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Induction of foci of phosphorylated H2AX histones and premature chromosome condensation after DNA damage in *Vicia faba* root meristem

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Abstract

Immunocytochemical analysis using antibody raised against human H2AX histones phosphorylated at serine 139 (γ-H2AX) demonstrates that root meristem cells of *Vicia faba* exposed to UV-radiation or incubated with hydroxyurea (HU) reveal discrete foci at the border of the nucleolus and perinucleolar chromatin or scattered over the whole area of cell nucleus. Western blots detected only one protein band at the position expected for the phosphorylated form of H2AX. The dose-effect relationship was demonstrated following treatment with 2.5 and 10 mM HU. Proteins extracted from root meristems incubated for 2 h either with HU and caffeine or with HU and sodium metavanadate showed unchanged amounts of bound γ-H2AX antibodies, as compared to root meristems treated with 2.5 mM HU. Higher quantities of phosphorylated H2AX histones were detected in proteins extracted from roots treated with HU and 2-aminopurine. All treatments were effective in producing evident aberrations of premature mitosis: broken and lagging chromatids, acentric fragments, chromosomal bridges and micronuclei. Our results show that phosphorylation of H2AX at the carboxy-terminal Ser-Gln-Glu sequence is among the earliest responses to double-strand breaks and, presumably, one of the key ATM/ATR-dependent signals indispensable for the repair of spontaneous and induced DNA damage in plant cells.

Additional key words: field bean, hydroxyurea, premature mitosis, UV-radiation.

Introduction

To cope with the intrinsic and extrinsic factors that could either disintegrate DNA at the G1 phase, interrupt DNA synthesis, or disturb the G2 functions preceding chromosome condensation, eukaryotes have evolved a series of inter-related cell cycle checkpoints that prevent entry into mitosis before the completion of the S-phase. These checkpoints allow time for the control of DNA repair and, in some cases, for the initiation of processes leading to cell death by apoptosis (Elledge 1996, Zhou and Elledge 2000). Ionizing or UV radiation and hydroxyurea-treatment, which impede replication fork movement during S-phase, activate two DNA-replication stress-response pathways, also referred to as the intra-S-phase checkpoints: *1)* the S-phase DNA damage checkpoint and *2)* the S-M replication checkpoint (Wang *et al.* 1999). The main role of these regulatory events is to sense fragmented and abnormally structured DNA molecules and to slow down or inhibit the progression of interphase until the cell has finished DNA replication and repaired the acquired damage.

Parallel pathways that respond to different types of stress activate three main classes of conserved proteins: *1)* the sensor kinases ATM (ataxia telangiectasia mutated) and ATR (ATM and Rad3-related), which perceive DNA damage or blocks in replication, *2)* signal transducers which relay the message, and *3)* effectors that convey the signal to target elements in the cell cycle machinery (Boddy and Russell 2001). In replication stress induced by hydroxyurea (HU, an inhibitor of ribonucleotide reductase; RNR), methyl methane sulfonate (MMS, a

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Abbreviations: AP - 2-aminopurine, ATM - ataxia telangiectasia mutated; ATR - ATM and Rad3-related; CF - caffeine; dNTPs - deoxyribonucleoside triphosphates; DSB - double-strand breaks; HU - hydroxyurea; IR - ionizing radiation; MMS - methyl methane sulfonate; MPF - M-phase promoting factor; PCC - premature chromosome condensation; PCNA - proliferating cell nuclear antigen; PI3K - phosphoinositide 3-kinase-like kinase; RNR - ribonucleotide reductase; ssDNA - single-stranded DNA; Van - sodium metavanadate.

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DNA-alkylating reagent), or physical factors, such as ionizing or ultraviolet (UV) radiation, the axial part of the S-phase checkpoint response system consists of the ATR kinase and the downstream effector Chk1 kinase (a member of the CHK kinase family). An important target in the S-M replication checkpoint is the Cdc25 phosphatase that can be phosphorylated by Chk1 at serine 216 (Zeng *et al.* 1998). This modification may negatively regulate Cdc25 by sequestering it in the cytoplasm. As a consequence, the cyclin B-Cdk1 (M-phase promoting factor; MPF) remains suppressed by Wee1/Myt1-mediated inhibitory phosphorylations on Thr-14 and Tyr-15 within the catalytic subunit of the complex (Liu *et al.* 1997).

Treatment of cells by HU results in two main classes of irregular DNA structures: *1)* hemi-replicated intermediate molecules with long single-stranded DNA regions (ssDNA), probably resulting from a defect in coordinating the replication of leading- and lagging-strands, and *2)* reversed forks (Sogo *et al.* 2002). The third and most severe type of genome damage - the DNA double-strand breaks (DSB) - is associated with specific ATM-mediated phosphorylation of the core H2A histone variant, H2AX. Accumulation of phosphorylated H2AX molecules clustered in megabase domains flanking structural abnormalities of chromatin participates in the recruitment of intra-S-phase checkpoint proteins and promotes mechanisms that block the entrance into mitosis to allow the DNA to be repaired (Burma *et al.* 2001).

Much of this research has focused on mammalian systems. Homologs of PI3K-related protein kinases have been found encoded in plant genomes (Hays 2002, Garcia *et al.* 2003), and most recently, ATM- and ATR-dependent γ-H2AX induction was shown in M-phase cells of *Arabidopsis thaliana* exposed to ionizing radiation (Friesner *et al.* 2005). Through immunocytochemistry and Western blot analysis of γ-H2AX (indicative of DSBs in cells exposed to IR), our present study has initiated the functional characterization of the signaling elements involved in HU-induced checkpoint pathways in root meristem cells of *Vicia faba*. Positive evidence for the presence of similar γ-H2AX-rich foci in cell nuclei of HU-treated plants has been correlated with short-term experiments using caffeine (CF), 2-aminopurine (AP) and sodium metavanadate (Van), chemical agents known to reverse the HU-induced cell cycle arrest and, consequently, to induce premature condensation of chromosomes (PCC).

Materials and methods

Plants: Seeds of field bean (*Vicia faba* L. var. *minor*) were germinated at 23 °C on wet blotting paper in Petri dishes. Four days after imbibition, dark-grown seedlings with primary roots ranging from 2.5 to 3 cm in length were selected for further experiments. During germination and incubations, roots were permanently aerated by gentle rotation of fluids in a water-bath shaker.

Cell irradiation: For each UV-irradiation, 10 intact seedlings were arranged so as to center their uncovered root tips in the same focal plane under the long-distance 4× objective of the epifluorescence *Optiphot-2* microscope (*Nikon*, Tokyo, Japan). Meristems were exposed to UV-radiation of *USH-102DH* (*Ushio Inc.*, Tokyo, Japan) mercury lamp (103 W), which was selected by a UV-2A filter emitting primarily 360 nm radiation at a fluency rate evaluated to be about 1 J m^{-2} s^{-1}. Following irradiation, roots were fixed according to immunocytochemical or Western blot protocols immediately or after appropriate post-treatment periods.

Immunocytochemical detection of H2AX: Apical parts of roots excised from the control, UV-irradiated or HU-treated seedlings (10 or 2.5 mM HU, *Sigma-Aldrich*, Munich, Germany) were fixed for 45 min (20 °C) in PBS-buffered 3.7 % paraformaldehyde (*Riedel-de-Haën*, Seelze, Germany). Then, roots were washed several times with PBS and placed in a citric acid-buffered digestion solution (pH 5.0) containing 2.5 % pectinase (*Fluka*, Seelze, Germany), 2.5 % cellulase (*Onozuka R-10*; *Serva*, Heidelberg, Germany) and 2.5 % pectolyase (*ICN*, Costa Mesa, USA), and incubated at 37 °C for 45 min. After 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*, Braunschweig, Germany). When air-dried at room temperature, the slides were pretreated with PBS-buffered 8 % BSA (bovine serum albumin, *Sigma*) and 4 % Triton X-100 (*Fluka*) for 50 min (20 °C) and incubated with rabbit polyclonal antibody recognizing human H2AX histones phosphorylated at serine 139 (anti-γ-H2AX antibody, *Upstate Biotechnology*, Lake Placid, USA), which was dissolved in PBS containing 1 % BSA (at a dilution of 1:750). Following an overnight incubation in a humidified atmosphere (4 °C), slides were washed 3 times with PBS and incubated for 1.5 h (18 °C) with secondary goat anti-rabbit FITC antibody (*ICN*) in PBS (1:500; v/v). Slides incubated solely with secondary antibody served as the negative control. In some experimental series, cell nuclei were counterstained with ethidium bromide (0.4 μg cm^{-3}, *Sigma*) and the total fluorescence emitted from cell nuclei served to evaluate relative DNA contents (2C, 2-4C, and 4C values, to distinguish G1, S, and G2 cells, respectively) (*LUCIA G*, *Precoptic Co.*, Warsaw, Poland, version 4,61). Following washing with PBS, slides were air dried and embedded in PBS:glycerol mixture (9:1) with 2.3 % DABCO (diazabicyclo[2.2.2]octane, *Sigma*). Observations were made using *Optiphot-2* epifluorescence microscope equipped with B-2A filter (blue light; λ = 470 nm) for

FITC and G-2A filter (green light; λ = 518 nm) for ethidium bromide. All images were recorded at exactly the same time of integration using *DXM 1200 CCD* camera (*Nikon*). In every experimental series, mean labeling index calculated as the ratio of cells showing specific fluorescence (at least two γ-H2AX foci per nucleus) to all cells within the examined population, was estimated from about 1000 cells (5 root meristems; 200 cells each). Mean number of fluorescing foci was computed from 30 labeled cells taken at random. The experiments were repeated twice.

Western blot analysis: Root meristem cells were lysed using *Tri Pure* (*Roche*, Westbury, NY, USA), according to vendors instructions, and the protein concentrations were determined using *Ultrospec 110 pro* (*Amersham*, Vienna, Austria). Identical aliquots of the samples dissolved in separation buffer (0.125 M Tris-HCl, pH 6.8, 4 % sodium dodecyl sulfate, 10 % glycerol, 10 % 2-mercaptoethanol) were incubated for 2 min at 95 °C and electrophoresed by standard SDS-PAGE (4 °C, 40 mA). Separated proteins were transferred onto a nitro-cellulose membrane (φ 0.45 μm, *Schleicher & Schüell*, Dassel, Germany) in a transfer buffer containing 20 % methanol, 192 mM glycine and 25 mM Tris (pH 8.3). After blocking non-specific protein binding using Tris-buffered saline containing 0.04 % Tween 20 (TBST) and 5 % fat-free dry milk, the membrane was incubated overnight with primary anti-γ-H2AX antibody (1:1500, *Upstate Biotechnology*) at 4 °C, followed by the reaction with a secondary goat anti-rabbit antibody conjugated with alkaline phosphatase (1:1000; *Cell Signaling*, Danvers, MA, USA), at room temperature for 1.5 h. After washing with TBST, signals were visualized with of NBT/BCIP (nitroblue tetrazolium chloride/5-bromo-4-chloro-3-indolyl phosphate, toluidine salt, *Sigma-Aldrich*) as a substrate.

Induction of PCC and Feulgen staining: Roots were incubated either in water (control) or in the mixtures of 2.5 mM hydroxyurea (HU) and 10 mM 2-aminopurine (HU+AP), HU and 5 mM caffeine (HU+CF) or HU and 0.02 mM sodium metavanadate (HU+Van). All reagents were obtained from *Sigma-Aldrich*. Following fixation in cold absolute ethanol and glacial acetic acid (3:1, v/v) for 1 h, excised root tips were washed 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, 1.5-mm-long apical segments were 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. All experiments were repeated twice.

Results

Preliminary analyses were designed to test whether antibodies raised against the gamma isoform of human histone H2AX may be applicable to intranuclear localization of DSBs in plants. Immunocytochemical observations revealed discrete fluorescent foci typical for γ-H2AX aggregates (Fig. 1) both in the population of root meristem cells exposed to UV or incubated under conditions restrictive to DNA replication (HU-treatment).

Irrespective of the experimental treatment or the phase of the cell cycle, the distribution patterns of phospho-H2AX foci ranges between the two types of cell nuclei, one with two or only few more fluorescing points situated at the border of the nucleolus and perinucleolar chromatin (Fig. 1*B*,*C*), and the other with numerous foci scattered over the whole area of chromatin (Fig. 1*D*). Within 1 min after the beginning of UV-irradiation, interphase nuclei showing phosphorylated form of H2AX histones could be found in about 1/3 of all meristematic cells, exceeding (by a factor of 6) the labeling index evaluated for the unirradiated control roots (Fig. 2*A*). The relative number of cells showing nuclear foci of phosphorylated H2AX increased after the prolonged exposure to UV-radiation, reaching nearly 40 % of the whole meristematic population of cells after 0.5 h and about 50 % after 1 h irradiation. Similar analyses of root meristems, which were allowed to recover for 30 min in water after the same periods of irradiation, demonstrated markedly higher labeling indices, yet the increased values did not correlate precisely with the results obtained from immunocytochemical examination of root meristems fixed immediately after their exposure to UV-radiation (Fig. 2*A*).

The very earliest fluorescing signals in root meristems treated with 10 mM HU was observed no sooner than 20 min after the start of incubation. Within 1 h, nearly 1/5 of the whole population of cells revealed at least two evident foci of phosphorylated H2AX histones (Fig. 2*A*). When roots were post-incubated in water for the next 30 min and cells were allowed to resume DNA synthesis, still new immunofluorescence signals appeared, increasing the proportion of cell nuclei with phospho-H2AX-specific foci to about 35 %.

In the control plants, none of the G1 cells revealed specific γ-H2AX fluorescence, and the labeling index estimated for the G2-phase was found slightly higher than that calculated for cell nuclei containing the 2-4 C DNA level (Fig. 2*B*). In root meristems exposed to UV radiation and incubated in water, the frequency of G1- and G2-phase cells indicating phosphorylated form of H2AX histones displayed highly positive correlation with the time of irradiation. This was in marked contrast to the subpopulation of root meristem cells containing DNA at

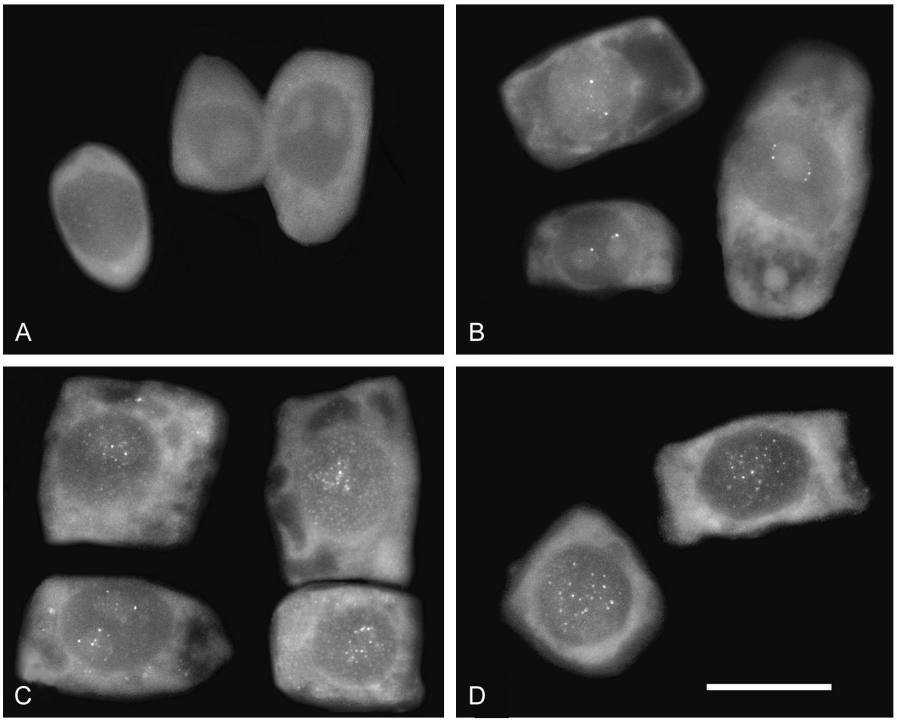

Fig. 1. Intranuclear localization of phosphorylated H2AX histones in root meristem cells of *Vicia faba*: *A* - untreated seedlings (similar images were obtained following the negative control procedure using only secondary goat anti-rabbit FITC antibody), *B* to *D* - distribution patterns of anti-γ-H2AX antibodies evidenced in seedlings treated with HU (*B*,*C*) or exposed to UV-radiation (*D*). *Bar* = 20 μm.

levels intermediate between the 2 C and 4 C, which revealed a substantial and fairly constant fraction of cells showing numerous intranuclear aggregates stained with anti-γ-H2AX antibodies, regardless of the duration of exposure to UV-radiation. In root meristems incubated with 10 mM HU for 1 h and recovered in water for 30 min, the labeling indices for S- and G2-phase cells were estimated to be 52 and 30 %, respectively. Surprisingly, response to HU-treatment has also been seen in about 20 % of the G1-phase cells (Fig. 2*B*). Furthermore, quantitative analyses of root meristem cells being at various stages of interphase clearly indicate that there is a close correlation between the number of aggregates of the phosphorylated H2AX histones and the labelling indices which characterize the cell cycle-dependent sensitivity to factors which harm to the integrity of cell nuclei (comp. Fig. 3 and Fig. 2*B*).

Recent experiments have shown that various treatments of root meristems with HU and CF or other related purine derivatives, such as 2-aminopurine and 6-dimethylaminopurine, give rise to a 'systemic' activation of mechanisms which increase the total level of protein phosphorylation and induce premature mitotic condensation of chromosomes. A remarkably similar effect induced by sodium metavanadate ($NaVO_3$) implied that the commitment of plant cells to PCC may appear not only as a consequence of suppression imposed upon the activity of protein kinases, but also as a product of the decreased activity of some of the tyrosine protein phosphatases (Rybaczek *et al.* 2002). To investigate possible correlations between the extent of HU-induced phosphorylation of H2AX histones and the amount of aberrations in cells entering PCC, root meristems of *Vicia* were incubated for 2 h with either HU (2.5 and 10 mM) or with the mixtures containing 2.5 mM HU and 5 mM caffeine (HU+CF), 10 mM 2-aminopurine (HU+AP) or 0.2 mM sodium metavanadate (HU+Van). Extracts of their proteins were immunoblotted with monoclonal anti-γ-H2AX antibodies and corresponding samples of root meristems were examined using standard Feulgen staining.

Immunofluorescence analysis demonstrated that the relative amount of cells revealing specific nuclear foci of phosphorylated H2AX histones in root meristems treated for 2 h with 2.5 mM HU is only slightly higher than that evidenced in the control plants and roughly 4 times smaller than in plants incubated for the same period of time with 10 mM HU (Fig. 4A). These data correlate well with the results obtained by Western blotting of total protein extracts from the control and HU-treated cells (Fig. 4B). In immunoblots of all samples, monoclonal γ-H2AX antibodies detected only one band at the position expected for the phosphorylated form of H2AX. Proteins extracted from root meristems incubated for 2 h with HU+CF and HU+Van mixtures revealed similar amounts of bound anti-γ-H2AX antibodies as those derived from root meristems treated with 2.5 mM HU. Surprisingly, significantly higher quantities of phosphorylated H2AX

histones have been detected in samples of proteins extracted from root meristems incubated with the mixture of HU+AP. This latter result did not correspond with either the increased labeling index or with the larger number of intranuclear aggregates of phosphorylated H2AX histones (data not shown). In HU+AP-treated root meristem cells, however, the sizes of individual foci (and, consequently, the total intensity of immunofluorescence estimated per cell nucleus) were slightly higher than those observed in root cells treated with HU+CF or HU+Van mixtures.

A series of parallel experiments was designed to determine the effects of short (2 h) treatments of 2.5 mM HU combined with CF, AP and Van on the induction of PCC (Fig. 4*C*). Incubations of root meristems in all these mixtures were effective in producing evident aberrations characteristic of premature mitosis: chromosomal breaks and gaps, lost or lagging chromatids and chromosomes, acentric fragments, and (in exceptionally rare cases of abnormal anaphase and telophase cells) chromosomal bridges and micronuclei. The highest percentage of cells indicating PCC-like morphology was observed in root meristems treated with HU+CF mixture (8.5 %; compared with 5.6 and 4.2 % in roots treated with HU+AP and HU+Van mixtures, respectively). Depending on the type of treatment, the mean number of visible chromosomal breaks observed in cells indicating evident symptoms of PCC ranged from 1 (HU+AP) to about 4 (HU+CF). However, the appearance of prematurely condensed chromosomes was remarkable similar in all experiments.

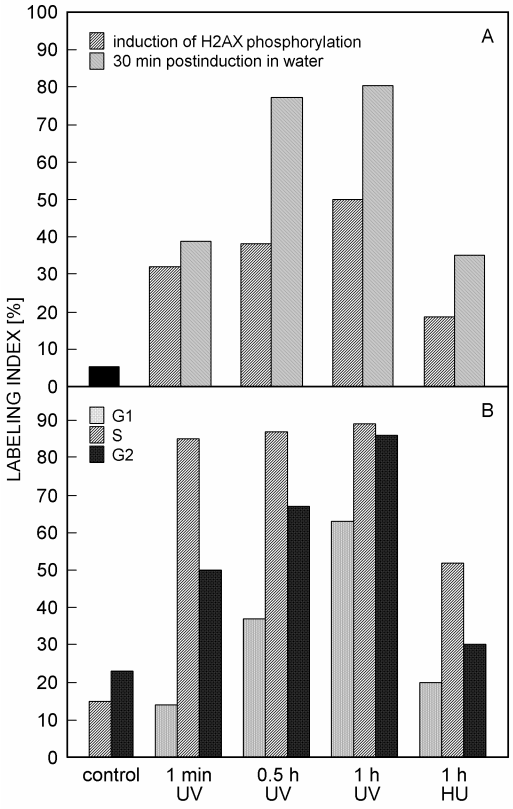

Fig. 2. *A* - Labeling indices calculated for root meristem cells stained immunofluorescently with anti-γ-H2AX antibodies in seedlings of *Vicia faba* following various exposures to UV-radiation or HU treatment. *B* - Labeling indices calculated for G1, S, and G2 cell populations following immunostaining with anti-γ-H2AX antibodies in root meristems of *Vicia faba* after exposures to UV-radiation or HU-treatment and 30 min postincubation in water.

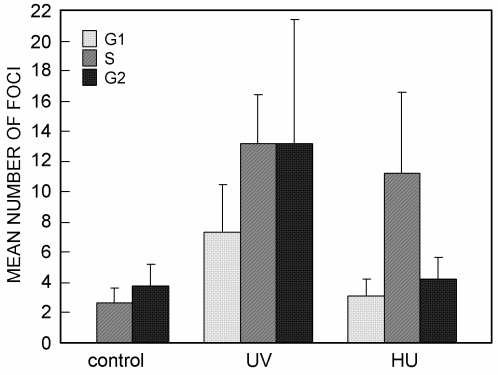

Fig. 3. Mean number (± SE) of fluorescent foci evidenced in G1, S, and G2 cell nuclei labeled with FITC-conjugated anti-γ-H2AX antibodies in root meristems of *Vicia faba*: untreated seedlings (control); roots exposed for 1 h to UV-radiation and kept for 30 min in water (UV); roots treated for 1 h with HU followed by 30 min postincubation in water (HU). Large SE values indicate significant intercellular variation.

Discussion

Our results suggest that phosphorylation of serine at the Ser-Gln-Glu amino acid sequence (SQE motif) of H2AX histones is among the earliest responses to DSBs and, presumably, one of the key ATM/ATR-dependent signals indispensable for the repair of spontaneous and induced DNA damage in root meristems of *V. faba*. Clusters of γ-H2AX foci have been shown to appear rapidly both after the exposure of root tips to UV radiation and HU-treatment suggesting that structural features of chromatin and the control mechanisms responsible for nuclear DNA integrity in yeasts and animals are similarly conserved in higher plants (Hays 2002, Friesner *et al.* 2005). Our data indicate that H2AX phosphorylation also takes place in response to S-phase arrest caused by the stalled machinery of DNA replication. It seems thus probable that the abnormally structured molecules generated by the UV- or HU-induced lesions activate overlapping biochemical pathways with ATM/ATR-mediated mechanisms to initiate signals of DNA damage (comp. Shiloh 2001, Melo and Toczyski 2002).

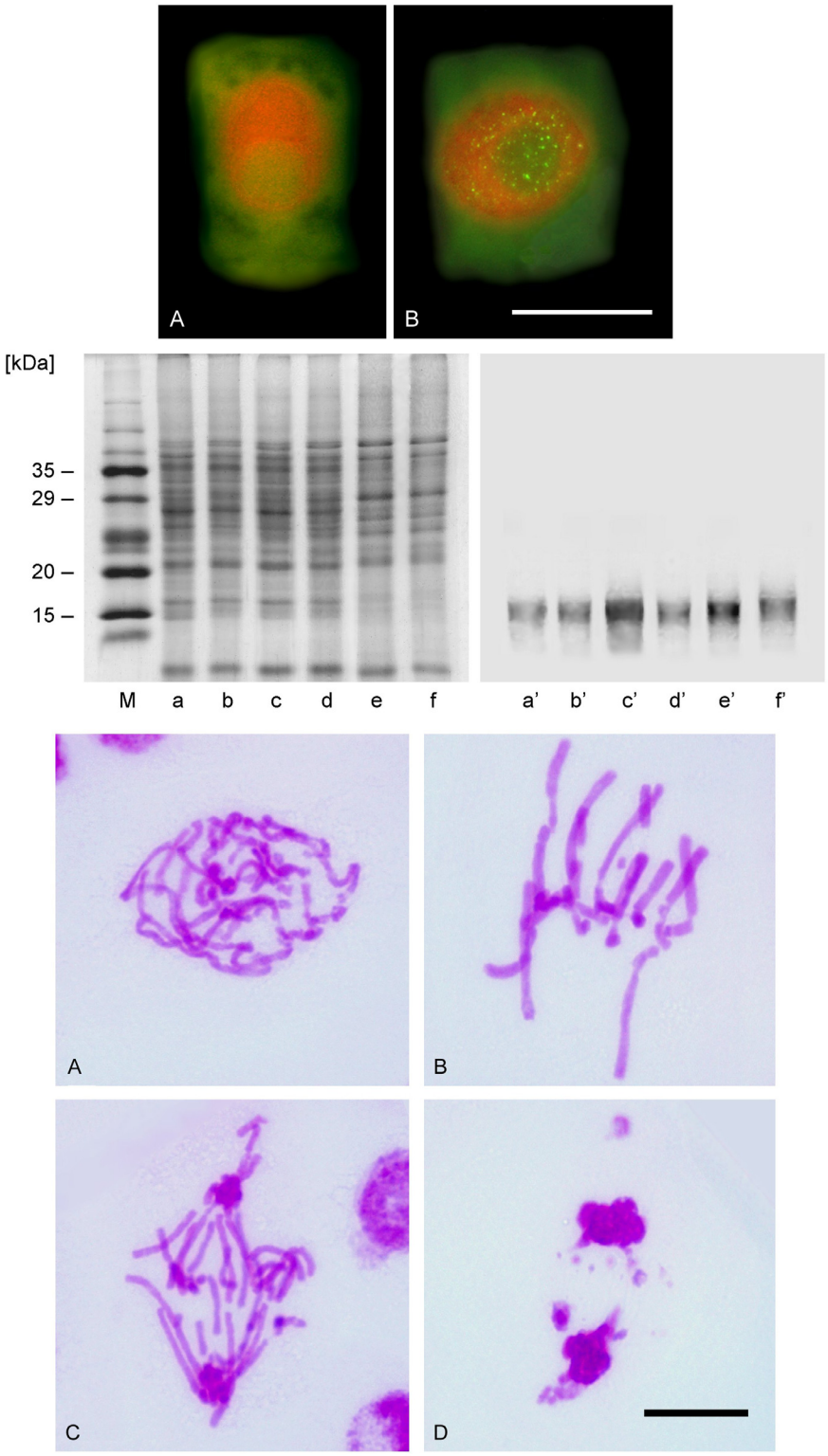

Fig. 4. *Upper* - Root meristem cells of *Vicia faba* double-stained with FITC-anti-γ-H2AX antibodies (green) and ethidium bromide (red): control (*A*) and 10 mM HU-treated seedlings (*B*). *Bar* = 20 μm. *Middle* - SDS/PAGE electrophoresis with Coomassie staining of proteins extracted from root meritem cells of *Vicia faba*: lane M - mass marker, lane a - control, lanes b to f - 2 h treatments with 2.5 mM HU (b), 10 mM HU (c), HU+CF (d), HU+2-aminopurine (e), and HU+Van (f). Western blots of separated and electrotranferred proteins performed using primary anti-γ-H2AX antibody and secondary goat anti-rabbit antibody conjugated with alkaline phosphatase (lanes a' to f' correspond with lanes a to f). *Lower* - HU/CF-induced premature mitosis in root meristem cells of *Vicia faba*: during prophase (*A*), metaphase (*B*), anaphase (*C*), and telophase (*D*). Feulgen staining. *Bar* = 10 μm.

A great number of results provide strong evidence that the formation of phosphorylated H2AX foci operate in recruiting the downstream factors critical for recognition and repair of nuclear DNA damage sites (Ward and Chen 2001). Although most of these data are readily pertinent to mammals, it seems reasonable to assume that at least two basic functions of γ-H2AX clusters may also be applicable to plants. First, they are likely to play a primary role in promoting structural changes of chromatin that allow for concentration of DNA repair and signaling factors. Second, the altered configuration of nucleosomal foci localized in the vicinity of domains surrounding the incurred lesions may tether the ends of DNA and, hence to prevent broken portions of chromosomes from a far-reaching separation and potential rearrangements (Paull *et al.* 2000, Fernandez-Capetillo *et al.* 2004).

Western blotting and immunocytochemical procedures revealed a relatively high level of H2AX phosphorylation in untreated root meristem cells of *Vicia faba*. This observation suggests that there may be either some fundamental requirement for the permanent functioning of the H2AX-dependent control over chromosomal structures throughout S- and G2-phases, or that the postreplicative repair of nascent DNA molecules requires some particular mechanism based upon the modified molecules of H2AX. Similarly to other experimental systems, root meristem cells of *Vicia* start out to reveal new nuclear foci of phosphorylated H2AX histones already within 1 min after the UV-induced damage or following a relatively short period of incubation with HU. As in the case of mammalian cells (Andegeko *et al.* 2001, Kobayashi *et al.* 2002), the immunofluorescent staining with anti-γ-H2AX antibody decreased gradually within the next few hours of treatment (data not shown).

Rapid kinetics of formation and dissolution of intranuclear γ-H2AX foci has prompted us to perform short-term experiments designed to evaluate the extent to which agents known to induce premature mitosis in HU-treated cells of *Vicia* (Rybaczek *et al.* 2002) may change an overall level of H2AX phosphorylation. The analysis made using Western blotting did not allow us to detect any significant difference in an overall level of H2AX phosphorylation between the extracts of proteins obtained from the control roots, HU-treated meristems (2.5 mM), or from samples of root tips of *Vicia* incubated with HU+CF and HU+Van mixtures. Unexpectedly, however, an evident increase in H2AX phosphorylation was found in proteins isolated from root meristems incubated for 2 h with HU+AP. This result may correspond either with the intensified phosphorylation of H2AX molecules associated with abnormal structures of DNA, or with a delayed degradation of the foci, implying some kind of AP-mediated protection over those areas of chromatin, which comprise the sites altered by the retarded replication of DNA molecules. At present, however, the possibility cannot be excluded that the rate of γ-H2AX focus formation exceeds the rate of AP-mediated suppression of ATM/ATR-dependent activities, and that once such formation had occurred, the presence of AP may promote rather, than restrain the state of H2AX phosphorylation at the site of DNA damage.

Checkpoint defects and such chemical agents as methylxanthines, aminopurines, phosphatase inhibitors and protein kinase antagonists, may abrogate the ATR-Chk1-Cdc25 pathway, advance mitotic events and, thus, activate the program of premature chromosome condensation (PCC). The ability of CF, AP and Van to attenuate prevention of mitosis in HU-treated root meristem cells of *Vicia* strongly supports the contention that elaborate regulatory mechanisms of the cell cycle are connected phylogenetically between different groups of eukaryotes (Vandepoele *et al.* 2002, De Veylder *et al.* 2003).

Most of the M-phase cells evidenced in root meristems of *Vicia* after 2-h incubations with HU+CF, HU+AP, or HU+Van represented various stages of normal cell division, with only a minor fraction corresponding to a 'true' state of premature mitosis. Induction of the PCC-like state (missegragated chromosomes, fragmented chromatids and chromosomal bridges) was restricted merely to a relatively small subpopulation of G2-phase cells having their DNA completely replicated. Although the mechanisms by which CF, AP, and Van can override the G2-damage checkpoint remain not clear, it is obvious that all these inhibitors may affect functional states of the regulatory factors engaged in the cell cycle progression. Several lines of evidence are consistent with the possibility that CF and AP suppress both ATM and the related ATR protein kinases (Sarkaria *et al.* 1999, Huang *et al.* 2003). As a consequence, the dephosphorylated (inactive) state of Chk2 and Chk1 effector kinases prevents Cdc25 tyrosine phosphatase from being phosphorylated and sequestered, which triggers the downstream processes correlated with the activity of cyclin B-CDK complex and, eventually, with the unscheduled condensation of chromosomes (Furnari *et al.* 1997, Shiloh 2001). Although PCC-like effects induced by Van, are remarkably similar to that found after incubations of root meristems with HU+CF or HU+AP, the mechanism by which HU+Van-treated cells can override the G2-damage checkpoint and become committed to enter premature mitosis still needs to be resolved, since the effects induced by vanadates comprise the suppression of Cdc25 phosphatase (Mailhes *et al.* 2003).

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