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OPINION PAPER

Moving forward in determining the causes of mutations: the features of plants that make them suitable for assessing the impact of environmental factors and cell age

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Abstract

Currently, the types of factors that impact the mutation rate is a controversial issue. The marked attention towards identifying the factors that impact the genomic mutation rate is justified because mutations are the source of genetic variation underlying evolution and because many mutations have deleterious effects and can cause diseases. Although data showing correlations between germ cell division number and mutation rates (from epidemiological studies and molecular evolutionary rate analyses) have suggested that most mutations in animals are replication errors, this notion is highly debated and inconsistencies in the correlations suggest that other, replication-independent factors, could play an important role. Likely candidates include environmental parameters and cell age, but these issues have proved to be difficult to study using animals and *in vitro* systems, and consequently, very few or no data currently exist. The specific features of plants that make them powerful model systems for revealing the influence of the environment (natural environmental factors) and cell age on the spontaneous genomic mutation rate are discussed here. Overall, the evidence suggests that plants could be key biological systems for advancing our knowledge about how and why heritable mutations arise.

Key words: Cell age, environment, genomic mutation rate, model system, plants.

Introduction

Given that the genomic mutation rate plays a critical role in many evolutionary processes, for example evolution of mating systems, sex, ploidy levels, Y chromosomes, and species extinctions (Charlesworth and Charlesworth, 1998; Kondrashov, 1998), and that many mutations cause diseases, it is of broad scientific interest to determine the factors that influence the rate of mutation. Currently, however, much remains unknown. Findings of correlations between the number of germ cell divisions (DNA replication) and mutation rates in humans and other organisms suggest that most germ line mutations are replication errors. Specifically, human epidemiological data and/or nucleotide substitution rates of selectively neutral DNA (which equals the mutation rate, Kimura, 1983; Miyata *et al*., 1987) have shown that more mutations occur in the male than in the female germ line for numerous animal taxa (e.g. humans, mice, chickens, and sheep) and in older rather than younger human males, patterns that each agree with the cell-division hypothesis (i.e. more DNA replications in males and in particular older males; Penrose, 1955; Risch *et al*., 1987; Becker *et al*., 1996; Moloney *et al*., 1996; Li, 1997; Green *et al*., 1999; Crow, 2000; Li *et al*., 2002; Makova and Li, 2002). Other data, however, have indicated that the mutation bias reported relative to gender and male age are not generally well correlated with the number of germ cell divisions and that other factors could explain these trends, such as methylation patterns, differential repair, metabolic rates, and preferential transmission of mutations to progeny from older males (Risch *et al*., 1987; Martin and Palumbi, 1993; Drost and Lee, 1995; Bromham *et al*., 1996; Hurst and Ellegren, 1998; Martin, 1999; Crow, 2000; Huttley *et al*., 2000; McVean, 2000; Sommer *et al*., 2001;

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Hebert *et al*., 2002; Hurst and Ellegren, 2002; Kumar and Subramanian, 2002; Li *et al*., 2002; Bartosch-Harlid *et al*., 2003). Regardless of whether one is, at present, more convinced by one argument or the other, it is apparent that most information about the factors that underlie spontaneous mutation rates has been limited to the detection of the presence or absence of correlations between the numbers of germ cell divisions and mutation rates. It is thus evident that further empirical data are needed regarding the relationship between replication-independent factors, such as environmental parameters and cell age, and the mutation rate. A first step in making progress on this issue is to consider why so few data currently exist. The challenges in assessing the impact of environmental parameters and cell age on genomic mutation rates using the relatively conventional *in vitro* and animal-based systems are described here and the innate advantages of plants for such research are highlighted.

Plausible reasons for the lack of data

Poor suitability of in vitro *research*

To date, most quantitative mutagenesis research has largely been based on *in vitro* analysis of bacteria, yeast, and isolated animal cell lineages. Although such research has played a critical role in current understanding of the mechanisms of mutation, including the molecular pathways involved in DNA damage and repair (Wabl *et al*., 1987; Rudd *et al*., 1990; Boesen *et al*., 1994; Friedberg *et al*., 1995; Miller, 1996; Bridges, 1997; Drake *et al*., 1998; Yang *et al*., 2004), it is generally not likely to reflect the types of parameters that impact the spontaneous *in vivo* mutation rate, which is most relevant for evolutionary and disease-related issues. This is because (i) few, if any, organisms in nature are subjected to the near homogenous and narrow environmental/growth conditions provided *in vitro*; (ii) most species are dependent on organism-level factors, not existing as isolated cell lines (Bridges, 1997); (iii) *in vitro* mutation rates have proved to be poor indicators of *in vivo* rates, even within a single species and thus are not likely to be effective models systems for mutational processes inherent to other organisms (Drake, 1991; Bridges 1997); and (iv) *in vitro* cells, can turn over in a single hour or day (Cullum and Vicente, 1978; Kuick *et al*., 1992), and thus, do not reflect the fact that most cells in nature, including those of bacteria, yeast, animals, and plants, are non-dividing for most of their lifespan (Loewe *et al*., 2003) [The human oocyte, for example, spends its entire lifespan, often decades, in the resting stage (Drost and Lee, 1995; Crow, 2000) while the male germ cells also spend substantial periods in the resting stage, with about one cell division per month on average, representing marked resting periods (Crow, 2000)]. With regard to the study of cell ageing, there are the additional difficulties in detecting mutations in non-dividing *in vitro* cells, as this process requires artificially imposed impediments to cell division (making it difficult to isolate replication-independent effects on mutation), and cloning, which inherently entails high numbers of cell divisions (DNA replications; Bridges, 1997; Heddle, 1998). In summary, *in vitro* analysis is not likely to represent the impact of environmental parameters or cell age on the spontaneous genomic mutation rate and thus has limited implications for this issue.

In vivo *research of mutation rates is challenging in animals*

Similar to *in vitro* analysis, there are innate challenges to investigating the impact of environmental parameters and cell age on the mutation rate *in vivo* in animal model systems. In particular, each of the main approaches to examine genomic mutation rates in animals, namely molecular evolutionary rate analysis, epidemiology, and short-term experimentation (Drake *et al*., 1998) is poorly suited to detecting these types of cause–effect relationships (Table 1). In terms of the impact of environmental parameters on mutation rates, for example, molecular evolutionary rate analysis and epidemiology are each unlikely to be effective for this purpose given that most animals are highly mobile, and that specific growth conditions/agents are likely not to be consistent enough to have a detectable impact, particularly for parameters that have a moderate or mild impact. Experimental methods, including mutation accumulation and observation of visible mutants, are challenged by the difficulty in quantifying the rate of spontaneous mutations *in vivo* over the time-course of an experiment (given the mutation rate is so low; for example 0.16 and 0.49 total mutations/genome/cell division in mice and humans, respectively, Drake *et al*., 1998), especially when assessed relative to a gradient of external environmental conditions. Another contributing factor is that there are very few animal taxa appropriate for experimental manipulation (Table 1). Similar to environmental parameters, there are innate challenges for the study of cell age in animal systems. Specifically, mutation-rate estimates obtained from molecular evolutionary rate analysis, epidemiology, and/or experimental methods, can generally only provide rates per generation (i.e. mutation rates per cell division are determined by dividing these values by the number of germ cell divisions per generation), and thus, do not provide any insight regarding the impact of replication-independent events including cell ageing (Drake *et al*., 1998; Lewis, 1999). Furthermore, germ line development has been described in only a very few animal species, making it difficult to conduct interspecies comparisons of the mutation rates of taxa that have germ cells with longer versus shorter periods of rest (non-dividing). This difficulty is confounded by the fact that there is no obvious benchmark for making comparisons of the impact

Table 1. *Summary of challenges to assessing the impact of environmental parameters and cell age on the mutation rate using* in vitro *and animal-based research*

Scientific approach	Basis of challenge	Resulting limitation(s) for determining the cause(s) of mutations
Environmental factors		
Molecular evolutionary rates	Mobility of animals	Mobility makes it unlikely that any parameter/agent that may alter the mutation rate (in the short term) will have a detectable impact on nucleotide substitution.
Epidemiology	Determining causation	Innate difficulty in determining the level of exposure to the parameter/agent of interest, identifying and distinguishing between confounding factors, and discerning the effects at mild or moderate dosages (Smith and Phillips, 1992; Smith, 2001).
Experimentation	Logistical	Few animal species are appropriate for *in vivo* research on effects of environmental stresses.
	Low response	Many environmental parameters have a subtle, and thus undetectable, effect on the spontaneous mutation rate over a single or few generations.
	Mutation rate estimation	General difficulty in measuring mutation rates in the short term as they are very low (Drake *et al*., 1998).
Cell age		
Molecular evolutionary rates	Few species with germ lines characterized	Comparison of mutation between species with short versus long resting stages in the germ cells is not possible (Vogel and Natarajan, 1995).
	No benchmark for age-based comparisons	The average germ cell age, for instance, is unlikely to be an effective standard for age-based comparisons across the germ lines among species because germ cells with particularly protracted resting periods are likely to have a far greater impact than the average (Sommer *et al*., 2001). No means to isolate impact of cell ageing from replication-dependent mutations within the male or female germ line in estimates of mutation rates per generation.
Epidemiology	Isolating impact of cell age	Epidemiology generally provides no information about what stages of germ line development spontaneous mutations arise, and thus it cannot be determined whether more/fewer mutations arise during stages with extended resting periods. Although the germ line stages in which mutations occur can sometimes be inferred from the pattern of mutations in F_1 and F_2 progeny, this is rarely achieved and is generally speculative (Lewis, 1999).
Experimentation	Isolating impact of cell age	*In vivo* experimental studies have primarily been limited to mice and are challenged by the inability to determine the stage of germ line development where mutations arise, and therefore, whether they occur in stages with extended resting periods (Lewis, 1999).

of cell age among species. The average germ cell age across the germ line, for example, is not likely to represent the effects of ageing as specific stages with a particularly long resting stage are likely to have a greater impact than the average (age-related DNA damage per unit time is proportionally higher as cell age progresses; Sommer *et al*., 2001). Short-term experimental approaches to the study of cell age are also challenged by the difficulty in measuring the mutation rate within a single resting cell (or a series of cells relative to time), and thus, such approaches have generally been limited to the examination of the onset of chemically induced mutations at different stages of male germ line development (as determined by its correlation to the time in the individuals development) or the study of the gametogenesis stage (Allen *et al*., 1995; Lewis, 1999; Russell, 2004). Altogether, the obstacles inherent to *in vitro* and in animal-based approaches likely explain the current absence of data regarding the impact of environmental parameters and cell age on the mutation rate. Other biological systems and approaches thus need to be explored.

Opportunities in plants

Although plants differ markedly from animals, most apparently in their development (including the lack of separation of the germ line and soma in plants) and cellular structure, they have consistently served as key model systems for the discovery of fundamental genetic processes inherent to all eukaryotes. Plants, for example, were the first to reveal the laws of genetics, the existence of transposable elements, and the ability to clone multicellular organisms (from a single somatic cell). Moreover, plant research has greatly contributed to our understanding of many genetic processes such as gene silencing, chromosome structure, and gene function (Table 2). The effectiveness of plant model systems for this purpose is probably attributable to the many genetic-based commonalities among eukaryotes, including genome organization and structure (Heslop-Harrison, 2000; Mayr *et al*., 2003), mechanisms and types of DNA damage, DNA repair and mutation (e.g. dimer bypass; Friedberg *et al*., 1995; Britt, 1996, 1999), processes of DNA replication and repair (Britt, 1999) and molecular pathways involved in DNA damage-induced cell cycle regulation and arrest (Huntley and Murray, 1999; Stals and Inzé, 2001; Vazquez-Ramos and Sanchez, 2003), mitosis (Criqui and Genschik, 2002), and cell-to cell interaction (Becraft and Freeling, 1992). Given the proven effectiveness of plants as model systems for genetics research for eukaryotes, they are an obvious alternative to be considered for the further study of the role of environmental parameters and cell age on the genomic mutation rate.

Table 2. *(a) Examples of major discoveries in genetics originating from plants. (b) Examples of genetic principles and processes that has been advanced by research in plants*

(a)

Discovery originating from plants	Species	Later reported in:
Laws of genetics	Peas (*Pisum sativum*), Mendel, 1865	All living organisms
Transposable elements	Maize(*Zea mays*), McClintock, 1951	Most organisms, e.g. *Drosophila* Pimpinelli *et al*., 1995, Kidwell and Lisch, 1997
Post-transcriptional gene-silencing	Petuna (*Ruelia* spp.), Napoli *et al*., 1990; Van der Krol *et al*., 1990	Taxa of the animal kingdom, such as *C. elegans* Fire *et al*., 1998, Plasterk, 2002
Paramutation	Maize, Brink, 1956; Stam *et al*., 2002	Other eukaryotes, e.g. mice Herman *et al*., 2003
Activity of catalytic viroids	Potato, Diener, 1971	Humans, underlies the Hepatitis D Branch *et al*., 1993
Successful cloning of an individual from an somatically differentiated adult cell	Carrot (*Daucus carota*), Steward *et al*., 1958	Sheep and others Campbell *et al*., 1996, Wilmut *et al*., 1997

(b)

Genetic principles and molecular processes aided by plant research	Citation
Genome structure	Meinke *et al*., 1998
Genes involved in genome maintenance, DNA repair and mutagenesis	Hays, 2002
Mechanisms of genome duplication and polyploidy	Chen *et al*., 2004*a*, *b*
Structure and function of centromeres	Copenhaver, 2003
Molecular structures of proteins such as those inherent to hemoglobins	Kundu *et al*., 2003
Molecular mechanisms of virulence of human/animal pathogens	Prithiviraj *et al*., 2005
Gene silencing	Meyer, 2000

Environmental parameters

One of the most apparent benefits of plants for revealing the impact of environmental parameters on the genomic mutation rate is that they are sessile organisms, and thus, are forced to endure their localized growth conditions. Specifically, because plants cannot escape their localized conditions, their environmental conditions are more likely to be consistent over the long term. This would act to enhance the relationship between mutation rates and environmental parameters, and improve the ability to detect their impact using experimental approaches and molecular evolutionary rate analysis. In addition to their sessile nature, the detection of natural environmental mutagens is also facilitated by the presence of indeterminate growth in plants (Gill and Halverson, 1984; Klekowski, 1998). As a result of this growth pattern, plants, unlike most organisms, are able to transmit mutations that arise in the soma to successive generations. In turn, because the soma in plants is constantly subjected to localized growth conditions, including topical (e.g. irradiation, UV, humidity) and soil-based agents (e.g. nutrients, water, minerals) as well as biotic agents (e.g. pathogens; Lucht *et al*., 2002; Kovalchuk *et al*., 2003), and because these mutations can be inherited by offspring, the effects of environmental agents on the mutation rate may be more readily evident in these than in other organisms using both molecular evolutionary analysis and experimental approaches. Plants should therefore be especially suitable for studying effects of environmental factors on mutagenesis where these factors are localized and consistent, and thus, reveal important factors affecting mutation rates in eurkaryotes. Although the impact of certain environmental agents will have plant-specific effects due to their distinct growth pattern, this is likely to be relatively rare given the fundamental nature of mutation. Notably, such mutation rate differences between plants and animals (relative to the environment), even when detected, would act to assist in revealing how and why environmental parameters influence the mutation rate.

Another highly valuable feature of plants for the study of environmental parameters is that, unlike animals, associations between environmental parameters and *in vivo* mutation rates can be readily detected using highly sensitive bioassay systems. Plants have consistently shown superior sensitivity (lower doses) and reliability (fewer false negatives) as environmental bioindictors than the comparable bacterial and mouse-based (*in vivo* and *in vitro*) systems (Heslop-Harrison, 1978; Zing and Zhang, 1990; de Serres, 1992; Grant, 1994, 1998, 1999; Rodrigues *et al*., 1997; Kovalchuk *et al*., 2001). For example, *Tradescantia* spp, have been used to detect ambient levels of natural conditions/agents such as irradiation, UV-B, temperature changes, and ozone (sensitivity is also demonstrated by the detection of extremely low doses of anthropogenic agents in the soil, water, and air; Grant, 1992, 1998; Ichikawa, 1992; Rodrigues *et al*., 1996, 1997; Wang and Wang, 1999; Klumpp *et al*., 2004). Mutations can be readily observed

through the observation of changes in flower colour (stamens) throughout the soma (based on the expression recessive mutations at a gene for flower colour in heterozygous plants) and chromosomal aberrations (micronuclei in the meiotic pollen mother cells (Rodrigues *et al*., 1997; Grant, 1998; Wang and Wang, 1999). These plant systems serve as a quick and effective means to identify those environmental parameters (non-anthropogenic) that have the potential to alter the *in situ* genomic mutation rate. In addition to these bioassay systems, plant species of many genera including *Allium*, *Arabidopsis*, *Crepis*, *Glycine*, *Hordeum*, *Nicotiana*, *Solanum*, *Rhizophora*, and *Pisum*, can and have been widely utilized for the detection of environmental mutagens (e.g. ozone, alkylating agents) based on chlorophyll mutation assays, pollen abortions, recessive visible mutations at heterozygous loci, chromosomal aberrations in root tips, and/or analysis of genetic markers (Stadler, 1930; Rodrigues *et al*., 1996, 1997; Grant, 1998, 1999; Kovalchuk *et al*., 2000; Proffitt and Travis, 2005). Overall, these highly sensitive and established systems provide an effective means to identify naturally occurring environmental parameters/agents (through experiments relative to environmental gradients) that have the ability to alter the *in vivo* mutation rate that is not as readily available for other organisms. In this regard, they could be key players in the determination of which environmental parameters are likely to have an impact on mutation rates among eukaryotes and thus to provide direction for future studies. Moreover, the wide array of mutants available in plants, particularly in *Arabidopsis thaliana*, could play a key role in the identification of genes and molecular pathways associated with environmentally induced mutations (Rhee *et al*., 2003).

It should be noted that, in addition to the identification of environmental parameters that could alter the *in vivo* genomic mutation rate, plants also offer the opportunity to reveal whether environmental fluctuation has an impact. Evidence indicates that this could be the case. A study of the impact of climatic conditions on the effectiveness of the *Tradescantia* bioassays, for example, incidentally revealed that high levels of temperature fluctuation have a greater impact on the *in vivo* mutation rate and the level of DNA damage (both with and without the anthropogenic mutagen) than specific high or low temperatures (Klumpp *et al*. 2004). In addition, plant systems could also reveal whether environmental parameters and/or fluctuations interact and influence the *in vivo* mutation rate. This has been suggested to be the case by the enhanced mutagenic activity of anthropogenic agents under low-humidity conditions in *Tradescantia* (Takahashi and Ichikawa, 1976; Klumpp *et al*., 2004). Unlike animals, where *in vivo* experimentation relative to environmental fluctuation is not appropriate and/or possible for most species, plants could reveal important patterns in the relationship between environment and mutation rates.

Age-related factors

In contrast to *in vitro* and animal-based research, where the study of cell age on spontaneous mutation rates is impeded by challenges in the quantification and manipulation of the duration of the resting stage of cells, plants provide a readily utilizable system for the investigation of age-related mutation. Specifically, embryo cells within plant seeds are non-dividing and are maintained in the G_0/G_1 stage of the cell cycle for extended time periods (Georgieva *et al*., 1994; Whittle *et al*., 2001; Vazquez-Ramos and Sanchez, 2003). The duration of the resting stage may thus be readily manipulated in seeds, allowing a means to assess the physiological and genetic impact of cell age on *in vivo* DNA damage and the onset of mutations. In this regard, plant seeds represent a naturally existing biological system where the impact of cell ageing on the rate of mutation can be readily studied.

Although a substantial argument has been made for the notion that many mutations in animals are replication errors (Crow, 2000), the evidence available to date from seed embryos indicates that significant levels of mutations result from age-related, replication-independent, events. Analysis of evolutionary rates of selectively neutral DNA among plant taxa, for example, has shown that nucleotide substitution rates at silent sites are higher for taxa with persistent (long-term) than transient (short-term) seedbanks, suggesting that more heritable base substitution mutations occur per unit time during seed (cell) ageing than during the lifetime of the plant (wherein the meristematic regions are constantly undergoing replication; Whittle and Johnston, 2006). In addition, there is increased variation in AFLPs and other genetic markers in naturally aged rye (*Secale cereale*) seeds that are inherited by the progeny for at least three generations (Chwedorzewska *et al*., 2002*a*, *b*). Individuals produced from older seeds have also been shown to contain higher levels of chromosomal and/or gene mutations in *Crepis* (Gerassimova, 1935), *Zea mays* (Peto, 1933), and *Triticum* (Floris and Melletti, 1972) and to have a higher frequency of pollen abortions, an indicator of lethal mutations (in haploid cells) in *Datura* (i.e. pollen abortion increases from one to more than 8% over 10 years; Cartledge and Blakeslee, 1934). It is thus evident that cell age plays a prominent role in determining the mutation rate in plants. Although these trends could be plant-specific, it seems unlikely given the fundamental genetic-based similarities between plants and other multicellular eukaryotes, and the fact that most other organisms have extended resting periods in the majority of their cells, including animal germ lines. Given the relative ease of study of plant seeds, compared with *in vivo* animal and *in vitro* systems, they offer valuable opportunities for better understanding the basic mechanisms underlying age-related mutations.

In addition to understanding quantitative relationships between cell age and mutation rate, seeds also offer a readily utilizable means to assess why age-related mutations arise.

Data obtained to date from plant seeds suggest that the age-related mutations could be caused by DNA replication across strand breaks and chromosomal aberrations, which have been found to accumulate in embryonic cells over time (with older embryos having a greater proportion of cells with damage and higher levels of damage per cell; Cheah and Osborne, 1978), and/or from the impairment of the DNA replication or repair machinery. It has been shown that older seeds also have a lower RNA and protein content (suggesting substantial degradation; Begnami and Cortelazzo, 1996; Reuzeau and Cavalie, 1997), reduced ability to translate RNA (Reuzeau and Cavalie, 1997), lowered activity of enzymes (Basavarajappa *et al*., 1991) such as RNA poly (A) polymerase (Grilli *et al*., 1995; Reuzeau and Cavalie, 1997), each of which might negatively influence the level and/or activity of molecules involved in DNA replication and repair. In this regard, seeds provide an effective system to assess the changes in the DNA (DNA damage), cell physiology, and gene expression, that are associated with age-related mutations. Estimates of mutation rates may also be obtained using genome-wide approaches such as mutation accumulation (where changes in fitness are believed to be proportional the mutation rate, Drake *et al*., 1998). For example, one may develop mutation accumulation lines (as has already been achieved in *A. thaliana*; Schultz *et al*., 1999; Shaw *et al*., 2000), where the seeds are aged between generations, and subsequently estimate the genomic mutation rate per generation as well as the proportion of the mutation rate that can be attributed to ageing (either based on fitness assays or from direct measurement of mutations using molecular mutation detection techniques; Del Tito *et al*., 1998). Given that the impact of seed ageing may depend on moisture and temperature conditions (Sivritepe and Dourado, 1998) such studies will need to be conducted under various natural and experimental environmental conditions to ascertain any possible differential effects. It is notable that the effectiveness of seeds for mutation research has been well established by the fact that they have been utilized in extensive mutagenesis studies, including ionizing radiation, UV, and ethyl methanesulphonate (EMS), which has led to the identification of genes and the mechanisms involved in DNA repair in plants (Britt, 1996; Preuss and Britt, 2003).

Conclusions

Much currently remains unknown about how and why mutations arise. In particular, there is a notable gap in the available data regarding the role of environmental parameters and cell ageing on the onset of mutations. The reasons why plants could be a more productive biological system than bacterial, yeast, and animal systems to advance our current understanding of the role of these factors on the mutation rate have been highlighted here. Although aspects of such research will be plant specific, it is likely given the fundamental nature of mutation, that such investigations will, at a minimum, provide insight into the types of environmental parameters that need to be further evaluated in other eukaryotes, the potential impact of cell ageing on the mutation rate, and the basic cellular events correlated to environmental and age-related mutagenesis. Overall, given the relative experimental advantages of plants, including low cost, ready availability, no ethical concerns regarding treatment, and their often short generation times, plus their innate benefits for the study of environmental parameters and cell age, it is believed that they will be powerful model systems for making advances in current understanding of how and why mutations arise.

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