

Stress-Induced Chromatin Changes: A Critical View on Their Heritability

Ales Pecinka^{1,*} and Ortrun Mittelsten Scheid²

¹Max Planck Institute for Plant Breeding Research, Cologne, Germany

²Gregor Mendel Institute of Molecular Plant Biology, Austrian Academy of Sciences, Vienna, Austria

*Corresponding author: E-mail, pecinka@mpipz.mpg.de, Fax, +49-221-5062-413.

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The investigation of stress responses has been a focus of plant research, breeding and biotechnology for a long time. Insight into stress perception, signaling and genetic determinants of resistance has recently been complemented by growing evidence for substantial stress-induced changes at the chromatin level. These affect specific sequences or occur genome-wide and are often correlated with transcriptional regulation. The majority of these changes only occur during stress exposure, and both expression and chromatin states typically revert to the pre-stress state shortly thereafter. Other changes result in the maintenance of new chromatin states and modified gene expression for a longer time after stress exposure, preparing an individual for developmental decisions or more effective defence. Beyond this, there are claims for stress-induced heritable chromatin modifications that are transmitted to progeny, thereby improving their characteristics. These effects resemble the concept of Lamarckian inheritance of acquired characters and represent a challenge to the uniqueness of DNA sequence-based inheritance. However, with the growing insight into epigenetic regulation and transmission of chromatin states, it is worth investigating these phenomena carefully. While genetic changes (mainly transposon mobility) in response to stress-induced interference with chromatin are well documented and heritable, in our view there is no unambiguous evidence for transmission of exclusively chromatin-controlled stress effects to progeny. We propose a set of criteria that should be applied to substantiate the data for stress-induced, chromatin-encoded new traits. Well-controlled stress treatments, thorough phenotyping and application of refined genome-wide epigenetic analysis tools should be helpful in moving from interesting observations towards robust evidence.

Keywords: Chromatin • Evolution • Stress • Trans-generational stress memory.

Abbreviations: ARP6, actin-related protein 6 (subunit of SWR1); CAF-1, chromatin assembly factor 1; FAS1,2, fasciated 1,2 (subunits of CAF-1); H2A, histone H2A (canonical

nucleosome subunit); H2A.Z, histone variant H2A.Z; MSAP, methylation-sensitive amplified polymorphism; qPCR, quantitative PCR; SWI2/SNF2, SWI2/Sucrose Non-Fermentable2 (remodeling complex); SWR1, Swi2/Snf2-related 1 (remodeling complex); TEs, transposable elements; TGS, transcriptional gene silencing; TSI, transcriptionally silent information (repetitive genomic sequence)

Introduction

Stress, in a biological context, refers to the consequences if organisms fail to respond adequately to unfavorable conditions. If stress cannot be avoided, e.g. by hiding or migration, physiological reactions are activated that help protect the organisms against deleterious effects, although a substantial impact on fitness, growth and development is often unavoidable. Plants, as sedentary organisms, have developed an impressive portfolio of stress responses. Nevertheless, pathogen attacks, drought, salinity or extreme temperatures can have a significant impact on vigor, including biomass production and yield in agriculture. Therefore, progress in plant breeding and biotechnology towards more stress-resistant cultivars requires better understanding of plant stress responses, to reduce such losses. Moreover, the need for greater insight into the stress defense mechanisms of plants will increase with the predicted rise of average temperatures and longer periods of extreme weather (Ahuja et al. 2010). The challenges of these changes will not only affect cultivated plants but will also have a tremendous impact on whole ecosystems including wild species. Thus, studying plant responses to abiotic stress may also be helpful in understanding plant ecology and evolution, the disappearance of species and colonization of new niches often with unfavorable conditions.

Approaches to understanding stress responses have been the focus of plant biologists for a long time and have provided extensive knowledge about various physiological stress responses and their molecular bases (Chinnusamy et al. 2004, Yamaguchi-Shinozaki and Shinozaki 2006, Huang et al. 2012).

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The early phases, and specificity, of stress perception have been of special interest to researchers, as these determine subsequent downstream reactions. Also, the return to the pre-stress physiology, once the adverse conditions are gone, has been well investigated. However, the long-term perspective, addressing the potential for a 'stress memory' or heritability of stress effects in case of lasting effects, is less well studied. This originates from the general consensus that most traits determining stress resistance have a genetic basis and are subject to Darwinian natural selection and Mendelian inheritance. While there is no doubt about the validity of these principles, supported by the successful introgression of stress resistance traits during plant breeding, the occasional rapid development of new, sometimes unstable, traits is not easily reconciled with this concept (Jablonka and Raz 2009). Therefore, other, 'faster' mechanisms for long-term adaptation have been postulated and often related to the idea of Lamarckian inheritance, assuming that 'an organism can pass on characteristics or potential that it acquired during its lifetime to its offspring' (http://en.wikipedia.org/wiki/Inheritance_of_acquired_characteristics). For a long time, this idea was rejected for two main reasons. First, in spite of many attempts, a Lamarckian type of inheritance could not be reproducibly confirmed. Secondly, the concept was heavily misused to perform pseudo-scientific experiments and eliminate the opponents of Trofim Lysenko and his colleagues in the first half of the last century in Russia. However, in recent years, the development of highly sensitive stress reporter systems and the discovery of epigenetic mechanisms have revived the idea of Lamarckian 'fast' inheritance (Koonin and Wolf 2009). Indeed, some epigenetic phenomena, e.g. paramutation (reviewed in Chandler and Stam 2004), lead to the quick loss or gain of novel phenotypes that are inherited in a non-Mendelian manner. Yet, although the genetic and molecular basis of paramutation is quite well understood and in agreement with classical paradigms, a connection with stress response is not obvious. Perception of stress in one part of the plant can cause increased resistance throughout the whole plant in the process of systemic acquired resistance, and, in a process termed priming, slight stress exposure of plants leads to faster and better responses upon subsequent, more severe treatments. Again, both phenomena are explicable by molecular effects on stress perception and signaling components (reviewed in Shah 2009, Conrath 2011), and there is no evidence for their transmission to the next generation. A more likely carrier of heritable information is chromatin, the complex of genomic DNA with specialized proteins that determine the organization and packaging of the long DNA molecules within the nucleus. DNA is wrapped around nucleosomes, which are abundant chromatin protein octamers consisting of 2×4 different histone molecules. The N-terminal tails of the histones protrude from the spherical nucleosomes and can be covalently modified by acetylation, methylation, phosphorylation, ubiquitination and other residues. Also the cytosine residues of DNA can be methylated. All modifications together change the physical and chemical properties of genomic DNA. Chromatin

controls the accessibility for DNA-interacting factors via condensation and provides information about gene expression potential in an epigenetic manner, i.e. in addition to DNA sequence information. Disturbances of chromatin structure result in de-regulation of gene transcription or hypersensitivity to DNA damage and can lead to abnormal development. As will be described below, there is growing evidence that stress responses can directly or indirectly modify epigenetic regulation and chromatin. As some chromatin changes are stable and become independent of the trigger, and in extreme cases form heritable epialleles (Cubas et al. 1999, Soppe et al. 2000, Manning et al. 2006), it is conceivable that stress induces persistent, or even heritable, chromatin modifications that alter gene expression and phenotypic traits, and thereby overrides Darwinian selection based exclusively on genome information. Here, we review recent literature on plant chromatin responses to abiotic stimuli and stress, their duration and functional significance, and discuss the criteria to claim their heritability.

Chromatin changes in response to stress

Short-term and transient responses

Reports on chromatin modifications upon external stimuli are numerous and diverse. Among abiotic stress factors, the best documentation exists for the effects of heat, which causes epigenetic deregulation and transposon activation (Lang-Mladek et al. 2010, Pecinka et al. 2010, Tittel-Elmer et al. 2010). This requires severe conditions and a certain duration of heat exposure, and it is enhanced by preceding cold treatment (Tittel-Elmer et al. 2010). The response is associated with loss of DNA-bound nucleosomes and transient heterochromatin de-condensation (Pecinka et al. 2010). Less drastic heat exposure affects histones more specifically: the transcript profile of mutants lacking *ACTIN RELATED PROTEIN 6* (*ARP6*) resembles that of heat-exposed plants even at ambient temperature (Kumar and Wigge 2010). *ARP6* is part of the *SWI2/SNF2* nucleosome assembly complex required for loading the histone H2A.Z variant onto DNA predominantly at transcriptional start sites. H2A.Z nucleosomes are more tightly associated with DNA than nucleosomes with canonical H2A but become evicted by higher temperature. Loss of *ARP6* function mimics the state after heat-induced H2A.Z dissociation and thereby results in similar transcriptional regulation and phenotypes. Thus, H2A.Z-mediated regulation of gene expression incorporates a thermo-sensing signal and represents a bona fide functional chromatin response to a change of an abiotic parameter (Kumar and Wigge 2010).

Heat, but also other abiotic stress types, leads to transcriptional activation of several transgenic and endogenous targets of transcriptional gene silencing (TGS)—a mechanism controlling repression and heterochromatinization of repetitive DNA regions in plants (reviewed in Madlung and Comai 2004, Chinnusamy and Zhu 2009, Mirouze and Paszkowski 2011,

Paszkowski and Grossniklaus 2011, Khraiweh et al. 2012). Here we focus on several recent studies with *Arabidopsis*, so far providing the deepest insight into *cis*- and *trans*-acting factors and mechanisms. Genome-wide expression analysis after prolonged heat or cold–heat stress revealed significant transcriptional up- and down-regulation of 1–2% of approximately 1,500 transposable elements (TEs) represented by probe sets on the ATH1 microarray (Pecinka et al. 2010, Tittel-Elmer et al. 2010). All TEs returned to their pre-stress expression level within <2 d of recovery at ambient temperature, with the exception of the *COPIA78* retrotransposon family. Transcripts of these TEs were detectable early (relative to other TGS targets) after onset of stress, and their high levels were still present up to 7 d post-stress. The potential for reintegration of new copies of this TE into the genome in the case of compromised epigenetic control (Ito et al. 2010, Ito et al. 2011) is discussed elsewhere (Mirouze and Paszkowski 2011, Paszkowski and Grossniklaus 2011). Chromatin analysis revealed transcriptional activation of these and other activated elements to be independent of DNA de-methylation and loss of histone H3 lysine 9 di-methylation (Lang-Mladek et al. 2010, Pecinka et al. 2010, Tittel-Elmer et al. 2010), two epigenetic marks reduced upon reactivation in the background of several TGS mutants. Instead, the genomic copies of the heat-induced TEs and many other genomic regions (including non-transcribed sequences) had reduced nucleosome occupancy, concomitant with the above-mentioned heterochromatin dissociation (Pecinka et al. 2010). A role for nucleosome loading, rather than specific modification marks, is further suggested by delayed re-silencing of heat stress-activated *TRANSCRIPTIONALLY SILENCED INFORMATION (TSI)*, an *ATHILA*-related retrotransposon (Steimer et al. 2000), in mutants with reduced *FASCIATA 1* and *2* proteins (*FAS1* and *FAS2*), the two largest subunits of the *CHROMATIN ASSEMBLY FACTOR 1 (CAF-1)* (Pecinka et al. 2010). Thus, interference of prolonged heat stress with epigenetic gene silencing may be due to transient changes of nucleosome loading and chromatin organization rather than DNA or histone methylation.

A direct connection between the temperature-sensing H2A.Z at transcription-competent start sites of genes (Kumar and Wigge 2010) and the heat-induced loss of nucleosomes from heterochromatic repeats (Pecinka et al. 2010) is unlikely as DNA methylation typical for the latter is mutually exclusive with H2A.Z domains (Zilberman et al. 2008). However, both responses have in common that the removal of histones does not increase expression of all genes equally and therefore is not sufficient for transcriptional activation. The occurrence of multiple histone variants, modifications, chaperones and different nucleosome loading make it likely that chromatin dynamics upon stress are the result of a complex interplay between physical factors, their perception, pre-existing chromatin structure and maintenance mechanisms.

Like abiotic factors, pathogen-induced stress can also result in chromatin responses, and different features of chromatin affect the defense against pathogens. Infections, or chemicals

mimicking pathogen attack, can change histone acetylation and methylation (Butterbrodt et al. 2006, Mosher et al. 2006, Jaskiewicz et al. 2011, Kim et al. 2012). Further, there is a correlation between the amount of a histone ubiquitin ligase and resistance to necrotrophic fungi (Dhawan et al. 2009), and loss of a histone methyltransferase results in enhanced susceptibility to bacterial pathogens (Palma et al. 2010). Involvement of chromatin remodeling in signaling of biotic stress is further suggested by decreased resistance to necrotrophic fungi of mutants with an impaired SWI/SNF ATPase (Walley et al. 2008). A role for histone variant placement is indicated by reduced salicylic acid-induced immunity in mutants lacking subunits of the SWR1 complex that installs histone variant H2A.Z (March-Diaz et al. 2008). Pathogens can also interfere with the hosts' chromatin in their favor (reviewed in Ma et al. 2011).

Memory effects reset upon reproduction

While changes in gene expression and chromatin triggered by the stressful conditions described above are largely transient, i.e. reconstituted to the pre-stress situation shortly after return to favorable conditions, there are several processes that indicate a 'memory' effect, sometimes lasting for the lifetime of the affected individual. The best documented case in connection with a chromatin signature is the process of vernalization, i.e. the control of flowering time by preceding exposure to low temperatures. Vernalization causes repression of flowering-inhibiting factors and, once installed, this suppression persists even upon return to higher temperatures. In *Arabidopsis*, this involves the recruitment of chromatin-modifying enzymes to specific target genes and their subsequent inactivation (reviewed in Adrian et al. 2009, Kim et al. 2009). There is no evidence that a 'memory' of vernalization is inherited from cold-exposed individuals to the next generation, but rather there is a well-documented resetting by renewed up-regulation of the flowering inhibitor during early embryo development (Sheldon et al. 2008). In addition, the cold temperature is necessary for an important developmental switch and cannot be considered as a stress in the sense of unfavorable conditions. This is different from the case of memory effects mentioned earlier, such as systemic acquired resistance, immunity, priming or acclimation. Perception factors and signal cascades are certainly key components in these processes, but growing evidence indicates that they can result in chromatin and DNA methylation changes at specific genes which, in turn, render these genes differentially responsive to later stimuli (reviewed in Jarillo et al. 2009, van den Burg and Takken 2009, Luo et al. 2011, Ma et al. 2011, Santos et al. 2011, Yaish et al. 2011, Grativol et al. 2012, Zhu et al. 2012). The enhanced or decreased susceptibility to renewed stress and the corresponding chromatin changes can persist for different periods beyond the primary exposure, sometimes for a long time, but there is no undisputed evidence that they are stably inherited by subsequent generations.

Lasting responses inherited by progeny

The last statement of the previous paragraph will not go unopposed, as there are numerous reports of experiments supposedly demonstrating stress-induced epigenetic states that are inherited by non-stressed progeny (recently, for example, Bilichak et al. 2012, Luna et al. 2012, Rasmann et al. 2012, Slaughter et al. 2012; more references reviewed in Boyko and Kovalchuk 2011). Rightly, these studies have received special attention as they propose a principally novel type of stress adaptation and revive the idea of inheritance of acquired characters. We, and others, have conducted an extensive literature review and identified several common issues that limit an unambiguous interpretation and acceptance of these studies. Based on this, we conclude that firm evidence for a role for chromatin modification in inheritance of stress-induced changes is still missing in plants. However, we agree that it is a very exciting field of research and, therefore, we propose criteria that we would like to see fulfilled during the analysis of trans-generational epigenetic memory effects. We believe that sharing these points with the research community may help to provide new, incontestable evidence for a direct and durable chromatin-encoded impact of environmental parameters on phenotype and adaptation.

- (i) Stress-induced expression changes of *trans*-acting chromatin modifiers do not unconditionally lead to quantitative changes of the respective chromatin mark. Lower expression of the DNA methyltransferase responsible for replication-associated maintenance methylation can only be effective if the inducing conditions do not arrest the cell cycle at the same time (Steward et al. 2000). Therefore, the analysis should include transcript and protein levels (in ideal cases protein activity), accessibility of the substrates and implementation of the chromatin changes at the specific targets.
- (ii) Transgenic reporter constructs for visualization of epigenetic effects have different expression levels, patterns and sensitivity, and need to be chosen carefully. The same reporter can be reactivated to various extents by different mutations (Elmayan et al. 2005), between strong expression in coherent cell lineages and weak, stochastic expression in individual cells. Trans-generational changes require the epigenetic change to occur in sectors or cells forming the germline, and must be significant enough to become permanent. Even genetically induced epigenetic switches can appear stable but revert after a few generations (Foerster et al. 2011), and lines containing transgenic homologous recombination substrates show occasional hyper-responsiveness and high variation even upon mock treatments. The variation between experiments can be of the same order as responses under inducing conditions within an experiment (Pecinka et al. 2009). Therefore, we suggest that data generated using transgenic constructs should be confirmed with experimentally different strategies, as with work with endogenous indicators, or independent quantification methods such as quantitative PCR (qPCR) analysis.
- (iii) Stress in nature often consists of several components, and plants have adapted to cope with multiple stress types simultaneously, as reflected in the many signaling components involved in different stress responses (Huang et al. 2012). While researchers usually try to apply one defined stress type at a time, this might not always be successful, due to incomplete control over growth conditions, undetected pathogen infestations, difficult dosing of stress or unavoidable side effects in experiments. Lack of reproducibility and different results between labs and/or experiments can be reduced by very carefully establishing the stress conditions prior to the actual experiments, recording as many parameters as possible, and repeating experiments with the same stress treatment under otherwise slightly different settings. Any trans-generational stress memory that is relevant under highly variable conditions in nature should be robust enough to be reproduced this way.
- (iv) DNA methylation is a well-established and important epigenetic mark in plants. However, it is not always the primary indicator of chromatin changes and depends in part on the level of small RNA molecules and other, already DNA-associated marks (Kanno and Habu 2011). DNA methylation differences can be indirect effects, or even be absent in spite of chromatin changes (Pecinka et al. 2010). Chromatin needs to be analyzed in a synoptic view on different features, including DNA methylation, small RNA quantification, specific histone modifications and DNA–histone association.
- (v) Analysis of DNA methylation is very popular as an indicator of stress-related changes, as it is relatively easy to investigate by various methods. However, many of the techniques [e.g. cytosine extension assays, methylation-sensitive amplified polymorphism (MSAP) and Southern blots with methylation-sensitive restriction enzymes] limit the experiments to certain genomic regions and cannot quantify or detect heterogeneity of methylation. They can provide preliminary evidence for genome-wide or region-specific differences, but these should be substantiated with bisulfite sequencing, offering either locus-specific or genome-wide single base resolution (Gupta et al. 2010).
- (vi) The role of DNA methylation can differ depending on its location within genes. In addition to functionally discrete modification of cytosines in different sequence contexts (CG, CHG or CHH), CG methylation in repetitive sequences, transposons and gene promoters is usually associated with transcriptional silencing, while methylated CG within exons and introns is prominent in the centre of moderately transcribed genes (reviewed in Saze and Kakutani 2011). Although the role of this gene body methylation is not clear, it is probably quite different from methylation at inactive parts of the genome (Saze and Kakutani 2011). This needs to be considered if stress-induced methylation changes are interpreted.
- (vii) Correlation is not causality: stress-related phenotypes or susceptibilities may appear connected with epigenetic changes (typically DNA methylation; see point iv) but these can be secondary effects or independent spontaneous variations (Becker et al. 2011, Schmitz et al. 2011), without relevance. Claims for a causal relationship between defined changes (see points v and vi) and stress responsiveness should be

proven by analysis including mutants, overexpressors, inhibitors, etc.

- (viii) In plants, trans-generational inheritance of induced chromatin changes is more difficult to define than in animals, due to the late separation of germline-forming cells from other somatic cells. Flowers containing the pre-meiotic cells are not more protected from stress exposure than other aerial plant parts, and differentiated somatic cells can re-differentiate into meristematic tissue and open a new germline via somatic embryogenesis (Verdeil et al. 2007). Re-establishment of a chromatin state after genetic interference may take more than one generation (Teixeira et al. 2009). Therefore, caution is required not to mistake such 'carryover' effects for proof of trans-generational inheritance. Claims for a memory effect should be documented by significant changes observed for more than two subsequent non-stressed generations, as in the case of the chromatin-based gene expression change in *Drosophila*, so far the best evidence for heritable effects after defined heat stress treatment (Seong et al. 2011). However, even here, the transcriptional activation is lost in the third non-stressed generation. Boosting the response by repeated treatments in subsequent generations makes the effect stronger, but not longer lasting (Seong et al. 2011), and can theoretically also be explained by additive carryover effects. Further, stress application restricted to the early part of the life cycle can help to reduce possible artifacts produced by affecting the progeny-forming cells while they are still contained within the exposed plant. At least in animals, a critical window for chromatin-related changes is limited to early developmental stages (Skinner 2011). A recent critical review of trans-generational epigenetic inheritance in mammals lists further arguments for transmission of diffusible molecules, rather than chromatin-based mechanisms (Daxinger and Whitelaw 2012).
- (ix) Many experiments addressing non-genetic trans-generational inheritance are not performed with actual stress treatments, but rather with different inhibitors or toxic compounds (Guerrero-Bosagna and Skinner 2012). While some of these might be good at mimicking stress by interfering with certain components in the signaling pathways, they may however, have unnoticed side effects that would not occur with the physical or pathogen-induced stress, or they could miss some targets of those more systemic treatments. Therefore, stress-inducing or stress-mimicking drugs should be used with caution and include validation of the results with more genuine stress.
- (x) Recent studies have shown that stress-induced chromatin effects can result in genetic changes (Ito et al. 2010, Ito et al. 2011, Matsunaga et al. 2012), or genetic changes can cause reprogramming of previously stable epigenetic states (Foerster et al. 2011). Any analysis of heritable chromatin change therefore needs to exclude simultaneous *trans*-acting genetic changes. With the exception of closely linked genetic and epigenetic changes, a proof of true breeding of the affected chromatin configuration upon outcrossing with non-affected plants could help to exclude such a connection.
- (xi) Finally, any transmitted stress-induced chromatin change is relevant for a discussion about inheritance of acquired characters

only if the change provides a benefit under specific conditions, i.e. affects the progeny's stress resistance, stress responsiveness or adaptability. Therefore, the progeny should be scored carefully for their performance under the same type of stress as applied to the ancestors, and for general fitness in comparison with progeny of unexposed plants.

According to these criteria, and to the best of our knowledge, no published data set unambiguously demonstrates trans-generational inheritance of an exclusively epigenetic and stable change induced by stress exposure of plants. Even severe conditions applied under laboratory conditions do not seem to be sufficient for permanent and/or complete erasure of pre-stress chromatin marks (Pecinka et al. 2010, Tittel-Elmer et al. 2010). Rather, a 'memory' function exists for maintaining existing or restoring disturbed chromatin states, as shown after genetic interference with DNA methylation (Teixeira et al. 2009), and not for remembering disruptions. Maintenance and restoration of chromatin states involves sophisticated, sometimes redundant and self-reinforcing mechanisms, for which quite a few components are known (Vaillant and Paszkowski 2007, Law and Jacobsen 2010, Kanno and Habu 2011, Meyer 2011, Saze et al. 2012). In addition, they can be determined by the DNA sequence itself, as shown by the autonomous installation of DNA methylation patterns independent from transcription, genomic location and neighboring sequences in fungi and mammalian cells (Miao et al. 2000, Lienert et al. 2011), or by partially sequence-determined nucleosome positioning (Segal et al. 2006, Chodavarapu et al. 2010).

Chromatin responses to stress in evolutionary perspective

In spite of the maintenance mechanisms, chromatin undergoes a lot of programmed or induced changes upon developmental and exogenous triggers, as described above. It is evident that individual stress-related genes in plants are also partially regulated at the chromatin level. Chromatin effects on other genes or genome-wide changes upon stress are less plausible. They could contribute to stress response in an as yet unknown way, or open a 'window of opportunity' for potentially beneficial changes (including a putative stress memory for future times or generations), thereby having a selective benefit. Alternatively, undirected effects could be a 'sign of imperfection' of the stress control. Maintenance of genome and epigenome stability under stress costs energy, and a limitation of resources under stress may allow this investment only locally. The less drastic effects of heat stress on higher order nuclear architecture in the shoot apical meristem, compared with differentiated tissues (Pecinka et al. 2010), might indicate such preferential protection, which would, in turn, reduce the chance for trans-generational chromatin changes even more. However, selection on the evolutionary scale, especially under adverse conditions, would certainly favor adaptive changes on all levels, including chromatin, even if they occur only with

minimal probability. Currently, they are not unambiguously substantiated, but plants are good candidates for a further, unprepossessed search. Constant refinement of chromatin analysis tools and growing genomic information, also for non-model species, together with the criteria listed here, will help answer whether it is time for a renaissance of Lamarck's ideas.

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