REVIEW

ARE TRANSGENERATIONAL EPIGENETICS AFFECTING GENETICALLY MODIFIED ORGANISMS?

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ABSTRACT

Due to the existence of traditional breeding methods (open pollination, mutation breeding, atomic farming) alongside recent technologies (CRISPR/Cas), the line is blurred between genetically modified organisms that require no regulation (as they have been produced by traditional methods), and GMOs (genetically modified organisms as defined by the European Union), which are created by genetic engineering. Although different breeding methods may lead to genetically identical organisms, recent progress in epigenetics and transgenerational epigenetics raises the question of whether plants possess a kind of consciousness that allows them to remember the method by which they have been created. Therefore, we cannot exclude the possibility that genetically identical organisms produced by different breeding methods exhibit different -omics (proteomics, metabolics, RNomics, epigenomics). In this paper, we summarize the recent literature on epigenetics, discuss the transgenerational epigenetic effects of different breeding methods, and emphasize the importance of strictly evaluating possible risks by assessing the final product.

Keywords: Adjusted organism; Genetically modified organism; Gene editing, GM food; Epigenetics; RNomics; Proteomics; Metabolics

INTRODUCTION

The simplistic view of the central dogma of genetics – that there exists a unidirectional flow of information from DNA to RNA to protein – has been challenged in recent years. Systems biology emphasizes the idea that different networks – genome, transcriptome, metabolome and proteome – interact with one another, with permanent information flow observed within and between the different networks **(Franklin and Vondriska, 2011)**. Furthermore, the Encyclopedia of DNA Elements project found that around 76% of human genomes are translated into around 21,000 protein-coding genes and 18,400 small or long noncoding RNAs **(Pennisi, 2012)**. Evidently, the genomics dark matter is much more important than previously thought. Steady progress has been made toward understand the rules of different noncoding RNAs. A recent review **(Jankowsky and Harries, 2015)** summarizes our current knowledge about the interaction between proteins and RNA. A novel network called RNomics has been proposed **(Huettenhofer, 2015)**, but how information is exchanged between this network and others is not yet well established. It is well known that post-translational modifications of proteins are essential **(Wang *et al.*, 2014)**. Similarly, with epigenetics, chemical modifications of DNA or histones **(Goldberg *et al.*, 2007)** are important for the function of a living organism, and RNomics plays an important role in the regulation of these epigenetic tags **(Heard and Martiensen, 2014)**. In our review, we will emphasize that epigenetics and transgenerational epigenetics can influence adjusted organisms (AOs). The term adjusted organism was recently introduced to revive unbiased discussion regarding genetically modified organisms **(Lassalle and Jarocki, 2016)** and to replace the meaningless pseudo-category of GMOs **(Tagliabue, 2016)**.

EPIGENETICS AND TRANSGENERATIONAL EPIGENETICS IN RODENTS AND MAMMALS

Epigenetics

According to **Weinhold (2006)**, "epigenetic literally means in addition to changes in genetic sequence." In recent years, we have seen a rapid increase in knowledge about epigenetics, but understanding the complex details of information flow between and within the different networks remains a challenge. A recent review by **Ranjan and Sharma (2016)** summarizes more than 250 recent studies that link epigenetic changes to environmental pollutions, including aluminium, arsenic, benzol, biphenol, cadmium, chromium, lead, mercury, nickel, and pesticides, and connects these epigenetic changes to diseases. Without a doubt, we are constantly exposed to environmental conditions that influence the chemical properties of our DNA and health. In a similar publication by **Ruiz-Hernandez *et al.* (2015)**, the possible mechanisms underlying the changes induced by environmental effects are explained. Chemicals can increase hypomethylation as the production of S-adenosylmethionine (a substrate for DNA methyltransferase) is decreased. It is also possible that chemicals can cause overexpression of DNA methyltransferase. Further targets include the enzymes responsible for acetylation, methylation, phosphorylation and ubiquitination at histone tails. Thus, no general mechanism exists; each environmental pollution factor causes a unique response. Consequently, it is difficult to determine which part of the chromatin

structure is altered. As an example, lead has been related to histone modifications that activate transcription, while nickel has the reverse effect. Environmental factors definitely influence the genomic and proteomic networks. Furthermore, as these factors influence the genomics, they directly influence the RNomics. Genome-wide DNA methylome analysis has led to the conclusion that the methylation pattern of long-noncoding RNA (lncRNAs) has been altered in breast cancer DNA leading to dysregulation of the cell cycle pathway **(Li *et al.*, 2015)**. As it has been shown that lncRNAs play a role in the epigenetic transmission of the effects of reprotoxicants **(Larriba and del Mazo, 2016)**, we can conclude that lncRNAs may be the inducers and targets of epigenetic changes. Feedback loops may also exist. In addition to connections to environmental factors and diseases, epigenetics could be important for depressive behaviours **(Januar *et al.*, 2014)**, although small sample sizes and lack of replicated data for the current data sets have prevented a conclusive answer on this. The possibility of transgenerational epigenetics, or epigenetic inheritance, is another question that is actively being researched. In rodents and mammals, epigenetic tags are acquired and erased during development. Between E11.2 and E12.5, during primordial germ-cell development, the genomic methylation pattern is erased and new sex-specific methylation patterns are established during gametogenesis **(Morgan *et al.*, 2005 and Messerschmidt *et al.*, 2014)**. Markers are erased, established and maintained during fertilization and implantation to ensure toti- and pluripotency. Thus, it seems to be unlikely that biotic or abiotic stress-induced epigenetic marks can survive these strict reprogramming events.

Imprinting

However, imprinting, or monoallelic gene expression according to parent-of-origin matter, which is linked to the parent-specific methylation pattern, is maintained. Many human diseases are connected to imprinted genes. From 150 imprinted genes verified in mice, half have been found in humans **(Peters, 2014)**. Importantly, these imprinted genes are not scattered throughout the genome, but are clustered into genomic regions that contains several genes **(Barlow and Bartolomei, 2014)**. Sixteen imprinted clusters that contain 12 or more imprinted genes and 80 up to 3700 kB of DNA have been characterized **(Barlow and Bartolomei, 2014)**. Typically, the clusters contain a differentially DNA-methylated region (gametic DMR), which is a methylation imprint maintained on only one parental chromosome in the diploid cell of the embryo. The gametic DMR can have a maternal or parental origin. If the gametic DMR at least partially controls the expression of the cluster, it is called an imprint control element (ICE). These clusters commonly contain mRNAs and at least one lncRNA. The gene is expressed from the methylated parental chromosome and the lncRNA from the opposite chromosome. Two models have been proposed to explain parental silencing. For the Igf2 cluster, the insulator model explains the expression pattern. The paternal chromosome is methylated at the ICE, preventing the CTCF protein from binding. The methylation represses the expression of the lncRNA and enables the cis-acting enhancer to activate expression of the mRNAs. On the maternal chromosome, the ICE is not methylated; thus, the CTCF protein can bind to block mRNA expression and the enhancer activates expression of the lncRNA. For the Igf2r cluster, the lncRNA model has been proposed. In this case, the maternal chromosome is methylated. Again, the methylation represses the expression of the lncRNA, which allows for expression of the mRNAs. On the paternal chromosome, the ICE is not methylated, allowing the lncRNA

to be expressed and to silence the mRNA in the chromosome. Further models have also been proposed **(Barlow and Bartolomei, 2014)**. For the Igf2 cluster, the expressed lncRNA acts as an miRNA (micro RNA) precursor, exerts epigenetic regulation, influences tumour progeny, and possesses myogenic properties **(Kanduri, 2016)**. During mammalian development, the imprints are erased in the germline (between E.8.5 and 11.5), but are maintained during development from zygote to blastocyst and remain in the somatic and extraembryonic tissues and in the placenta **(Barlow and Batolomei, 2014 and Li and Sasaki, 2011)**. How do the imprints escape the reprogramming? Most likely, cis- and trans acting factors ensure the protection of the methylation pattern. A recent article by **Matsuzaki *et al.* (2015)** demonstrates that a segment of the H19 ICR (imprinting control regions; cis-regulatory control elements) is essential for de novo postfertilization DNA methylation, supporting the maintenance of imprinted methylation during early embryogenesis. In summary, imprinting genes can survive extensive reprogramming events because they are clustered into genomic regions, where control elements ensure the maintenance of the methylation pattern during embryogenesis. However, biotic and abiotic stress-induced chromatin modifications are not clustered. Consequently, it seems unlikely that this methylation pattern can survive the strict reprogramming.

Transgenerational epigenetics

Transgenerational epigenetics, however, whereby genetics marks are inherited through the germline, has been reported. Agouti mice provide the most prominent example of this. As described by **Lassalle *et al.* (2003)**, two subtypes of melanin can be distinguished: the black coloured eumelanin (a) and the reddish-yellow pheomelanin (A). When the pro-viral intracisternal A particle (IAP) retrotransposon was inserted into the pseudoexon 1A gene upstream of the agouti signalling protein (ASP) involved in the switch from eumelanin to pheomelanin, the following pattern was observed. A pseudoagouti mouse was obtained when the maternal nutrition was changed during pregnancy to a diet with potential methyl donors. A pseudoagouti mouse is heavily methylated, while the LTR (long terminal repeats) of the IAP is unmethylated in yellow mice. Intermediate states exhibit a variety of methylation patterns. **Cropley *et al.* (2006)** asserted that the F2 population is influenced by nutritional effects from the F0 maternal generation. However, this might not qualify as transgenerational epigenetics **(Skinner, 2008 and Heard and Maertienssen, 2014 and Hanson and Skinner, 2016)**. As the F1 generation could be influenced through the germline, if a female or male is exposed to biotic or abiotic stress in the F0 generation, at least the F2 generation should be observed. In the case of gestating females, the F1 generation (embryo) and the F2 generation (through the germline of F1) might be influenced. Thus, at least the F3 generation must be observed. Especially in the A^{vy} (agouti viable yellow*)* allele of agouti mouse, the primordial germ cells (which will eventually become the F2 generation) might be influenced. Thus, to ensure that the observed effect is truly transgenerational and not intergenerational, the F3 generation females should show inheritable epigenetics. There have been some examples of true transgenerational inheritance in rats **(Hanson and Skinner, 2016)**, but the majority of studies have been limited to the F3 generation, and in only 3 cases (out of 15 reports) has the F4 generation been influenced. Consequently, once abiotic or biotic stress is released, the effect seems to fade out. Furthermore, the results are disputable. For example, Vinclozolin was shown to have a transgenerational effect on the F3 generation **(Hanson**

and Skinner, 2016), but another study was unable to reproduce these results **(Schneider *et al.*, 2008)**. Therefore, circumstances (species, strain, germ cell development stage, exposure route, dosage) need to be better defined to ensure reproducibility of the data. As pointed out by **Hanson and Skinner (2016),** a so-called critical window was defined, and negative results **(Whitelaw, 2015 and Iqbal *et al.*, 2015)** are an effect of non-suitable experimental design conditions. One can argue that there is no error in the design of the experiment, because the main purpose of the study is different. In the former case **(Hanson and Skinner, 2016)**, the researchers aimed to show the possibility of transgenerational epigenetics; consequently, experimental conditions that would maximize the outcome (transgenerational epigenetics) were chosen. In the latter case **(Schneider *et al.*, 2008)**, the probability of transgenerational epigenetics under realistic conditions was investigated. Certainly, in normal development, the environmental stress factors will not always occur at the optimal induction concentration and timing. Therefore, transgenerational epigenetics is possible, but its relevance under non-laboratory conditions is disputable. In a similar publication **(Aiken and Ozanne, 2013)**, a Pubmed search revealed 45 studies with possible transgenerational epigenetics, but again less than 5 studies detected an effect on the F3 generation. Overall, the results support **Mayr's (1980)** definition of soft inheritance: "the genetic basis of characters could be modified either by direct induction by the environment, or by use and disuse, or by an intrinsic failure of constancy, and … this modified genotype was then transmitted to the next generation." Soft inheritance, however, should not be a one-way street. Once the biotic or abiotic stress is released, the organism should be able to return to its original condition; otherwise, transgenerational epigenetics would first create an advantageous situation but then a disadvantageous situation would arise for later generations. Indeed, soft inheritance might be recognized as an immediate, fast response to a thread. In a recent publication by **Burggren (2016)**, three main scenarios of trait inheritance during the sudden appearance and disappearance of environmental stress are distinguished. If there is no change in genetics, the population will decline during the environmental stress, and it will take several generations following the release of stress to restore a healthy population. If, however, mutations in the genome are created during the environmental stress, the population will regain vitality, but once the stress disappears, the population will be at a disadvantage. In the case of epigenetics, the population can react immediately to the stress factor, and once the stress dissipates, it can quickly return to its original condition. Other scenarios include "wash in" and "wash-out" scenarios, whereby transgenerational epigenetics might take some generations to appear or disappear.

Summary

We propose that epigenetics and transgenerational epigenetics provide testing grounds for a variety of phenotypes. As epigenetics can alter chromatin structure **(O`Dea *et al.*, 2016)**, thus influencing mutation rates, favourable phenotypes and their epigenetics could facilitate mutations in subsequent generations that enable stable inheritance of this phenotype. Consequently, epigenetics could increase the plasticity of evolution and increase the adaptation of the organism to fast changing environmental conditions. To understand epigenetics and transgenerational epigenetics in greater detail, we need to obtain a clearer picture of their underlying molecular mechanisms. **Lim and Brunet (2013)** discovered that environmental stimuli can influence chromatin structure via

noncoding RNAs – including siRNA (small interfering RNA, worm), piRNA (Piwi-interacting RNA, worm and fly), viRNA (small interfering RNAs derived from virus, worm), miRNA (micro RNA, mice) – DNA methylation (mice, rat) and histone modification (with the help of histone methyltransferase proteins H3K4me2/3 (worm), H3K36me3 (worm), H3K36me3 (worm, fly), H3K9me2/3 (worm, fly), and H3K27me3 (mice, human). Prion proteins (yeast) might also play a role. These changes might influence metabolics, which change the expression of different chemicals. As these chemicals are themselves potential environmental stress factors, they could initiate epigenomic changes. Chromatin modifications have far reaching consequences. A recent review by **Allis and Jenuwein (2016)** summarizes the current knowledge regarding chromatin's contribution to the epigenomic function, including RNAi repression, constitutive heterochromatin (histone methyltransferase SUV39H1, heterochromatin protein HP1), repeat element silencing (methyltransferase ESET), DNA repair (death domain-associated protein 6, DAXX, which is a histone chaperone), histone variant exchange (histone variant 3.3), remodelling (SWI/SNF nucleosome remodelers), boundaries 3D architecture (transcriptional repressor CTCF), transcript quality control (RNA polymerase), promoter activity (TF promoter), enhancer functions (histone acetyltransferase), enhancer-promoter communication (lncRNA), trithorax stimulation, polycomb silencing (polycomb protein EZH2), bivalent chromatin (H3K4me2/3), DNA methylation (DNA methyltransferase) and inactive X (Xist [X-inactive specific transcript] is an RNA gene on the X chromosome). Consequently, it is possible that there are feedback loops that alter chromatin marks at a genomic locus expressing noncoding RNAs, which can be transmitted to the germline and guide H3K9me3 deposition at the same genomic locus **(Lim and Brunet, 2013)**. This could be a mechanism for the progression of active transgenerational epigenetics. However, we cannot deny the recent data. Transgenerational epigenetics might be mainly passive as a result of the incomplete erasure of epigenetic marks through the development stages. As mentioned earlier, evidence has been limited mainly to the F2 generation, with only some reports showing transgenerational epigenetics reaching the F3 and F4 generations; therefore, a strong positive feedback loop seems to be absent. If, however, epigenetics are a test bed during evolution, an intersection will exist. Either the new trait is beneficial and will thus be included in the genome and transformed into a hard inheritance in later generations, or the new trait is non-beneficial due to the dissipation of biotic or abiotic stress and the soft inheritance will thus fade out.

EPIGENETICS AND TRANSGENERATIONAL EPIGENETICS IN PLANTS

Epigenetics

Similar to rodents and humans, epigenetics in plants represent a vital mechanism. The onset of flowering is controlled by different pathways **(Kim and Sung, 2014)**, which include the photoperiod pathway, with clock genes and light receptors sensitive to different wavelengths suppressing or inducing flowering. This pathway acts on the floral integrator gene FT (flowering locus T). Light-wave-independent pathways exist, such as the autonomous pathway, which includes FLD (flowering locus D), FPA (flowering time control protein FPA), FY (flowering time control protein FY), FCA (flowering time control protein FCA), FVE (classical flowering time locus) and FLK (flowering locus K homology (KH) domain). This pathway can act on FLC (flowering locus C), and therefore activate or suppress floral integrator genes, including FT, FD (flowering time gene FD), and SOC1 (suppressor of overexpression of constans 1). Another pathway is the gibberellin pathway; gibberellin hormones influence many aspects of plants. For flowering control, the interaction with SOC1 is important. Vernalization, the control of flowering by required exposure to extended periods of cold ensures that flowering occurs after the wintertime **(Achrem *et al.*, 2012)**. This is a well-established mechanism that highlights the importance of epigenetics. Vernalization includes VIN3 (vernalization insensitive 3), VIL1 (VIN3-LIKE 1), VRN5 (vernalization 5) and VRN2 (vernalization 2), acting on FLC and FLC-like proteins and influencing FT, FD and SOC1. This mechanism was first described in 1960 **(Chourard, 1960)**, but due to recent developments in molecular genetics, the molecular details have just been revealed. The process at flowering locus C (FLC), which contains a MADS-type transcriptional repressor that prevents activation of genes responsible for floral growth, involves the following key players. FRIGIDA is a protein that activates FLC and forms a transcription activator complex (FRI-C) with SUF4 (binds to the FLC promoter), FLX and FES1 (transcriptional activators), and FRL1 and FES1 (stabilize the complex) **(Choi *et al.*, 2011)**. Once FRI-C is assembled, a TAF14 homolog (transcription factor) and the SWR1 complex and SEt2 homolog (chromatin modification factors) are recruited. The mechanisms that antagonize FRIGIDA include a) COOLAIR (cold induced long antisense intragenic RNA), transcribed from the 3-prime end of FLC in an antisense direction, and b) COLDAIR. COOLAIR reaches maximum expression after 10 days of vernalization. Its transcripts are polyadenylated at distal sites by FY (polyadenylation factor), and complexed with CSTF64 and CSTF 77 (cleavage stimulation factors) in the absence of FCA (*Arabidopsis* RNA-binding protein). If the concentration of FCA is increased, the polyadenylation sites change from distal to proximal sites, which triggers FLD (flowering locus D protein)-dependent demethylation of FLC and transcriptional repression **(Ietswaart *et al.*, 2012 and Hawkes *et al.*, 2016)**. COOLAIR is able to recruit the polycomb complex (PRC2) to chromatin. It has been shown that COOLAIR negatively affects H3K36-methylation (Histone methylation). COLDAIR is another noncoding RNA that transcribes from the 5-prime end of the FLC in the sense direction. Its maximum expression is reached three weeks after vernalization. COLDAIR supports the recruitment of proteins such as the CURLY LEAF protein, a component of the polycomb repressive complex (PCR2), which is important for ensuring stable silencing after vernalization. Thus, COLDAIR is

important for silencing FLC after vernalization **(Andres and Coupland, 2012)**. Parallel to the expression of COOLAIR and COLDAIR, which silence FLC through antisense RNA, the vernalization process (flowering acceleration by cold induction) is established. COOLAIR and COLDAIR (RNomics) connect the autonomous pathway with the vernalization pathway. This emphasizes that different pathways interact with one another **(Franklin and Vondriska, 2011)**; consequently, to understand the entire mechanism, one must not focus on only one particular pathway. During prolonged cold induction, the PHD-PRC2 complex (polycomb repressive complex with plant homeodomain finger proteins), including VIN3 and VRN5, is accumulated near the first intron. The polycomb complex enhances PHD-PRC2-mediated H3K27 trimethylation (**Csobra *et al.*, 2014)**. During temperature increase, VIN3 is lost and another PHD-PRTC complex is formed that increases the level of H3K27me3 (histone methylation) across the FLC genome and maintains a low FLC mRNA level **(Baulcombe and Dean, 2016)**. In summary, before the onset of cold induction the mRNA FLC concentration is higher than after 1 to 2 weeks exposure to cold temperature, as, through COOLAIR, the mRNA level decreases. Further cold induction lead to expression of COLDAIR, and the polycomb complex (PHD-PCR2) is formed in the nucleation region. The FLC chromatin is demethylated, but methylated at specific sites to ensure silencing of the gene. After prolonged exposure to cold temperature, and once the temperature increases, genome wide methylation, mediated by the PHD- PRTC complex, ensures stable silencing and a low mRNA level of FLC **(Song *et al.*, 2012)**. Further factors important for this process have also been identified **(Ietswaart *et al.*, 2012 and Zhu *et al.*, 2015)**. As the process depends on the species **(Achrem *et al.*, 2012)** – the above is valid for *Arabidopsis thaliana* – differences in the mechanism and variations in information flow between networks (epigenomics, RNomics, proteomics, genomics) are common. Is it possible that epigenetic changes can be transmitted to the next generation?

Imprinting

The mechanism of imprinting, or monoallelic gene expression according to the parent-of-origin, is different in rodents or mammals because the plant's mechanism of sexual reproduction is completely different. A flowering plant will first develop female and male reproductive organs. During sporogenesis, pollen mother cells are generated (2n, diploid) from the male reproductive tissue, and these divide through meiosis into microspores (n, haploid). In a later step of the development process, during gametogenesis, these microspores develop further into bicellular pollen, including vegetative (n) and generative cells (n). The generative cells divide further to create sperm cells (n). During sporogenesis, from the female reproductive tissue, the megaspore mother cell develops (2n), and this divides further through meiosis into megaspores. After three rounds of nuclear cell division, an eight-nucleated female gametophyte is formed. This gametophyte further develops into the mature female gametophyte containing the egg cell (n), the central cell (2n), the antipodals (3 each n) and synergids (2 each n). The endosperm (3n) and zygote (2n) are produced during fertilization, when the central cell and the egg cell fuse each with one sperm cell (*A. thaliana*). The zygote develops into an embryo and then into a mature seed **(Kawashima and Berger 2014 and Dickison and Scholten, 2013)**. The question arises of whether the endosperm (central cell) or the zygote (egg cell, embryo) is the target of

the imprinting. It has long been suggested that the central cell is the main target through the activity of DME (Demeter gene) and through the repression of the maintenance methyltransferase MET1. Consequently, while the methylation pattern is retained in the embryo, the endosperm possesses a parent-of-origin imprint and maternal-specific gene activation **(Feil and Berger, 2007)**. As the endosperm has the function of providing nutrition to the developing embryo, the endosperm imprint influences seed size and affects the embryonic development (**Bai and Settles, 2015 and Costa *et al.*, 2014)**. Imprinting, however, is not limited to the endosperm. **Nodine and Bartel (2012)** identified more than 100 transcripts with parent-of-origin expression in *Arabidopsis thaliana* embryos. It has been assumed that hypomethylated regions in the endosperm can produce siRNAs (small interfering RNAs) that can move to the embryo and enable ROS1 (repressor of silencing 1) demethylation at specific regions **(Jiang and Koehler, 2012)**. Imprinting in *Arabidopsis* embryos has revealed that PRC2 (polycomb repressive complex 2) might regulate these events **(Raissig *et al.*, 2013)**. In the endosperm, the majority of genes are maternally expressed (MEGs), but paternally expressed genes can be also detected (PEGs). Generally, imprinting is far more common in the endosperm than in the embryo, and in both cases there is a preference for maternal expression **(Ikeda and Nishimura, 2015 and Jiang and Koehler, 2012)**. Examples of endosperm imprinted genes with maternal expression include MEA (*Arabidopsis*), FIS2 (*Arabidopsis*), FWA (*Arabidopsis*), SDC (*Arabidopsis*), MOP9.5 (*Arabidopsis*), Mez1 (*maize*), Fie1 (*maize*), Mee1 (*maize*) and OsFIE1 (*rice*); for paternal expression, an example is PHE1 (*Arabidopsis*). As in the case of MEA, DNA methylation and histone modification are imprinting mechanisms that frequently work in parallel. The methylation of lysine residue 27 of histone 3 (H3K27) by polycomb complex activity silences the expression of the parental gene, and DME activity in the female demethylates and activates the gene. A negative feedback loop in the endosperm ensures the silencing of the parental gene and expression of MEA from the maternal gene **(Jullilen *et al.*, 2006)**. Furthermore, RNA-directed DNA methylation (RdDM) plays a vital role **(Xi and Yu, 2015 and Jing *et al.*, 2016 and Zhang *et al.*, 2011)**. It is important to note that although hypomethylation is related to activation, the opposite can be true, as shown for the PHERES1 locus **(Makarevich *et al.*, 2006)**. In contrast to rodents and mammals, the imprints in plants are not clustered into large clusters with control elements (**Pires and Grossniklaus, 2014 and Feil and Berger, 2007)**, but rather microclusters have been identified **(Zhang *et al.*, 2011).** Therefore, plant imprints show higher plasticity. A possible explanation for this is the role of transposons (TEs) in the imprinting process of plants **(Pignatta *et al.*, 2014)**. **Gehring *et al.* (2009)** found that differentially methylated regions (DMRs) are preferentially located in genomic regions related to transposons. Therefore, genes that depend on promoter strength will be a target of TEs. According to this theory, imprinting is a result of a defence mechanism against foreign DNA. During the late embryonic stage and vegetative life phase of the flower, the TEs are methylated and silenced, but the DNA methylation mechanism in the fertilization state enables gene activation or silencing in a parental-specific manner **(Koehler and Weinhofer-Molisch, 2010)**. As transposons can be inserted relatively simply into existing genes, imprinting could evolve genetically via transposon insertion, which could enable epigenetic mutations involving a change in methylation pattern without any DNA change **(Rodrigues and Zilberman, 2015)**. Recently, the importance of TEs for the evolution of related species has been revealed **(Gao *et al.*, 2015)**. As the majority of TEs in plants are class I elements **(Lisch, 2013)**,

the plasticity of imprinting should be enhanced. It has been also suggested that RdDM activity and ROS1 activity antagonize each other to influence gene transcription **(Hsieh, 2016 and Tang *et al.*, 2016)**. In summary, while the sexual reproduction pathway and imprinting target sites in plants differ from mammals or rodents, the underlying molecular mechanisms for imprinting are similar, including DNA methylation, histone modifications and RNomics. To further understand the similarities and differences between rodents/humans and plants, the different methylation patterns must be considered. As summarized, in human and rodents, epigenetic marks are erased during the primordial germ-cell development, are modified during gametogenesis and are maintained (as imprints) and erased during the development from zygote to blastocyst. The imprints remain in somatic and extraembryonic tissues and in the placenta **(Smallwood and Kelsey, 2012 and Barlow and Batolomei, 2016 and Li and Sasaki, 2011)**. Thus, very strict reprogramming exists. In mammals, the methylation occurs mainly at CG sites, while in plants CG, CHG and CHH (H= A, T, C) methylation is important. CG site maintenance in plants is similar the process in humans; the methylated sites are recognized in *Arabidopsis thaliana* by a VIM (variant in methylation) that recruits MET1 (DNA methyltransferase 1) to maintain methylation. CHG and CHH maintenance is more complex and requires H3K9me2 (histone 3 lysine 9 dimethylation), which interacts with CMT3 (chromomethylase 3), while SUVH4 (SU(VAR) homologue) creates a positive feedback loop that maintains H3K9me2 dimethylation. De novo methylation of CG, CHG and CHH includes H3Kme2, CMT2, SHH1 (sawadee homeodomain homologue), PolIV (RNA polymerase IV) and RDR2 (RNA-dependent RNA polymerase 2), creating 24-nt siRNAs (including components of the RdDM pathway). This pathway recruits DRM1 and DRM2 (domains rearranged methyltransferase). In the absence of DDM1 (decreased DNA methylation1), another pathway enables methylation; it involves PolII (RNA polymerase II) and RDR6 (RNA-dependent RNA polymerase 6), creating 21-nt siRNAs (including components of the RdDM pathway), and DRMs (domains rearranged methyltransferase) that interact with siRNAs **(Kawashima and Berger, 2014 and Yang *et al.*, 2015)**. In *Arabidopsis*, 24% of CpG, 7% of CHG and 2% of CHH sites have been methylated, whereas in the unfertilized eggs of *maize* the pattern is quite different, with 86% CpG, 74% CHG and 5% CHH **(Yong *et al.*, 2016)**. As methylation is closely linked to the RdDM pathway, 24-nt siRNAs are produced that resist in the endosperm and could integrate into the embryo to enforce epigenetic marks. Consequently, imprinting in plants will differ from human imprinting because of the differences in the sexual pathway, involvement of transposons, and the abundance of methylation patterns in plants. Furthermore, the reprogramming is less stringent. For the development of sperm cells and vegetative cells from the microspore, the CG methylation pattern does not change significantly for sperm cells, while it decreases for DNA transposons in the vegetative cell while the mature pollen develops from the bicellular pollen. CHH methylation, however, significantly decreases during microspore development and remains at this lower level in the sperm cells. In the vegetative cells, during the period in which the mature pollen develops from the bicellular pollen, the LTR retrotransposons (long terminal repeats retrotransposons) are CHH-methylated. For the embryo (egg cell), CG methylation decreases during gametophyte development, but increases during the later development stage (zygote development stage) to reach a similar percentage of CG methylation in the heart-stage seed than before gametophyte development. For the endosperm (central cell), CG methylation is decreased during gametophyte development and remains at this

level during further development. The latter case is similar to the CHH methylation pattern. In the development of the embryo (egg cell, zygote), CHH methylation decreases until the globular-stage seed, after which it increases again to finally reach a similar percentage of CG methylation to before gametophyte development (**Kawashima and Berger, 2014**; in *Arabidopsis thaliana*). Further changes to methylation pattern can occur at the histone **(She and Baroux, 2014)**. Complex methylation pattern changes from the meristem to the reproductive organs, stamens and pistils, have recently been investigated **(Yang *et al.*, 2015)**. The reprogramming is less strict than in human/rodents and this has far reaching consequences. One can expect a higher occurrence of transgenerational epigenetics.

Transgenerational epigenetics

Considering that transgenerational epigenetics results from incompletely erasure of epigenetic marks, transgenerational epigenetics would be a largely passive process. Of course, this "error" in erasing marks may be an example of evolutionary design to enhance the likelihood of adapting to environmental stress **(O`Dea *et al.*, 2016)**. We use the same definition as for human/rodents: to ensure that the effect is really transgenerational, the F3 generation should display inheritable epigenetic marks. The most prominent example of transgenerational epigenetics has been obtained for the so-called epi-RILs (epigenetic recombinant inbred lines). Plants homozygous for ddm1 (mutant of the ddm gene) are severely hypomethylated. These plants have been crossed with plants homozygous for DDM1 (the normal methylation pattern). In the F2 generation, homozygous DDM1 plants were chosen and self-crossed for six generations to create recombinants. Stable DNA methylation patterns were inherited **(Heard and Martienssen, 2014)**. However, “the observation that about one half of DNA hypomethylation variants induced by ddm1 systematically regain wtDNA methylation over two to five generations illustrates the potentially transient nature of many epialleles" **(Johannes *et al.*, 2009)**. Further investigations **(Teixeira and Colot, 2010 and Teixara *et al.*, 2009)** have shown that RdDM is important for restoring the DNA methylation pattern at severe loss, and as RdDM DNA methylation is limited, it takes several generations to regain the wild-type methylation pattern. Consequently, the transgenerational effect on epi-RILs is induced by the organism’s limited possibility to respond to such a dramatic change rather than being an active mechanism that strives to maintain the methylation pattern. At the same time, not every methylation site is able to regain the wild-type pattern. Another example of transgenerational epigenetic variation has been found for drought resistance in *rice plants* **(Zheng *et al.*, 2013)**, and the effect of UV treatment has been detected in the F4 generation of *Arabidopsis* **(Molinier *et al.*, 2006)**. **Holeski *et al.* (2012)** summarizes the results of resistance to herbivores or pathogens. Evidently, the majority of studies have been limited to transgenerational epigenetics that lasted only one or two generations. Recently **Iwasaki and Paszkowski (2014)** identified a negative feedback loop for stress-induced transcriptional changes. DDM1 (DNA methylation 1) and Mom1 (Morpheus Molecule 1, a regulator of transcriptional gene silencing) act redundantly to prevent transgenerational effects in *Arabidopsis*. This provides evidence that an active mechanism exists to prevent extensive transgenerational epigenetics. Similar results have been obtained for transgenerational retrotransposition, where small interfering RNAs (siRNAs) play a crucial role in preventing transgenerational effects **(Ito *et al.*, 2011)**. Evidently, siRNAs

are crucial for preventing the progression of transgenerational epigenetics, and plants possess an active mechanism for restoring the wild-type pattern **(Iwasaki and Paszkowski, 2014 and Ito *et al.*, 2011; Teixara *et al.*, 2009)**. Furthermore, research on iron deficiency in *Arabidopsis thaliana* has suggested an active mechanism for preventing transgenerational transmission **(Murgia *et al.*, 2015)**.

Summary

As we have suggested for rodents/humans, epigenetics might be a test bed during evolution. Either the new trait is beneficial and will be transformed into a hard inheritance, or the biotic or abiotic stress dissipates and the new trait is non-beneficial, leading to the soft inheritance fading out. A similar idea was proposed by **Vannier *et al.* (2015)**, who suggested that plasticity mediated by transgenerational epigenetics could be followed by hard inheritance, or integration into the genome. Interestingly, there exist a few examples of natural transgenerational epigenetics. In Linaria vulgaris, bilateral-to-radial floral symmetry depends on the methylation pattern of the so-called Lcyc (Cycloidea-like) gene **(Pecinka *et al.,* 2013)**. Also, fruit ripening of tomatoes can be epigenetically controlled **(Manning *et al.*, 2006).** Consequently, there exists the possibility of maintaining epigenetics over many generations. Once we better understand the mechanism by which transgenerational epigenetics are established and maintained, a novel method for manipulating plants could be established. The target would not be the gene itself but the methylation pattern of specific genes. Importantly, as mentioned for rodents/humans, the exact timing of the application of biotic or abiotic stress is crucial. If the stress is applied to young sporophytes, the chimeric nature of their organs leads to a mixture of tissues. As a result, the microspores and macrospores might not contain the epigenetic mark and the corresponding phenotype will not be obtained. However, if the stress is applied to the flowering flower, all parts of the flower will be affected by the stress, including the pollen/grain and the embryo, and the epigenetic change might remain **(Kumar *et al.*, 2015)**. Furthermore, plants can reproduce asexually. Since meiosis can be bypassed, the likelihood of transgenerational epigenetics should increase **(Verhoeven and Preite, 2014)**. It has recently been shown that apomixis (asexual reproduction through seeds) allows for transgenerational fixation of phenotypes **(Sailer *et al.*, 2016)**, but only the F2 generation was investigated. Although epigenetic studies of asexual plants and comparison with sexual reproduction are limited, for some plants, such as the *Fallopia japonica*, landraces have been developed that show more epigenetic differences than genetic differences **(Verhoeven and Preite, 2014)**. Finally, recent data suggest that not only are intraindividual epigenetics important, but also interindividual epigenetic differences. **Xu *et al.* (2016)** showed that airborne plant-plant or even plant-plant-plant communication using jasmonic acid (JA) or salicylic acid (SA) pathways can transfer stress-induced epigenetic marks from a stressed to an unstressed neighbouring plant; the bystander effect is real **(Yao *et al.*, 2016)**. To summarize, compared to humans/rodents, transgenerational epigenetics in plants is more likely. However, similar to humans/rodents, these epigenetic marks are unstable. Nevertheless, through a yet unknown mechanism, natural transgenerational epigenetics are indeed stably inherited; and therefore we cannot rule out the possibility that a biotic or abiotic stress applied to the parents might be transferred to the future generations. Transgenerational epigenetics are important for enhancing the plasticity of the genome so that the organism can

quickly adjust to environmental changes but are rarely observed in F4 to F6 plant generations if the environmental stress is applied solely to the F0 generation.

ADJUSTED ORGANISMS, EPIGENETICS, AND TRANSGENERATIONAL EPIGENETICS

Most of our daily food has been created by mutations. As natural and spontaneous mutations are rare, mutation breeding has been the most economic choice of achieving such changes. New technologies like CRISPR/Cas techniques can produce fruit/crops that are genetically identical to those previously produced by traditional methods. The question arises, however, of whether these new technologies should be regulated under EU directive and be called GMOs **(Lassalle and Jarocki, 2016)**. According to the **National Academies Press (2016)**,

"prior National Research Council reports have argued that there is no strict dichotomy between genetic engineering and other forms of plant breeding with respect to risk. Recent developments in genome editing and other emerging genetic-engineering technologies make it even more apparent that regulatory approaches that focus on some form of breeding "process" as an indicator of risk are less and less technically defensible. Some emerging genetic-engineering technologies are likely to create new crop varieties that are indistinguishable from those developed with conventional plant breeding, whereas other technologies, such as mutagenesis, that are not covered by existing laws could create new crop varieties with substantial changes to plant phenotypes. The size and extent of the genetic transformation has relatively little relevance to the extent of the change in the plant and consequently to the risk that it poses to the environment or to food safety. The committee recommends the development of a tiered approach to regulation that is based not on the breeding process but on considerations of novelty, potential hazard, and exposure as criteria."

The new term "Adjusted Organism" provides a first attempt to such a unifying approach **(Lassalle and Jarocki, 2016)**. Increasing knowledge in epigenetics points out that subtle differences resulting from the different breeding methods might influence the metabolics of the plants. In this chapter, we will describe some of the traditional and novel breeding methods and examine the possibility of transgenerational epigenetics. The evolution of corn dates back more than 10,000 years, with landraces developed using unconscious selection and open pollination. According to **Mercer and Perales (2010)**, "landraces tend to possess significant phenotypic variability and some have developed tolerances to particular abiotic and biotic stresses." Thus, landraces are a direct consequence of chemical- and radiation (UV)-induced stress. Consequently, the EU Recital 17 "states that the Directive should not apply to organisms obtained through certain techniques of genetic modification which have conventionally been used in a number of applications and have a long safety record. Mutagenesis–a method used in traditional plant breeding, where variations in the plant genome are introduced using radiation or chemicals–is explicitly exempt from the scope of GMO legislation, on the basis that it has a long history of safe use" **(European Parliament, 2016)**. For example, the Japanese Gold Nijisseiki, a mutant induced by gamma radiation to prevent black spot disease, is not a GMO **(Saito, 2016)**. Organic food made from Rio Star Grapefruit, which was created by atomic gardening, is also exempted from the GMO regulation. The same is true for rice breeding where it has been shown that ion beams are more effective than gamma rays **(Yamaguchi *et al.*, 2009)**. Natural mutations will occur by UV-A and UV-B radiation. As the ozone layer becomes increasingly depleted, UV-C-

induced mutations are also likely. IR-induced mutations are also possible. Consequently, radiation-induced mutation breeding methods are exempted from the GMO regulation because the methods reassemble natural mutation strategies. Obviously, the problem with the EU regulation is defining a threshold that divides techniques into safe and risky categories. A recent article by **Mueller-Xing *et al.* (2014)** summarizes the effects of UV on *Arabidopsis thaliana*. UV activates the MAP kinase signalling pathway (MPK3, MPK6), reacts with UV photoreceptor UVR8, which can lead to increased flavenoid and anthocyanin biosynthesis, and upregulates ROS-induced cell death. Of course, DNA double strand breaks can occur, which would lead to activation of DNA repair pathways. **Mueller-Xing *et al.* (2014)** suggest a potential link between epigenetic memory and DNA repair. Furthermore, the UVR8 pathway and the MAP kinase pathway could potentially establish transgenerational epigenetic memories in addition to the upregulated phytohormones (SE) that influence H3K4me3. Data from **Migicovsky and Kovalchuk (2016)** support transgenerational changes in response to UV-C, but these epigenetic changes were measured only until the F2 generation. UV-B-induced epigenetic changes have been followed until the F4 generation in *Arabidopsis thaliana* **(Molinier *et al.*, 2006)** for constructs that measure the increase in homologous recombination in response to UV radiation. However, the reproducibility of these results is poor; transgenerational stress memory is not a general response mechanism of *Arabidopsis thaliana* **(Pecinka *et al.*, 2009)**. In contrast to **Molinier *et al.* (2006)**, no transgenerational effect of UV-B or UV-C could be detected. In another study by **Lang-Mladek (2010)**, transgenerational epigenetics were observed, but this effect faded out before the F3 generation. As discussed in the previous sections, not only is the intensity of the stress crucial, but also the exact timing of application of the biotic or abiotic stress. Consequently, the results from different research groups vary widely. Unfortunately, investigation on other crops or plants remains rare, with *Arabidopsis thaliana* being the main plant studied. Certainly, the studies are few and the results are contradictory. However, one cannot exclude the possibility that plants created by radiation-induced mutation breeding (using x-ray, ion beams or other high energy radiation such as UV), as traditional non-GMO methods, carry an epigenetic mark as a kind of memory of the abiotic stress that was used to create the mutation. This might influence the plant's metabolics and potential allergenic properties. The lack of data in this area emphasizes the need to evaluate the product and not the method. The number of studies is not sufficient to enable unbiased prediction based solely on the method. Another non-regulated method uses chemicals (abiotic stress). Ethyl methanesulfonate and sodium azide, for example, have been used by BASF to create their Clearfield crops and DuPont produces herbizide-resistant crops using chemical mutations with methanesulfonate. Both of these examples are non-GMO products per EU regulation. For biotic and abiotic stress, the asexual variants of *Taraxacum officinale* exhibited transgenerational epigenetics **(Verhoeven *et al.*, 2010)**. Unfortunately, only the F2 generation was investigated and, as pointed out in the previous chapter, the possibility of transgenerational effects is increased in apomictic plants. In another publication, it was shown that the amount of salt, as an abiotic stress factor, influences the following generation via DNA methylation and dicer-like proteins **(Boyko *et al.*, 2010)**. Again, only the F2 generation has been investigated. The effects of stress induction by heavy metals can also be passed to the following generation, but if the stress is relieved, the transgenerational effect fades out **(Rahavi *et al.*, 2011)**. This result is similar to the outcome of the research from **Wibowo *et al.* (2016)**, which emphasizes the memory

effect of short-term stress: "Our main conclusion is that intergenerational priming responses to hyperosmotic stress are triggered by recurrent exposure to stimuli, but that this response is rapidly lost in the absence of stress." In contrast, **Groot *et al.* (2016)** concludes: "we showed that exposure to salt treatment can have persistent consequences for offspring phenotypes not only one generation after exposure, but also two or even three generations later. These transgenerational effects were often expressed as 'dose' effects where multiple-generation exposure has different impact on offspring traits than single-generation exposure. Our results suggest that transgenerational effects commonly occur and may have a considerable impact on plant phenotype, performance and the way plants respond to their environment." Thus, we cannot exclude the possibility of transgenerational epigenetics induced by chemical stress. We should be aware that both chemical- and radiation-induced stress has the possibility of inducing transgenerational epigenetics, which could eventually upregulate metabolites that might increase allergenic effects. While 10,000 years of safe use provides strong evidence of a lack of risk, we still lack the knowledge to understand the process in detail. The existence of transgenerational epigenetics seems to be possible, but is it likely? In summary, we should have a strict risk assignment for the product itself and not judge it by the mechanism used to create it. Modern breeding methods use genome editing. This includes TALENs (transcription activator-like effector nucleases), ZFNs (zinc finger nuclease) and CRISPR (clustered regularly interspaced short palindromic repeats)/Cas (CRISPR-associated) **(Xiong *et al.*, 2015)**. Compared to the traditional breeding method, which is a sledgehammer method of randomly creating mutations in the DNA, the new methods are minimally invasive and work like a scalpel. However, should these new technologies be regulated as GMOs? The Swedish Board of Agriculture has exempted some of the CRISPR/Cas methods from GMO regulations **(Swedish Board of Agriculture, 2015)**; however, future decisions will be made by a case-by-case basis. The EU decision regarding the CRISPR/Cas methods enhanced tough GM laws. CRISPR/Cas not only more efficiently creates mutations identical to those created by unconscious or conscious selection, but could enable a new form of mutation breeding – epigenetic engineering **(Vora *et al.*, 2016)**. Until now, CRISPR/CAS has mainly been used to create site-specific double-strand breaks in connection with the cell's own repair system (non-homologous recombination, homologous recombination). These double-strand breaks can induce epigenetic changes. In cells, they promote the methylation of histone H3 on lysine 9 **(Ayrapetov *et al.*, 2014)**. Furthermore, they can permanently alter the methylation pattern of the DNA and the histone **(Russo *et al.*, 2016)**. Consequently, the novel breeding methods have the possibility of inducing epigenetic changes; however, it is currently unknown whether this leads to transgenerational effects. Is it possible that genetically identical organisms produced by different breeding methods show differences in their proteomics, metabolics, RNomics and epigenomics? As the traditional methods (mutation breeding) and novel methods are all closely linked to epigenetic changes, and the epigenetic response mechanisms differ, it is possible that genetically identical organisms produced by different breeding methods possess different -omics. However, if transgenerational epigenetics are mainly a short-term stress response, the differences will fade out after several generations (F3~F4).

CONCLUSION

Recent progress in transgenerational epigenetics has emphasized the idea that the traditional breeding methods used for more than 10,000 years might induce inheritable epigenetic changes. For novel breeding methods (including genome editing), data on such transgenerational epigenetic changes are missing, but we cannot exclude the possibility that epigenetic marks are transferred to the next generations. The ability to simulate the molecular mechanisms that enable mutation breeding is steadily increasing but is not yet sufficient. Consequently, a threshold that divides methods into beneficial/safe and potentially dangerous does not exist. Placing the emphasis on the product rather than on the method used to create the organism provides the only strict scientific approach. Transgenerational epigenetic effects cannot be ruled out. Therefore, identical mutations, created by two different breeding methods, could exhibit subtle differences, including metabolic changes that might increase allergenic effect. To investigate the frequency and importance of these hypothetical subtle changes, we suggest that identical mutations be created by different methods (e.g. radiation breeding and CRISPR/Cas) and be analysed in terms of all the available -omics.

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