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Phosphorylation of H2AX histones in response to double-strand breaks and induction of premature chromatin condensation in hydroxyurea-treated root meristem cells of *Raphanus sativus*, *Vicia faba*, and *Allium porrum*

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Summary. Histone H2A variant H2AX is rapidly phosphorylated on the induction of DNA double-strand breaks by ionizing radiation and hydroxyurea-mediated replication arrest, resulting in the formation of γ-H2AX foci along megabase chromatin domains nearby the sites of incurred DNA damage. In an attempt to establish a relationship between species-specific nuclear architecture and H2AX phosphorylation in S/G_2 phase-arrested root meristem cells, immunocytochemical comparisons using an antibody raised against human γ-H2AX were made among three plants differing with respect to DNA contents: *Allium porrum*, representing a reticulate type of DNA package, *Vicia faba*, having semireticulate cell nuclei, and *Raphanus sativus*, characterised by a chromocentric type of chromatin. Another approach was aimed at determining possible correlations between the extent of hydroxyurea-induced phosphorylation of H2AX histones and the quantities of root meristem cells induced by caffeine to enter aberrant mitotic division (premature chromosome condensation). It was concluded that the higher-order structure of chromatin may contribute to the accessibility of molecular factors engaged in the recognition and repair of genetic lesions. Consequently, in contrast to *A. porrum* and *V. faba*, a diffuse chromatin in chromocentric cell nuclei of *R. sativus* may become more vulnerable both to generate DNA double-strand breaks and to recruit molecular elements needed to arrange the cell cycle checkpoint functions, and thus, more resistant to factors which allow the cells to enter premature chromosome condensation spontaneously. On the other hand, however, caffeine-mediated overriding of the S-M checkpoint control system resulted in the typical appearance of premature chromosome condensation, irrespective of the genomic content of DNA.

Keywords: Hydroxyurea; H2AX phosphorylation; Premature chromosome condensation; *Raphanus sativus*; *Vicia faba*; *Allium porrum*.

Abbreviations: DSB double-strand breaks; HU hydroxyurea; PCC premature chromosome condensation.

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Introduction

To maintain genome integrity and to preserve hereditary determinants of an organism, molecular surveillance mechanisms assemble into higher-order networks, called cell cycle checkpoints, that block or delay transitions in interphase and mitosis until certain monitored event(s) are completed. Chromatin-based processes seem to be linked to the checkpoint control system in at least two complementary ways. First, some of the nucleoplasmic factors may arrange an early signal to start the response, and hence to initiate the checkpoint pathway. Later, the altered properties of chromatin may support the assemblage of the downstream biochemical elements needed for both a more effective inhibition of cell cycle progression and a more efficient repair of chromosomal DNA molecules (Koundrioukoff et al. 2004).

Biochemical pathways engaged in the intra-S-phase checkpoints comprise a number of elaborate mechanisms which detect unreplicated and damaged DNA. The control system over the genome integrity in higher eukaryotes consists of sensors that recognise the sites where chromosomes became injured, specific transducers that relay appropriate signals, and effectors that reply to these signals so that the cell triggers a suitable physiological reaction (Boddy and Russell 1999, Osborn et al. 2002, Lukas et al. 2004). To arrange an ordered sequence of events in which the various determinants contribute to the fitness of such kind of surveillance pathways, there is a need both for timely switching on of numerous genes and for precise cooperation of diverse proteins. An important clue concerning the nature of the competitive interactions between these elements comes from looking at the response to DNA double-strand breaks

(DSBs) and the role that the phosphorylated form of histone H2AX (γ-H2AX) plays in those regions of cell nucleus which contain altered chromatin structures generated by ionizing radiation (Rogakou et al. 1998) or replication forks stalled during the S phase (Fernandez-Capetillo et al. 2004).

H2AX, a ubiquitous variant of the H2A histone family, is rapidly phosphorylated at the C-terminal SQ(D/E)(I/L/F/Y)-(end) motif (Ser139 of the SQE sequence [Redon et al. 2002]) in a very early stage of response to either DNA lesions or DSB intermediates produced during programmed cell death (Rogakou et al. 2000), meiotic recombination (Mahadevaiah et al. 2001), and V(D)J rearrangements of immunoglobulin genes (Chen et al. 2000). A highly significant positive correlation between the appearance of γ-H2AX clusters and aggregates containing ATM (ataxia telangiectasia mutated) and ATM-related kinases, such as DNA-PK (DNA-dependent protein kinase) and ATR (ATM and Rad3-related), suggests a tight linkage between H2AX phosphorylation and the retention of factors that monitor structural and functional properties of replicating chromosomes. DNA damage and/or spatial rearrangements of chromatin, induced primarily by H2AX phosphorylation (and possibly also by other covalent modifications of histones), are considered as the basis for additional activities recruited to the lesion sites as indispensable tools to change the surrounding chromatin by ATP-dependent remodeling. Such factors comprise SWI/SNF and Rad54, histone acetyltransferases (HATs) like SAGA/NuA4-related complexes and p300/CBP, which have been evidenced to occur in different molecular pathways responsible for the maintenance of genome integrity (for references, see Allard et al. 2004). The emergence of phospho-H2AX tracks along megabase chromatin domains flanking DNA damage sites has also been implicated to facilitate recruitment of intra-S-phase checkpoint substrates engaged in mechanisms that allow replicating DNA to be repaired and, concurrently, that prevent the cell from entering mitotic division (Bassing et al. 2002, Momota et al. 2003). In addition, the altered arrangement of nucleosomal foci confined to small areas in the vicinity of incurred damages may join the broken ends of DNA molecules and hence protect adjacent domains of chromosomes from extensive rearrangement (Paull et al. 2000, Fernandez-Capetillo et al. 2004).

For the most part, the spatial and temporal relationships between DNA damage and γ-H2AX formation have been explored in animal and human cells. Experiments using root tip cells of *Arabidopsis thaliana* exposed to ionizing radiation revealed discrete γ-H2AX foci located in the vicinity of M-phase chromosomes (Friesner et al. 2005). The likely basis for this phosphorylation is the ATR kinase, involved in the transduction of information that commences at broken DNA and ends at the target elements in the cell cycle machinery. Induction of γ-H2AX foci in *A. thaliana* was observed at only one-third the rate observed in mammals, which may correlate with the relatively high resistance of plants to the adverse environmental factors that lead to disruption of nuclear functions (Hays 2002, Garcia et al. 2003). Our recent immunocytochemical analysis demonstrated that root meristems of *Vicia faba* exposed to UV irradiation or incubated with hydroxyurea (HU) reveal discrete fluorescent foci typical for γ-H2AX histones (Rybaczek and Maszewski 2006). These data correlated well with the results obtained by immunoblotting of total-protein extracts from the control and HU-treated cells. Western blots detected only one band at the position expected for the phosphorylated form of H2AX. In root meristems exposed to UV light, the frequency of G_1- and G_2-phase cells showing the phosphorylated form of H2AX histones displayed a highly positive correlation with the time of irradiation. This was in marked contrast to the subpopulation of root meristem cells having intermediate DNA levels (between the 2C and 4C), which – regardless of the duration of exposure to UV light – revealed a substantial and fairly constant fraction of cells showing numerous intranuclear aggregates stained with anti-γ-H2AX antibodies (Rybaczek and Maszewski 2006).

Over the years, a variety of comparative analyses has established a more or less evident relationship between the nongenic characters of nuclear DNA (referred to as the nucleotype) and a number of ontogenetic processes, including those having far-reaching biological consequences for plants, such as minimum generation time, endopolyploidy, cell cycle timing, or M- and S-phase durations (Bennett 1998). Plant cell nuclei differ with respect to their supramolecular organisation, ranging from a reticulate (chromonematic) type, with high levels of genomic DNA arranged into large strands of condensed chromatin, to chromocentric (diffuse) type, with a small amount of DNA localised mostly in the decondensed chromatin and few or several chromocentres dispersed in the vicinity of the nucleoplasmic space (Barlow 1977, Nagl 1985, Lingua et al. 2001). Although principal morphological characters correlated with varying nuclear DNA contents and chromatin architectures account for a number of changes in the dynamics of cellular activities, such as the rates of cell divisions and DNA endoreplication, differences in metabolic processes, production of splicing factors (Barlow 1983, Moreno Díaz de la Espina et al. 1992), the relationship between the "global" or "innate" nuclear features and the events correlated with the recognition and repair of DNA damage in plants is largely unknown. The present study attempts to determine what fundamental characteristics of H2AX phosphorylation induced

in S/G_2-phase-arrested root meristem cells are associated with varying DNA contents and differing nuclear architectures in three plants species: *Allium porrum* and *Vicia faba*, representing a reticulate and semireticulate type of DNA package, respectively, and *Raphanus sativus*, characterized by a chromocentric type of chromatin. The obtained results have emerged from two different kinds of experimental approach. In the first, conversion of ribonucleotides to deoxyribonucleoside triphosphates was blocked by HU, an inhibitor of ribonucleotide reductase. Further immunocytochemical observations of HU-treated cells were designed to resolve macromolecular foci arranged at the sites of incurred DSBs. Another approach was aimed at determining possible correlations between the extent of HU-induced phosphorylation of H2AX histones (DSB-dependent formation of γ-H2AX foci) and the quantities of root meristem cells induced to enter premature condensation of chromosomes (PCC). A highly specific morphology of such chromosomes (with numerous breaks and lagging fragments) seems to be reminiscent of the former defects incurred during S phase of the cell cycle. Accordingly, in the second series of experiments, S/G_2-phase-arrested cells were treated with caffeine, a methylxanthine known to inhibit ATM/ATR kinases (Blasina et al. 1999, Kaufmann et al. 2003). The mechanisms invoked by suppression of sensors that detect fragmented and abnormally structured DNA molecules allowed some of the blocked cells to override the "S-M dependency control system" and, thus, to advance an unscheduled and aberrant mitotic division.

Material and methods

Plant material

Seeds of cultivated radish (*Raphanus sativus* L.), field bean (*Vicia faba* subsp. *minor* L.), and leek (*Allium porrum* L.) were sown and germinated at 23 °C on wet blotting paper in petri dishes. Three to four days after imbibition, dark-grown seedlings with equally sized primary roots (about 1 cm long for *R. sativus*, 2.5 to 3 cm for *V. faba*, and about 2 cm for *A. porrum*) were selected for further experiments. During germination and incubations with inhibitors, roots were permanently aerated by gentle rotation of fluids.

For calibration of the absolute DNA content per nucleus, absorbance of Feulgen-stained nuclei at early prophase was measured at 565 nm for both the reference standard (root meristem cells of *Allium cepa*; assuming a DNA value of 33.5 pg per 2C nucleus according to Johnston et al. [1999]) and the three selected plant species. The estimated 2C DNA levels were about 1.3 pg for *R. sativus*, 26.1 pg for *V. faba*, and 65.3 pg for *A. porrum*.

Immunocytochemical detection of H2AX

Immunocytochemical assays were carried out as described earlier (Rybaczek and Maszewski 2006). Seedlings were incubated for 24 h with 1.0, 2.5, and 10 mM HU (Sigma-Aldrich). Excised apical parts of roots were then fixed for 45 min (20 °C) in 3.7% paraformaldehyde in phosphate-buffered saline (PBS), washed several times with PBS, and placed in a citric acid-buffered digestion solution (pH 5.0; 37 °C for 45 min) containing 2.5% pectinase (Fluka), 2.5% cellulase (Onozuka R-10; Serva) and 2.5% pectoliase (ICN). After incubation, the digestion solution was removed, root tips were washed 3 times (as before), rinsed with distilled water, and squashed onto Super Frost Plus glass slides (Menzel-Gläser). Air-dried slides were pretreated with PBS-buffered 8% bovine serum albumin (BSA) and 4% Triton X-100 (Fluka) for 50 min (20 °C) and incubated overnight in a humidified atmosphere (4 °C) with a rabbit polyclonal antibody raised against human H2AX histones phosphorylated at serine139 (anti-γ-H2AX antibody; Upstate Biotechnology), dissolved in PBS containing 1% BSA (at a dilution of 1 : 750). Following incubation, slides were washed 3 times with PBS and incubated for 1.5 h (18 °C) with secondary goat anti-rabbit antibody conjugated to fluorescein isothiocyanate (FITC) (ICN) in PBS (1 : 500, v/v). To distinguish late S/G_2 phase cells, nuclear DNA was stained with 4′,6-diamidino-2-phenylindole (DAPI) (0.4 μg/ml; Sigma-Aldrich). Following washing with PBS, slides were air dried and embedded in a PBS-glycerol mixture (9 : 1) with 2.3% diazabicyclo[2.2.2]octane. Observations were made with a Optiphot-2 fluorescence microscope (Nikon) equipped with B-2A filter (blue light; $\lambda = 470$ nm) for FITC and UV-2A filter (UV light; $\lambda = 518$ nm) for DAPI. All images were recorded at exactly the same time of integration with a DXM 1200 charge-coupled-device camera. Mean labelling indices were calculated as percent of G_2 cells that carry γ-H2AX foci, and each value was determined from the analysis of 5 (*A. porrum* and *V. faba*) to 10 (*R. sativus*) squashed root meristems. Nuclear DNA contents in DAPI-stained cells were evaluated in arbitrary units, assuming the total intensity of fluorescence emitted from posttelophase and early-prophase cells as reference standards for the 2C and 4C DNA values, respectively.

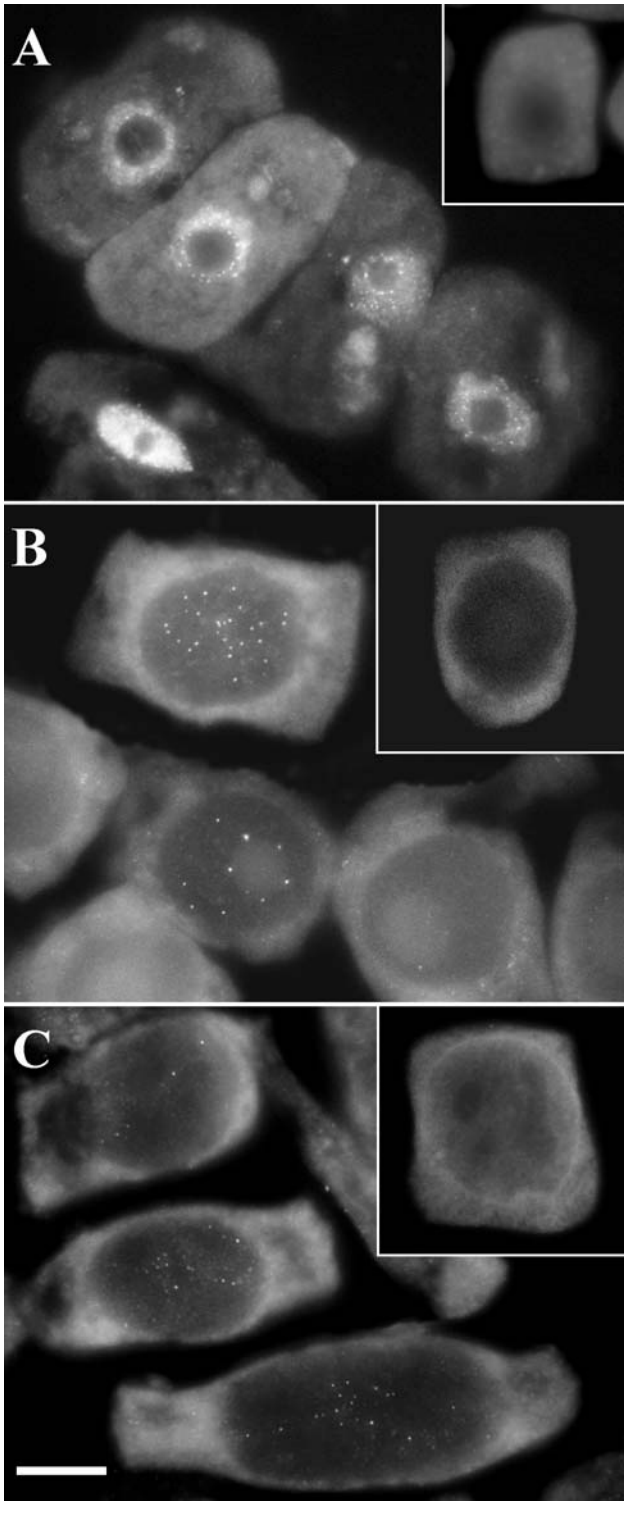
Induction of PCC and Feulgen staining

Seedlings pretreated for 24 h with 2.5 mM HU were transferred into petri dishes containing a mixture of 2.5 mM HU and 5 mM caffeine (Sigma). After every 2 h, root tips were excised and fixed in cold absolute ethanol and glacial acetic acid (3 : 1, v/v) for 1 h, washed several times with ethanol, rehydrated, hydrolysed in 4 M HCl (1.5 h), and stained with Schiff's reagent (pararosaniline; Sigma-Aldrich) according to standard methods. After rinsing in SO_2-water (3 times) and distilled water, 1 to 1.5 mm long apical segments (depending on the plant species) were cut off, placed in a drop of 45% acetic acid, and squashed onto microscope slides. Following freezing with dry ice, coverslips were removed and the dehydrated dry slides were embedded in Canada baume. Slides were analysed under the light (*A. cepa* and *V. faba*) or fluorescence microscope equipped with G2A filter (green light, $\lambda = 470$ nm; *R. sativus*) to count mitotic cells that had characteristic features of either a normal mitosis or PCC (resolved by chromosomal breaks and gaps, lost or lagging fragments of acentric chromatids). Mean mitotic and PCC indices (with standard deviations) were calculated from 10 root meristem cell populations for each sampling period.

Results and discussion

The combined information from immunocytochemical and biochemical studies supports the concept that the whole repertoire of cellular reactions to the incurred genomic defects or blocks of DNA replication requires a highly specific organisation of chromatin, prearranged by the phosphorylated form of H2AX histones. Accordingly, the local change in spatial configuration of intranuclear structures is considered as a basis for recruitment and sequestration of surveillance and repair factors and for the new relations between

these elements, which may, in turn, cover both tight control over the cell cycle progression and the induction of activities needed to restore the damaged molecules of DNA or to commence the programmed cell death by apoptosis (Fernandez-Capetillo et al. 2004). The above ideas have prompted the search for relations between structural properties of cell nuclei (correlated with genome size) and the appearance of γ-H2AX foci formed in response to replicational stress induced by HU (Fig. 1). A number of side effects associated with HU-mediated inhibition of ribonucleotide reductase comprise dissociation of the replication complex (Lundin et al. 2002), accumulation of hemireplicated intermediates, single-stranded DNA interruptions at stalled replication forks, which may then become converted into DSBs (Merrill and Holm 1999), formation of Holliday junctions through fork reversal (Sogo et al. 2002), and other specific DNA damage by forming nitric oxide and hydrogen peroxide (Sakano et al. 2001).



The inclusive work on the complete DNA sequences in *A. thaliana* revealed orthologs to most human genome-maintenance proteins that prevent mutations, repair broken chromosomes, and perform specialised functions associated with the intra-S-phase checkpoints and apoptosis (Hays 2002, Garcia et al. 2003). Previous studies on roots of *Vicia faba* exposed to UV light have shown that γ-H2AX foci may appear in about one-third of all meristematic cells already within 1 min after the start of irradiation (Rybaczek and Maszewski 2006). Moreover, DSB-specific H2AX phosphorylation was also observed in response to HU-mediated replication arrest, indicating that, irrespective of the origin and character, a wide array of abnormally structured DNA molecules can arrange overlapping biochemical pathways to initiate and convey appropriate signals needed to stop the cell cycle progression and to rescue the genome from further damage.

So far, the arrest of DNA replication induced by exposing cells to HU treatment has been shown to generate extensive ATR-dependent phosphorylation of H2AX in mammals (Ward and Chen 2001, Kumar et al. 2005). The data presented by Lundin and her co-workers (2002) suggest that the retardation of DNA synthesis by HU generates substrates for both homologous and nonhomologous recombination and the analysis of DNA from these cells by pulsed-field electrophoresis revealed that the DNA fragments released by HU-induced DSBs contained a high proportion of newly replicated DNA. Furthermore, the most abundant DNA fragments generated by HU-induced DSBs corresponded well to the size of a single replicon or a few contiguous replicons.

The relative numbers of cells showing fluorescing anti-γ-H2AX antibodies clustered at the presumed DSB sites in the untreated root meristems of *R. sativus*, *V. faba*, and *A. por-*

Fig. 1 A–C. Immunofluorescence of phospho-H2AX foci formed in root meristem cells after 24 h replicational stress induced by 2.5 mM HU. **A** *Raphanus sativus*; **B** *Vicia faba*; **C** *Allium porrum*. Examples of G_2 phase cells from the untreated root meristems are given in the insets. Bar: 10 μm

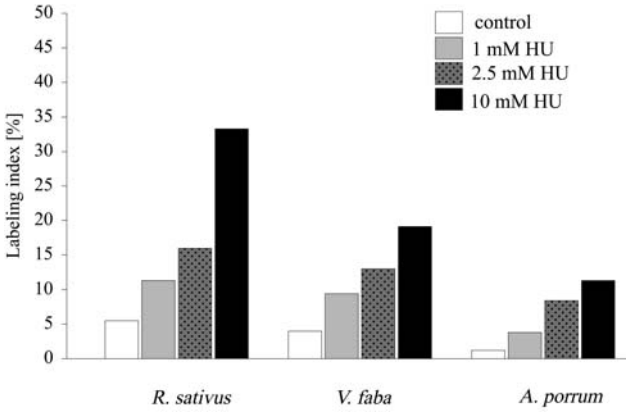


Fig. 2. Labelling indices (%) estimated by immunofluorescence of phospho-H2AX foci in root meristem cell populations of *R. sativus*, *V. faba*, and *A. porrum* in the control seedlings and in seedlings exposed to the 24 h replicational stress induced by 1, 2.5, and 10 mM HU treatments

rum indicate an opposite relationship with the species-specific DNA C values (Fig. 2 and Table 1). The labelling index calculated for *R. sativus* is greater by some 20% than that for *V. faba* and almost 4 times higher than that for root tips of *A. porrum*. In general, quite similar differences among the three plant species can be established after 24 h of incubations with 1, 2.5, and 10 mM HU solutions. Comparisons of labelling indices clearly show a range of decreasing values correlated inversely with the nuclear DNA contents. Concurrently, the treatments with various concentrations of the inhibitor demonstrate an apparent dose–effect relationship, with the maximum induction of intranuclear γ-H2AX foci occurring in *R. sativus* 24 h after the start of incubation with 10 mM HU.

To perform further immunocytochemical comparisons, root meristems from the control and HU-treated seedlings (24 h of incubation with 2.5 mM HU) were double-stained with FITC-conjugated anti-γ-H2AX antibodies and DAPI, and late S/G_2 cells were selected by means of cytofluorimetry. In both *V. faba* (Fig. 1B) and *A. porrum* (Fig. 1C), the allocation of phosphorylated H2AX foci has been found varying between two different patterns: one with only a small number of points fluorescing at the border of the nucleolus and its perinucleolar regions and the other with numerous accompanying foci scattered all over the area of reticulate chromatin. While some of the nuclei in root meristem cells of *R. sativus* (Fig. 1A) show similar distribution patterns of γ-H2AX foci, most of them reveal peripheral dispersion of immunofluorescent sites, having no specific association with either large nucleolar regions or chromocenters. Furthermore, the analyses of S/G_2 phase cells labelled with FITC-conjugated anti-γ-H2AX antibodies indicate that there is an apparent inverse correlation between the amount of fluorescent aggregates and the basic nuclear DNA content of the examined plants (Table 1). Taking into account both values, the average number of γ-H2AX foci per 1 pg of nuclear DNA calculated for the replicated genome of *R. sativus* (G_2 phase cells) has been found about 125 and 44 times greater than that estimated for the G_2 cells of *A. porrum* and *V. faba*, respectively.

Two methodological problems are encountered during immunocytochemical studies of the examined plants. Firstly, due to the negative relationship between the genome size and the tendency to endoreplication (Nagl 1976), the population of root meristem cells with 4C DNA values may comprise a small fraction of endopolyploid cells that cannot be discriminated from late S or G_2 phase cells by fluorimetric or morphological observation. The second and fundamental difficulty relates to the nucleotypic dependency of the genome size and several rate-limiting factors underlying DNA replication, including species-specific frequencies of replication origins and various numbers of their families activated sequentially during S phase (Maszewski 1991). During the 24 h period of HU treatment, each cell (irrespective of the plant species) must have been exposed to the inhibitor and, consequently, blocked either at the G_1/S transition point or at one of the succeeding stages of the S phase. Consequently, cell nuclei having larger DNA C values and higher numbers of replication origins (e.g., *A. porrum* and *V. faba*) should have produced larger quantities of stalled replication forks and, theoretically, give rise to more γ-H2AX foci

Table 1. Phospho-H2AX foci formed in root meristem cells (G_2 phase) of *R. sativus*, *V. faba*, and *A. porrum*

Treatment	Mean nr. of phospho-H2AX foci per:[a]					
	Nucleus (G_2)			1 pg of DNA		
	R. sativus	*V. faba*	*A. porrum*	*R. sativus*	*V. faba*	*A. porrum*
Control	0	2.0 ± 0.5	1.0 ± 0.4	0	0.04 ± 0.02	0.01 ± 0.01
10 mM HU	35.6 ± 4.3	20.0 ± 2.4	13.8 ± 4.6	16.18 ± 2.10	0.37 ± 0.05	0.13 ± 0.02

[a] Values are means with standard deviations; n = 50

than cell nuclei from plants with smaller genomes (e.g., *R. sativum*). A different relationship observed among the three analysed plant species leads, thus, to an alternative hypothesis. Despite considerable variation in cell cycle times and the total duration of DNA synthesis, it may be more productive to correlate the evaluated differences in the quantities of γ-H2AX foci with nuclear architectures, rather than with other kinds of different parameters having reference to the dynamics of cellular proliferation.

By analogy with their animal homologs, the prime role that macromolecular assemblages of phosphorylated H2AX histones are likely to play in plants is the foundation of structural changes of chromatin needed to accumulate signalling and DNA repair factors and to allow information to be transmitted among the functionally separate protein complexes responsible for controlling diverse aspects of the cell cycle regulation (Burma et al. 2001, Shroff et al. 2004). Consistent with cytological observations and cytofluorimetric data, the nature of differences evidenced in our current study must surely be also understood in the context of chromatin structure in which the foci of phosphorylated H2AX histones are embedded. The organisation of cell nuclei in root meristems of the selected plant species reflects considerable variation and fits into one of the two general configurations termed as (1) chromocentric (*R. sativus*) or (2) reticulate type of DNA package (*V. faba* and *A. porrum*). It seems highly probable that the overall phenotypic characters correlated with small genome size of *R. sativus* are remarkably similar to those observed in the physical organisation of interphase chromatin in *Chirantodendron pentadactylon* (Echeverría et al. 1999) or *A. thaliana* (Heslop-Harrison 2003). The main functional properties in common may rely on the specific distribution of highly condensed blocks of repetitive DNA sequences with low amounts of interspread genes (chromocentric heterochromatin), and large amounts of fine fibrils containing diffuse euchromatin loops which emanate from the chromocentres into the perichromatin regions of the nucleus. It may be, then, that in contrast to DNA-rich genomes containing both a significant fraction of euchromatin mixed together with noncoding interstitial heterochromatin, the specific compartmentalisation of chromatin domains in plants with low 1C DNA content renders them more susceptible for extensive HU-induced lesions. However, the diverse effects correlated with the two contrasting nuclear architectures may consist in an unequal accessibility of large molecules of ATM and ATR kinases (or other signaling factors) to those regions of chromatin which contain irregular DNA structures encountered in HU-treated cells at the sites of stalled replication forks: (1) hemireplicated intermediate molecules with long single-stranded DNA regions (ssDNA), (2) reversed forks, and (3) occasionally, DSBs (Osborn et al. 2002, Sogo et al. 2002).

The S-phase checkpoint response initiated by sensor kinases allows the generated signal to be branched out and to produce downstream effects on both replication forks and the components which stimulate the onset of nuclear division (Boddy and Russell 1999). Temporal coordination of these control mechanisms establishes an interconnected network which prevents mitotic condensation of chromosomes until DNA replication and DSB repair are completed. A variety of genetic lesions or experimental treatment with chemical agents (such as methylxanthines, aminopurines, phosphatase inhibitors, and protein kinase antagonists) may disrupt the checkpoint response system and advance the timing of mitotic events, thus leading the cells to PCC (Ghosh

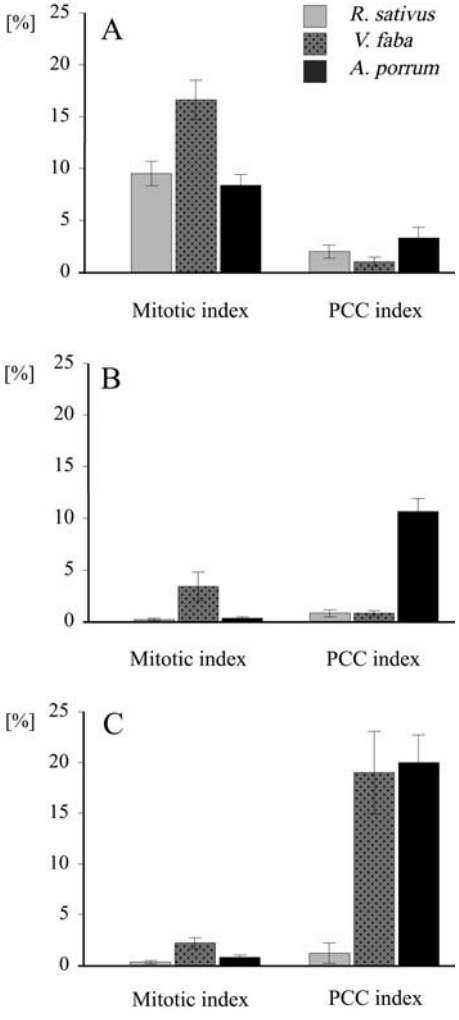


Fig. 3 A–C. Mitotic and PCC indices (%) recorded for root meristem cell populations of *R. sativus*, *V. faba*, and *A. porrum*. **A** Control roots; **B** roots treated for 24 h with 1 mM HU; **C** roots treated for 24 h with 2.5 mM HU

et al. 1998, Nghiem et al. 2001, Nishijima et al. 2003). It seems now commonly accepted that caffeine-mediated inhibition of ATM/ATR precludes the downstream Chk1 protein kinase from being phosphorylated and activated. As a consequence, cells preserve the ability to enter mitotic division owing to the lack of constraints imposed upon the activity of Cdc25 tyrosine phosphatase, the most proximal activator of cyclin B-CDK complex (Graves et al. 2000).

Examination of root meristem cells induced to undergo caffeine-mediated PCC has been preceded by the analysis of mitotic indices both in the control and HU-treated seedlings (Fig. 3). In accord with the expectations, 24 h incubation with either 1 or 2.5 mM HU brought about a significant decrease in the dynamics of cell divisions in *R. sativus* and *A. porrum* and led to a more than 10-fold reduction of the mitotic index in *V. faba*. However, a surprising effect observed in all samples of HU-treated roots was the emergence of M phase cells with evident PCC-like abnormalities, commonly encountered following incubation with a mixture of HU and caffeine. As compared with the relative quantities of aberrant mitotic divisions estimated in the control cell populations, their frequencies in root meristems of *A. porrum* have been found 4 and 8 times greater after incubation with 1 and 2.5 mM HU, respectively, and nearly 25 times greater in 2.5 mM HU-treated roots of *V. faba*. On the other hand, no increase in the fraction of PCC-like mitotic cells was found after incubation with either 1 or 2.5 mM HU for root meristems of *R. sativus*.

DSBs are among the most severe lesions of DNA molecules in the eukaryotic genome that may often lead to chromosomal abnormalities. Although H2AX histones are not indispensable for irradiation-induced cell cycle checkpoints, mice lacking H2AX were radiation sensitive, growth retarded, immunodeficient, and males were found infertile. Furthermore, metaphases from $H2AX^{-/-}$ mouse embryo fibroblasts revealed a significant increase in chromatid breaks and dicentric chromosomes (Celeste et al. 2002). To assess the possible impact of DNA defects (evidenced by the frequencies of H2AX foci formed in HU-treated root meristem cells) on the frequency of PCC events and the amount of chromosomal damages, root meristems of *R. sativus*, *V. faba*, and *A. porrum* were incubated for 24 h with 2.5 mM HU and transferred into medium containing both HU and caffeine. The observations of Feulgen-stained cells confirmed a wide range of aberrations reported earlier (Sen and Ghosh 1998, Rybaczek et al. 2002), such as chromosomal breaks and gaps, lost or lagging fragments of acentric chromatids, and the formation of micronuclei (Fig. 4). Whereas the appearance of prematurely condensed chromosomes was remarkably similar among the three chosen plant species, the relative quantities of cells appearing with evident symptoms of prematurely advanced mitosis revealed substantial differences (Fig. 5). The analyses of root meristem cells in *R. sativus* and *A. porrum* resolved maximum indices of PCC (less than 30% of all mitotic cells) 10 h after the start of

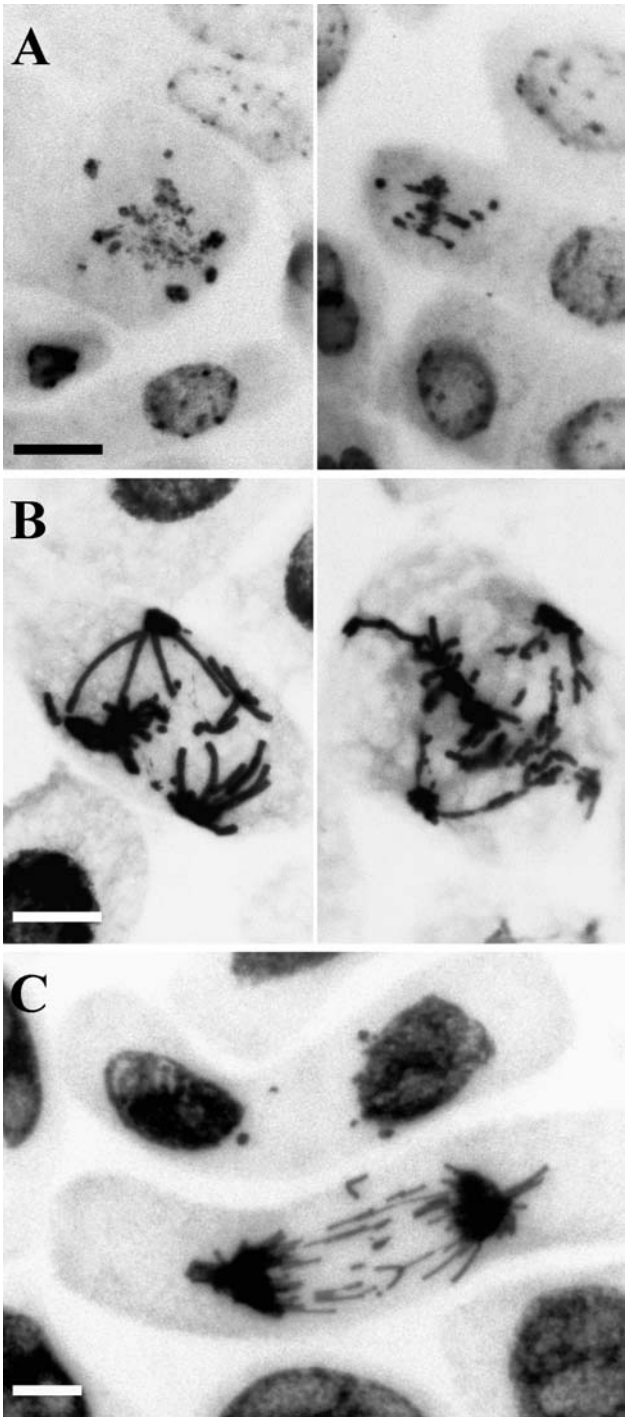


Fig. 4 A–C. PCC in Feulgen-stained root meristem cells exposed to the 24 h replicational stress followed by 8 h of treatment with 2.5 mM HU plus 5 mM caffeine. **A** *Raphanus sativus*; **B** *Vicia faba*; **C** *Allium porrum*. Bar: 10 μm

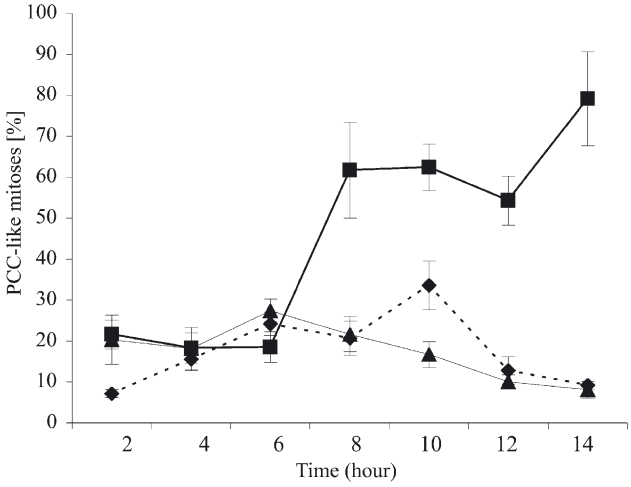


Fig. 5. Percentage of mitotic cells showing symptoms of PCC in root meristems of *R. sativus* (◆), *V. faba* (■), and *A. porrum* (▲) exposed to the 24 h replicational stress, recorded at different times (hours) of treatment with 2.5 mM HU plus 5 mM caffeine

incubation with the mixture of HU and caffeine. In root tips of *V. faba*, the proportion of cells showing a morphology typical for PCC increased rapidly between 6 and 8 h of treatment and attained the highest level (nearly 80%) by the end of the sampling period (14 h).

Apparently, at least two noticeable conclusions can be drawn from the data presented in our study that imply a correlation between genome size, the mode of response to HU-mediated DNA damage, and the ability of cells to override the S-M checkpoint controls. First, the differences among root meristem cells of the three plant species must inevitably point to the role of the specific higher order of chromatin that provides a more or less permeable barrier to the immense number of molecular factors engaged in the recognition and repair of genetic lesions. Although our approach is not readily applicable to any of the various hypotheses concerning the way the γ-H2AX foci might be arranged relative to the DNA lesions (Rogakou et al. 1998), it may be that the sizeable areas of diffuse chromatin in chromocentric cell nuclei of *R. sativus* may become more vulnerable to produce DSBs (marked by nuclear foci of phosphorylated H2AX histones) than the fairly dense nuclei in root meristems of *A. porrum* and *V. faba*. Second, it seems probable that HU-treated cells can have some intrinsic ability to override the S-M dependency, which allows them to prematurely enter mitotic division, irrespective of the unrepaired DNA molecules. It may be, then, that plants characterised by a small genome size (associated with efficient means to recruit factors needed to arrange the checkpoint functions) become protected from spontaneously entering PCC more efficiently than plants having large genomes. On the other hand, however, there seems to be no objective means of reconciling the physical (nucleotypic) nature of chromatin or the species-specific type of nuclear arrangement with the caffeine-induced facilities of root meristem cells to override the S-M checkpoint control system in plants that differ with respect to the genomic content of DNA.

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