 2004), the finding that tomato natural 74
mutant *Colorless non-ripening* (*Cnr*) is due to an epimutation was unexpected 75
(Thompson et al. 1999). Although the dominant pleiotropic mutation *Cnr* was 76
described in tomato more than a decade ago, only recently its epigenetic "nature" 77
was revealed (Thompson et al. 1999; Manning et al. 2006). *Cnr* epiallele inhibits 78
normal ripening and produces a severe phenotype by which fruits develop a 79
colorless, mealy pericarp. Such phenotype is due to an absence of ripening-related 80
carotenoid biosynthesis and modifications in the cell wall structure of the pericarp 81
(Eriksson et al. 2004). *Cnr* epiallele corresponds to the *SBP3*-like (*SQUAMOSA* 82
promoter binding protein3-like) gene (Solyc02g077920), a tomato SBP-box family 83
member (Salinas et al. 2012). The SBP-box family of transcription factors is unique 84
to plants and their members are characterized by a highly conserved SBP domain of 85
approximately 76 amino acid residues, involved in DNA binding and nuclear 86
localization (Preston and Hileman 2013). 87

In *Cnr* mutant, the epigenetic allele of *SBP3*-like/*CNR* gene is heavily methyl- 88
ated mostly in a 300 bp region located approximately 2 kb upstream of the ATG 89
(Fig. 5.1), while its wild-type counterpart is not. Given that hypermethylation in 90
upstream sequences is generally associated with gene silencing (Seymour 91
et al. 2008), modifications in the methylation status likely explain the reduced 92

SBP3-like/*CNR* expression in *Cnr* fruits. Moreover, in non-mutant or wild-type plants, the promoter of *SBP3*-like/*CNR* appears to be demethylated just prior to the onset of ripening. Such observation led to the hypothesis that DNA methylation contribute to the regulation of fruit ripening (Seymour et al. 2008). *Cnr* epimutation is stable over generations as few revertants were observed (Manning et al. 2006), implying that epigenetic modifications were inherited in a Mendelian fashion and resulted in the suppression of *SBP3*-like/*CNR* transcription during fruit development. While the nature of the epimutation in the *Cnr* mutant is well established, the possible causes for the appearance of this epialelle are less understood. Interestingly, in the mutant, most of the methylated cytosines are in a symmetrical sequence context (CpG, CpHpG, where H is A, C or T), which is generally maintained by METHYLTRANSFERASE1 (MET1) and CHROMOMETHYLASE3 (CMT3) methyltransferases in Arabidopsis, respectively (Martienssen and Colot 2001; Lindroth et al. 2001).

In silico survey in Sol Genomics (http://solgenomics.net) suggests that tomato has one *MET1* homolog, which is located at chromosome 11. Two possible homologs of *CMT3* in the tomato genome are located at chromosomes 1 and 12 (Table 5.1). Expression profiles retrieved from RNA-seq data of the Tomato eFP Browser (http://bar.utoronto.ca/efp_tomato/cgi-bin/efpWeb.cgi) showed that *MET1* and *CMT3* homologs are lowly expressed in "Breaker fruit" stages while *SBP3*-like/*CNR* is highly expressed (Fig. 5.2). Future studies are needed to address whether tomato MET1 and CMT3 enzymes are indeed involved in the generation of the natural *Cnr* epialelle.

Some clues regarding possible causes of the epimutation in the *Cnr* mutant allele may come from evaluating *CNR*, *MET1*, and *CMT3* loci in different genetic backgrounds. For example, *Cnr* epialelle arose in tomato Liberto background, in which the DNA in the *SBP3*-like/*CNR* genomic region showed an increased predisposition for methylation in comparison with that from Ailsa Craig background (Thompson et al. 1999; Manning et al. 2006). Therefore, one can speculate that the Liberto cultivar is more likely to give rise to *Cnr* mutant plants than the Alisa Craig cultivar. Additionally, Liberto cultivar is more similar in this respect to fruits from *Lycopersicon cheesmanii* (Manning et al. 2006). *L. cheesmanii* is one of the wild tomato species endemic to the Galapagos archipelago and exhibits a range of peculiar phenotypes when compared with cultivated tomato (Arkive 2013). Particularly, *L. cheesmanii* '*long*' displays bright orange-yellow fruits (Nuez et al. 2004). It will be fascinating to evaluate whether fruit phenotype in this wild relative is a result of *SBP3*-like/*CNR* genomic region being more prone to changes in methylation status during fruit development and ripening than cultivated tomato. It is feasible that the fruit phenotype in this species may be a result of epigenetic-driven modifications in the expression of *SBP3*-like/*CNR* locus. Assuming that such modifications can be confirmed, they must be the product of Darwinian evolution, which would have produced the (epi)genetic mechanisms that underlie these effects on DNA methylation status in specific loci.

Is it possible that other tomato loci are also prone to changes in methylation status during fruit development? In other words, could we identify novel epialelles

Table 5.1 Tomato cytosine-5 DNA methyltransferases

Protein name	Putative function	Locus no.	Chromosome
MET1	Maintenance of CpG methylation	Solyc11g030600	11
CMT3-like	CpHpG methylation in repetitive DNA and transposons in heterochromatin	Solyc12g100330	12
		Solyc01g006100	1
DRM-like[a]	De novo: CpG, CpHpG, CpHpH	Solyc02g062740	2
	Maintenance: CpHpG, CpHpH	Solyc10g078190	10

[a]Domains-rearranged methyltransferases-like proteins

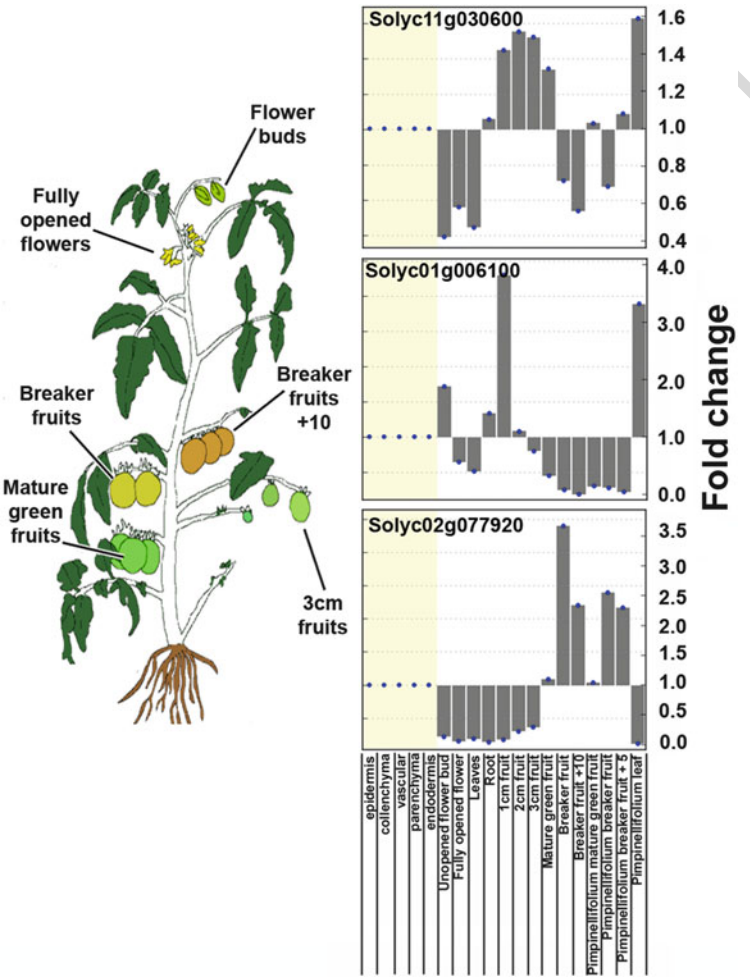

Fig. 5.2 Expression profiles of *SBP3*-like/*CNR* (Solyc02g077920), *MET1* (Solyc11g030600), and *CMT3-like* (Solyc01g006100) genes in different tissues and organs. The figure was generated using RNA-seq data from Tomato eFP Browser (http://bar.utoronto.ca/efp_tomato/cgi-bin/efpWeb.cgi). Adult tomato plant showing tissues/organs analyzed is shown in the *left panel*

associated with natural changes in fruit development and ripening? A promising answer for this important biological and agronomical question may come from genome-wide analyzes of the DNA methylation status during fruit development and ripening. Recently, Zhong et al. (2013) provided the first insights into the link between the fruit ripening genetic program and DNA methylation state. After injecting a chemical inhibitor of cytosine methylation, 5-azacytidine, the authors performed whole-genome bisulfite sequencing in four stages of fruit development, from immature to ripe, identifying more than 50,000 differentially methylated regions (representing 1 % of the tomato genome). The sequencing of these epigenomes provided, among others, one crucial finding: in wild-type fruits, the degree of methylation of promoter regions decreased progressively along fruit development (Zhong et al. 2013). Several of these promoters belong to typical ripening-related genes, implying that potential epialleles associated with ripening and fruit quality might arise during breeding programs that use distinct genetic backgrounds and growing conditions.

Evidence so far suggests a key role of the epigenome structure and developmental dynamics in coordinating tomato fruit ripening. Such evidence include data showing that binding of the MADS-box transcription factor RIPENING INHIBITOR (RIN)—a key regulator of ripening (Vrebalov et al. 2002)—to a set of promoters was inhibited in the *Cnr* background, suggesting that promoter hypermethylation blocks RIN binding (Martel et al. 2011). Progressive demethylation of ripening-related gene promoters seems to be necessary for binding of transcriptional regulators (such as RIN), thus triggering the accumulation of ripening-related transcripts (Martel et al. 2011). Intriguingly, Zhong et al. (2013) observed that binding sites for the RIN transcription factor are hypermethylated in the *rin* loss-of-function mutant, which suggest that promoter methylation status of some genes may be altered by the binding of the transcription factors themselves. Similar results were observed for the mouse epigenome (Stadler et al. 2011). Nonetheless, the mechanism(s) underlying demethylation of gene promoters during wild-type fruit development remain(s) unclear and further efforts are needed to unravel additional endogenous and/or exogenous cues that contribute to this epigenetic modification. In summary, it seems that tomato fruit cells take advantage of epigenome reprogramming along with fruit-specific transcription factors to regulate the fruit transition into a ripening-competent state when the seeds become viable.

Among the three main phases that precede tomato fruit ripening (Gillpasy et al. 1993), phase III corresponds to the developmental stage in which fruit grows basically due to cell expansion concomitant with a dramatic increase in nuclear ploidy level, a process termed endoreduplication (Joubès et al. 1999). Endoreduplication could lead to variation in DNA methylation in specific fruit tissues. To evaluate the possible correlation between endoreduplication and methylation status in fruit tissues, Teyssier et al. (2008) employed Southern experiments with methyl-sensitive restriction enzymes along with HPLC analysis to demonstrate tissue-specific variation in DNA methylation levels. The authors observed an increase in CpG and/or CpHpG methylation at specific loci (mostly repetitive sequences and retransposons) in pericarp genomic DNA during fruit development.

Interestingly, a sharp decrease of the global DNA methylation level was also observed in pericarp during the onset of the fruit ripening, which is consistent with the methylome data from Zhong et al. (2013). Conversely, no major variation of DNA methylation either global or locus-specific was observed in locular tissue, which could reflect tissue-specific variations of DNA methylation during fruit development and ripening (Teyssier et al. 2008). The reasons for tissue-specific differences in DNA methylation are still obscure, but it is unlikely to be triggered by the induction of endoreduplication in fruit tissues. For instance, cytosine methylation did not increase significantly in locular tissue at the loci analyzed by the authors, although their nuclei were highly endoreduplicated (Teyssier et al. 2008). Therefore, it seems that an increase in endoreduplication is not necessarily followed by an increase in DNA methylation in all tomato fruit tissues, though the authors did not verify this fact by using whole-genome bisulfite sequencing. As mentioned before, the mechanisms underlying the differential DNA methylation in developing fruits are still not elucidated. However, it is possible that differential and tissue-enriched expression of specific DNA methyltransferases (Table 5.1) during fruit development (Fig. 5.2) may be partially responsible for the DNA methylation patterns observed (Teyssier et al. 2008).

An appealing connection between plant epigenetics and stress was hypothesized by the Kovalchuk group in Arabidopsis and experimentally supported in rice, in which at least some stress-induced phenotypes depend upon altered DNA methylation (Boyko and Kovalchuk 2008; Wang et al. 2011). Recent findings in tomato are consistent with such conjectures. González et al. (2011) investigated DNA methylation within gene bodies by evaluating the distribution of cytosine methylation in *Abcisic acid stress and ripening1* (*Asr1*), a tomato water stress-inducible gene of the *LEA* (*late embryogenesis abundant*) superfamily. Similarly to data from Arabidopsis, it was found in tomato that DNA methylation at CpG sites within plant gene bodies is not necessarily associated with silencing as it is in animals (Zhang et al. 2006; González et al. 2011). Indeed, dehydration stress incited higher CpG methylation levels in the first exon of the *Asr1* gene, concomitant with enhanced gene expression. However, tomato plants under drought stress displayed removal of methyl marks at approximately 70 % of asymmetric CpHpH (where H is A, C or T) sites and a decrease of the repressive histone H3K27me3 epigenetic mark and an induction of expression of the same gene. Interestingly, most demethylated sites were present in intronic regions of the *Asr1* gene (González et al. 2011). These sites may be targets for RNA-directed DNA methylation (RdDM) as it has been demonstrated that intron-derived siRNAs mediate DNA methylation of their host genes (Chen et al. 2011). Although the authors did not check whether intronic regions of the *Asr1* gene have potential to form internal hairpin structures, these structures—if present—could produce siRNAs to mediate RdDM of *Asr1* in *cis*.

The same research group has recently published a related study on the *Asr1* paralog, *Asr2*, which has been a target for positive selection during the evolution of the *Solanum* genus in arid environments (González et al. 2013). Similarly to *Asr1*, loss of DNA methylation and the repressive histone H3K27me3 epigenetic mark were observed in the gene body and regulatory regions of *Asr2* under stress

conditions. Taken together, these two studies suggest that rapidly acquired novel epialleles of stress-related genes due to desiccation might be an alternative mechanism for plant adaptation to environmental drought conditions, not only in Arabidopsis but also in species with larger and more complex genomes such as tomato.

The finding that CpHpH methylation in tomato can occur in the body of stress-associated genes lacking repeated sequences, may represent an alternative mechanism for the stress-driven gain or loss of epigenetic marks that regulate gene expression in plants. DNA methylation within gene bodies in plants is emerging as an important epigenetic modification, as it regulates gene expression and plant development in some cases, though how those mechanisms operate remains elusive (Teixeira and Colot 2009).

How epigenetic states of gene activity are maintained steadfastly throughout consecutive rounds of cell division is one of the central questions in developmental biology. Investigations in metazoans, plants and microorganisms suggest an important and conserved role of the DDB1-CUL4-based ubiquitin E3 ligase complex in perpetuating epigenetic marks on chromatin, most likely via regulating histone modification or/and DNA methylation (Higa et al. 2006). This complex contains the adapter protein DDB1 (UV-damaged DNA binding protein 1) that binds to UV-damaged DNA and participates in DNA repair pathways at the stage of binding and recognition (Chu and Chang 1988). Recently, a study on tomato DDB1 suggested that this protein plays an important role in regulating the epigenetic state of genes controlling organ size, growth habit, and photosynthesis (Liu et al. 2012; Tang et al. 2012). Transgenic plants overexpressing an alternatively spliced tomato *DDB1* transcript, $DDB1^F$, displayed reduced organ size and a decrease in DNA methylation level at the *SlWEE1* gene (*Solanum lycopersicum WEE1*), a negative regulator of cell division. Reduced DNA methylation in the *SlWEE1* promoter was shown to be correlated with high expression levels of this gene in the transgenic plants, likely leading to growth arrest of the fruits (Liu et al. 2012; Tang et al. 2012).

Another interesting finding was that some of the phenotypes (reduced organ size and high shoot branching) observed in transgenic tomato plants overexpressing $DDB1^F$ are independent of the presence of the transgene in subsequent generations. For example, plants of the T2 and T3 generations containing no $DDB1^F$ transgene showed reduced organ size and higher axillary branching, similarly to phenotypes present in T1 plants containing the transgene (Liu et al. 2012; Tang et al. 2012). However, at later generations (T4 plants), fruit weight and shoot branching phenotypes reverted to wild-type phenotypes (Tang et al. 2012). Based upon these observations, the authors concluded that both phenotypes are epigenetically controlled and can be transmitted over three generations (Liu et al. 2012; Tang et al. 2012).

Although the results on tomato DDB1 are exciting, the mechanism(s) leading to such heritable epigenetic changes in specific loci remain(s) to be determined. In Arabidopsis, DDB1-CUL4-based ubiquitin E3 ligase interacts with components of the Polycomb Repressive Complex 2 (PRC2), required for epigenetic silencing of

chromatin, thus indicating a novel role of ubiquitylation in epigenetic regulation of gene expression (Dumbliauskas et al. 2011). Assuming a conserved role of DDB1 in tomato, one can speculate that overexpression of *DDB1*^{F} may lead to degradation of epigenetic regulators, such as DNA methyltransferases, consequently reducing methylation levels of target genes. As observed by Liu et al. (2012), *DDB1*^{F} transgene seems to be responsible for the initiation of the decreased methylation of the *SlWEE1* gene, but not for its maintenance across generations. This observation implies the action of additional epigenetic "players" on the maintenance of the methylation levels of *SlWEE1* and likely other genes encoding negative regulators of cell division, which could have an impact in multiple traits of agronomic importance in tomato (Tang et al. 2012).

Grafting is a significant technique to improve performance of horticultural plants including several agronomically important woody fruit trees and vegetables. This method is generally performed by grafting the shoot part of a plant (scion) onto a root part of another plant (rootstock), often with distinct genetic backgrounds, even different species or genera (Burge et al. 2002). The recently documented mobility of various genetic components including DNAs and RNAs between the scion and stock (Haroldsen et al. 2012) have risen the question whether phenotypic traits altered in the grafted products have a heritable basis as a result of the exchanging of genetic information. Although DNA exchange has been documented, it only occurred at very low frequencies (Thyssen et al. 2012; Stegemann et al. 2012). Small RNAs of 21–24 nucleotide (nt) in size were also reported to be able to move across the graft union via plasmodesmata and phloem. Significantly, movement of 24-nt siRNAs was capable of directing DNA methylation in the genome of the recipient cells (Molnar et al. 2010), tantalizingly suggesting that epigenetic modifications may take place in the grafted products, probably resulting in heritable new characteristics passing to the next generation of non-grafted plants.

To test this hypothesis, Wu et al. (2013) analyzed relative DNA methylation levels by using methylation-sensitive amplified polymorphism (MSAP) and locus-specific bisulfite-sequencing in seed plants, self- and hetero-grafted scions/rootstocks, selfed progenies of scions and their seed-plant controls of pure-line cultivars of tomato, eggplant (*Solanum melongena* L.), and pepper (*Capsicum annuum* L.). Extensive alterations in two DNA methylation contexts (CpG and CpHpG) were observed in all independent samples of multiple interspecific graftings tested involving these three *Solanaceae* species. Importantly, such alterations seem to be heritable for some loci, which is surprising if taken into consideration that the induced epigenetic modifications would have to affect primordial cells that are destined to form gametal cells. Based on gene expression analyzes, the authors suggested that methylation pattern alterations and their inheritance induced by grafting were at least in part due to perturbed expression of the cellular machinery required for DNA methylation. Therefore, it seems that, at least in *Solanaceae* species, inter-species hetero-grafting produces heritable alteration in DNA methylation patterns that may produce functional developmental consequences in the graft hybrids. Such functional consequences could help to generate hetero-grafted scions/rootstocks with agronomic relevance. Moreover, we can hypothesize that

these alterations in DNA methylation constitute an important genetic component underlying the Darwinian concepts of graft hybridization and graft hybrid, concepts of which were put forward by Charles Darwin more than two centuries ago (Darwin 1868).

In addition to DNA methylation, nucleosome remodeling and histone posttranslational modifications contribute to modulate different chromatin states that control transcription and other chromatin-based nuclear processes (Sadeh and Allis 2011; Kouzarides 2007). While DNA methylation status and its modifications have been fairly documented in tomato, studies on histone modifications are missing for this crop. To initiate these studies, Aiese Cigliano et al. (2013) identified and performed expression profiling analyzes of *histone modifier* genes (*HMs*) in tomato. This in silico study identified over 100 *HMs* loci including 32 histone acetylases, 14 histone deacetylases, 52 histone methylases, and 26 histone demethylases. Putative roles of these genes in tomato development were addressed by analyzing the expression data of all the *HMs* identified in distinct organs and developmental stages. Differential expression of members of the distinct classes of *HMs* suggests a complex regulatory network of histone modifications and likely transcriptional control during tomato development. By taking advantage of the existing *Solanum pennellii* introgression lines (ILs), in near future it will be possible to integrate the map position of *HMs*, their expression profiles and the phenotypes of ILs in order to select candidate *HM* genes involved in the process of interest to be used in tomato breeding programs.

5.2.2 Small RNAs

Small RNAs and enzymes involved in their biogenesis and function are also important components of the plant epigenetic machinery. Plant sRNAs are produced either by double- or single-strand RNA precursors (dsRNAs or ssRNAs, respectively). Depending on the nature of the precursor RNA, sRNAs are classified into microRNAs (miRNAs) that are produced from stable ssRNA hairpin structures and small interfering RNAs (siRNAs) that are processed from long dsRNAs (Brodersen and Voinnet 2006). Formation of long dsRNAs requires the activity of RNA-dependent RNA polymerases (RDRs), while their processing depends upon the activity of distinct members of Dicer-like (DCL) family. In the case of miRNA precursors, their processing is generally initiated by the DCL1 enzyme. The 19–25 mer imperfect duplexes produced by DCL are unwound and one of the strands binds to Argonaute (AGO) proteins. The AGO-containing complexes (sometimes referred to as "silencing complexes") are then guided by the incorporated sRNAs to target RNA or DNA that are recognized by sequence complementarity (Brodersen and Voinnet 2006). Multiple copies of *DCL*, *AGO* and *RDR* genes are found in plants. For instance, the Arabidopsis genome contains 4 *DCL*, 10 *AGO* and 6 *RDR* genes, whereas a total of 32 and 28 genes (including *DCLs*, *AGOs* and *RDRs*) in rice and maize, respectively, have been identified thus far (Kapoor

et al. 2008; Qian et al. 2011). Functional analyzes of these genes revealed that different sRNA-associated enzymes play multiple roles in regulating growth and development as well as in response to abiotic and biotic stresses.

In tomato, 7 *SlDCL*, 15 *SlAGO*, and 6 *SlRDR* genes have been identified so far (Bai et al. 2012). One recent study conducted by Xian et al. (2013) analyzed in details the localization and expression patterns of all tomato *AGOs*, showing that some *SlAGOs* have unique expression patterns during fruit development. For instance, *SlAGO7* expressed extremely high in −2 dpa (2 days before anthesis) fruits but was downregulated in 8 dpa to red fruits. This observation suggests that *SlAGO7*, which is a homolog of Arabidopsis *AGO7*, might regulate early stages of fruit formation, presumably through regulating synthesis of 21-mer trans-acting siRNAs (tasiRNAs) to maintain proper expression of the *AUXIN RESPONSE FACTOR* (*ARF*) genes (Montgomery et al. 2008). Such hypothesis is supported by the fact that *ARF3* and *ARF4* mediate reproductive organ asymmetry as shown by mutations in both genes that led to strong flower phenotypes in Arabidopsis, likely due to alterations in auxin signaling (Pekker et al. 2005). Interestingly, one of the mutants of the tomato wiry leaf syndrome (*w2*) was identified as having mutations in the *SlAGO7* locus, therefore renamed as *w2-ago7*. *w2-ago7* mutant plants fail to produce tasiRNAs, resulting in misregulation of *SlARF3* and *SlARF4* genes and leading to the formation of shoestring leaves that lack leaf blade expansion (Yifhar et al. 2012). An interesting finding in this study was that, unlike Arabidopsis AGO7, *SlAGO7* is not only dedicated to generate tasiRNAs but also is required for the biogenesis of numerous tomato small RNAs. The source and functions of the sRNAs requiring AGO7 are presently unknown. However, this phenomenon illustrates the complexity of tomato small RNA biogenesis and our limited appreciation of its significance. Notably, *w2-ago7* plants display flowers with narrow organs that are fused at their base, while wild-type tomato flowers have five sepals, five yellow fused petals and stamens, and two to three fused carpels (Yifhar et al. 2012). Although the authors did not analyze reproductive phenotypes in this particular study, it would be of economical importance to evaluate the effect of tomato wiry leaf syndrome and tasiRNAs on flower and fruit development.

As expected, tomato small RNA population is vast and complex and, although a subset of sRNAs is conserved across different families, several sRNAs are family and species-specific (Moxon et al. 2008; Mohorianu et al. 2011). The most conserved class of tomato sRNAs is the miRNA class, but even miRNAs are not well conserved. Moxon et al. (2008) cloned quite a few novel miRNAs that seems to be tomato-specific. However, the authors failed to validate most predicted targets for these novel miRNAs. One possible explanation is that some of the newly identified sRNAs were mistakenly classified as miRNAs. Many putative nonconserved miRNAs, which are not supported by biogenesis data (demonstration of DCL1 dependency or cloning of perfect miRNA* sequences, which represent the opposite strand of the mature miRNA forming the imperfect small RNA duplex), could be siRNAs rather than miRNAs. In fact, current computational approaches to predict non-conserved miRNAs and targets from RNA-seq data produce a considerable quantity of false positive and an unknown amount of false negative results, and thus

the need for better prediction algorithms is evident (Moxon et al. 2008; Hamzeiy et al. 2014).

Transposon-specific sRNAs are usually abundant in small RNA libraries. A particular class of transposons, miniature inverted-repeat transposable elements (MITEs), has been shown to be able of generating sRNAs and regulating gene expression in a genome-wide fashion (Lu et al. 2012). Moreover, MITE-derived sRNAs may represent the evolutionary link between miRNAs and siRNAs in humans and plants (Piriyapongsa et al. 2007; Zanca et al. 2010; Ortiz-Morea et al. 2013). In the *Solanaceae*, including tomato, a number of MITE families were identified and some are capable of affecting gene function and regulation potentially through physical genome changes and by generating small RNAs that are primarily 24-mer in length (Kuang et al. 2009). In *Solanaceae* species, Kuang et al. (2009) showed that these MITE-associated 24-mer sRNAs are generated by RDR2, DCL3, and possibly DCL4. This study and others proposed that the amplification and diversification of MITEs and other transposable elements (TEs) in plant genomes may contribute to evolution of networks of coordinately regulated genes via insertion and subsequent selection of homologous elements in many protein-coding genes. These homologous mobile elements may became target sites for co-regulation by silencing complexes loaded with target-specific MITEs and other TE-associated small RNAs.

By evaluating the accumulation patterns of sRNA populations during tomato fruit development, it was possible to determine that there are various genomic regions that give rise to differentially expressed sRNAs during this process and only a small fraction of these sRNAs are miRNAs (Mohorianu et al. 2011). Furthermore, it was also found that, in contrast to Arabidopsis, most tomato sRNAs that are not strand biased (e.g., heterochromatin siRNAs) have perfect matches with protein-coding genes or regions annotated as protein-coding genes (Mohorianu et al. 2011). Along with data from tomato genome and methylomes, sRNA profiles in fruits point out a scenario in which several ripening-related genes or loci may be co-opted for using sRNA-based regulation (The Tomato Genome Consortium 2012; Zhong et al. 2013). One such example are three loci that show homology to the ethylene-responsive factors, *EIN3* and *EIN4*. sRNAs matching these loci were mainly 22-mer and showed no strand bias, suggesting that they were produced by DCL2 from RDR-generated dsRNAs (Mohorianu et al. 2011). Although it is currently unknown how sRNAs are produced from these loci, it is possible that they regulate their genomic region of origin in *cis* or even other mRNAs in *trans*, thus contributing to complex regulatory networks during fruit development and ripening. Nonetheless, the final proof that ripening-associated genes are either sources of these sRNAs or their targets can only come from experiments using *DCL*-deficient tomato mutants.

Similarly to other species, several families of conserved miRNAs and targets were identified in tomato by using bioinformatic and cloning techniques (Moxon et al. 2008; Mohorianu et al. 2011; Zhang et al. 2008; Karlova et al. 2013). Some miRNA families showed differential accumulation during fruit development, suggesting a particular role in this developmental process in tomato. For instance, miR159, miR162 and miR165/166 were abundantly expressed during early fruit

development and the expression of miR156, miR164 and miR396 was shown to increase during ripening (Mohorianu et al. 2011). My research group has recently generated transgenic tomato plants ectopically expressing miR156 and miR164 (Silva 2012). Both miRNAs seem to affect early stages of flower and fruit development, as their overexpression in transgenic plants led to disorganization of floral organs and therefore to the formation of fruits with odd shape and less seeds. By using degradome-coupled to deep sequencing analysis, Karlova et al. (2013) identified known ripening regulators, such as *CNR* and *APETALA2a* (*SlAP2a*), with developmentally regulated degradation patterns. The levels of the intact messenger of both *CNR* and *SlAP2a* seem to be actively modulated during ripening, by miR156/157 and miR172, respectively. microRNA modulation of these two central regulators of tomato ripening adds another layer of complexity to the regulatory networks taking place during this developmental process. According to our data and others, the function of miR156/157 in fruit ripening is still unclear as fruits of miR156/157-overexpressing plants still ripe normally (Zhang et al. 2011; Silva 2012). However, one can speculate that the main function of miR156/157 and likely miR172 in wild-type plants is to fine-tune the expression of *CNR* and *SlAP2a* to appropriate levels in particular stages of fruit ripening. Along with DNA methylation levels, miRNA regulation may contribute to the proper balance of gene expression during tomato fruit development and ripening.

Although functional studies are still necessary to precisely determine the roles of conserved and non-conserved miRNAs during fruit development, their functions in tomato leaf development are well documented. By cloning the miR319-insensitive version of *LANCEOLATE* (*LA*) gene from the partially dominant mutant *Lanceolate* (*La*), Ori and coworkers (2007) demonstrated that regulation of *LA* by miR319 defines a flexible window of morphogenetic competence along the developing leaf margin that is required for the elaboration of compound leaves. In another study, Berger et al. (2009) analyzed *goblet* (*gob*) loss-of-function mutants, in which primary leaflets are often fused, and secondary leaflets and marginal serrations are absent. *GOB* encodes a NAC-domain transcription factor that is negatively regulated by miR164. Accordingly, leaf-specific overexpression of the miR164 also led to loss of secondary-leaflet initiation and to smooth leaflet margins in transgenic plants. Along with phenotypic and molecular analyzes of the dominant mutant *Gob*, which contains a miR164-insensitive version of the *GOB* gene, the above mentioned observations indicate that the miR164/*GOB* module is crucial for the proper development of leaflet boundaries in tomato. Considering the discoveries presented thus far, the future surely holds novel and exciting breakthroughs regarding the roles of miRNAs and targets in tomato development. Such knowledge may become crucial for breeding programs aimed at modifying developmental parameters in tomato, such as leaf patterning and ripening.

5.3 How Knowledge on Epigenetics Can Contribute to Tomato Breeding?

The crossing between genetically distinct parents provides the mixing of genomes in the resulting hybrids that is essential for the generation of new, favorable genetic combinations, known as breeding. Together with genetic natural variation, epigenetic regulation may be a genome-wide phenomenon that contributes to increasing the yield in many hybrids commercialized today. For example, epigenetic mechanisms can account, at least in part, for the extreme phenotypes found in hybrids when comparing with their parents. Such phenotypes are sometimes heritable and go beyond the F1 generation. The heritability of these phenotypes indicates they are different from those associated with heterosis or hybrid necrosis (Bomblies and Weigel 2007; Birchler et al. 2010). The expression "transgressive segregation" was coined to describe the phenotypic novelty of these hybrid lineages that transgress the parental range. Many eukaryotes exhibit transgressive segregation, though it is more frequent in plants than animals (Rieseberg et al. 1999).

Shivaprasad et al. (2012) investigated the possibility that stable transgressive phenotypes in the progeny of crosses between cultivated tomato and a wild relative (*Solanum pennellii*) were associated with genome-wide epigenetic modifications. The initial hypothesis was that transgressive segregation in the progeny would be affected by epistatic interactions between small RNAs and their targets from the opposite parent. To support this hypothesis, siRNAs corresponding to *S. pennellii phenylalanine ammonia-lyase* (*PAL*) mRNAs were highly represented in some hybrids relatively to the parents. The presumption was that these siRNAs acted in *trans* (perhaps like tasiRNAs) and led to the observed increase in DNA methylation on *PAL* loci in late generations. As neither siRNA accumulation nor DNA methylation alterations were evident in the F1 progeny but rather in subsequent generations (Shivaprasad et al. 2012), the authors suggested that the epigenetic effects observed in late generations were initiated by interactions occurring during gametogenesis of the F1 progeny and that they were subsequently reinforced by RNA-directed DNA methylation (Fig. 5.3).

In addition to changes in siRNAs and DNA methylation, Shivaprasad et al. (2012) observed that transgressive phenotypes in the progeny can also be mediated by alterations in the expression of specific miRNAs. miR395 was highly expressed in some of the hybrid progeny, suggesting that one of the parents contributes an allele at a trans-regulatory locus that can specifically increase the abundance of the miRNA generated from the miR395 allele contributed by one or both parents. A possible explanation could be this trans-regulatory locus encodes a transcription factor that regulates expression of the miR395 precursor, being present or more efficiently expressed only in one of the parents (Fig. 5.3). This microRNA has been shown to be induced by salt stress in different species (Ding et al. 2009; Jia et al. 2009). Accordingly, there was a positive correlation between elevated accumulation of miR395 in particular tomato progenies and their higher tolerance to salinity stress (Shivaprasad et al. 2012).

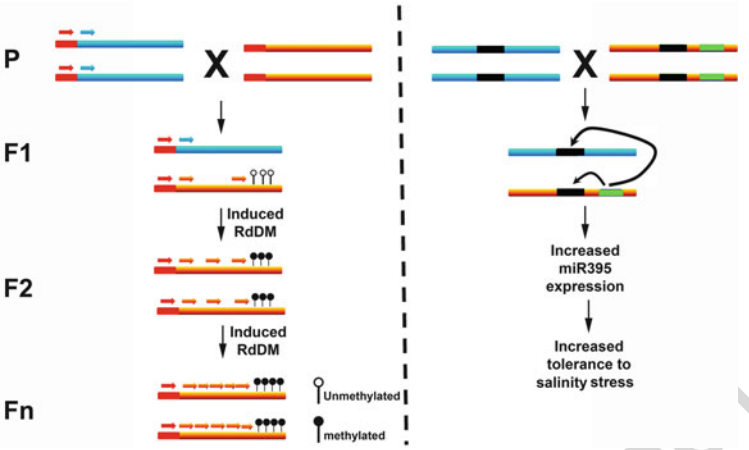

Fig. 5.3 Possible scenarios for epigenetic-based transgressive segregation. *Left panel*: interaction between allelic or non-allelic loci that share only limited sequence identity (*red box*) in F1 can lead to generation and spreading of siRNAs (*small arrows*), perhaps through a phenomenon called transitivity. As a result, this sRNA production may direct gradual small RNA amplification and RNA-dependent DNA methylation (RdDM) over several generations. *Right panel*: introduction of an allele at a trans-regulatory locus (*light green box*) in F1 leads to the enhancing of transcription of *MIR395* locus (*black box*) and possibly increases salt tolerance in particular hybrids. *P* parents

This study in tomato provides some of the first concrete evidence for epigenetic phenomena generating entirely new allelic states not easily explained by Mendelian laws. However, these findings are just a flavor of what kind of genetic and epigenetic variations we may achieve by combining the genomes of cultivated tomato and wild relatives, creating not only the classical ILs but also "epigenetic inbred lines" or epi-ILs. Based on the wide variety of close wild relatives and easy crossing, tomato will probably became a model for studying epigenetic basis of transgressive segregation, allowing for its more efficient utilization in plant breeding.

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