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Lack of mitotic delays at the onset of proliferation in dormant root primordia challenged by ionizing radiation

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Abstract

X-rays at doses between 2.5 and 20 Gy were applied to *Allium cepa* L. bulbs containing either dormant root primordia (before water imbibition) or actively proliferating meristems. Irradiation of the primordia that were enriched in G_0 cells neither delayed proliferation onset nor root sprouting. Under both protocols, irradiation decreased the final length of the roots to about 60 % (at 20 Gy) of that reached by the unirradiated controls. Irradiation of the proliferating meristems increased the mitotic index at some fixation times. This could not be due to a rise in the cell entry into mitosis, as the rate of root growth decreased simultaneously. The increased mitotic index should be the consequence of a delay in the relative time taken by mitosis in the whole cycle time. Lengthened mitosis probably allows the post-replicative repair of most DNA lesions, as the frequency of interphases with micronuclei was higher in the cells which were irradiated when still dormant than in those irradiated when cycling. Thus, the mitotic delays should be the consequence of a checkpoint pathway activated by the presence of DNA damage. This feedback mechanism seems only to develop after cell proliferation is restored.

Additional key words: aberrant mitosis, activation of proliferation, *Allium cepa* L., checkpoint delays in cell cycle, interphases with micronuclei, mitotic index, onion, proliferating meristems.

Introduction

Physiological induction of proliferation in the cells of root primordia found in dormant seeds or bulbs takes place after their water imbibition. Dormancy of root primordia in *Allium cepa* L. bulbs seems to be analogous to the attenuated dormancy displayed by some, but not all *Avena fatua* seeds. Both cell types have a moderate moisture content and retain some metabolic activity. Thus, there is some unscheduled DNA synthesis for DNA repair in the attenuated dormant *Avena* (Elder and Osborne 1993), and some semiconservative replication in the dormant *Allium* root primordia. In these latter cells, labelling and mitotic indexes are about 84- and 24-fold lower, respectively, than in proliferating roots (Sans and De la Torre 1979).

The embryos of ageing seeds accumulate DNA lesions (Cheah and Osborne 1978) that limit their germination. The present work evaluated any possible delaying effect of the induced DNA damage in the sprouting and growth of roots from onion bulbs, as well as in the processing of the primary DNA lesions up to their transformation in chromatid/chromosomal breaks in mitosis, after moderate X-ray doses (2.5 to 20 Gy). For this, irradiation was applied either immediately before water imbibition of the dormant root primordia or during proliferation in their growing roots (3 d later).

The presence of any checkpoint mechanism controlling an irreversible transition can be asserted by the presence of a delay or lengthening in the cycle phase

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previous to such a transition (Hartwell and Weinert 1989). Checkpoints are enzymatic subpathways of the general cellular response to DNA damage, that are able to delay an irreversible cycle transition in the presence of some unrepaired lesion. As a result, the proliferating cell gets some additional time to either repair the damage (Zhou and Elledge 2000) or become adapted to its presence (Paulovich *et al.* 1997).

Materials and methods

The root primordia and the meristems of the enlarging roots of *Allium cepa* L. cv. "Francesa" bulbs were used. Onion bulbs were allowed to sprout their roots and later grow in the dark at a constant temperature of 25 °C, in a *Refritherm Struers* incubator (Copenhagen, Denmark), in cylindrical glass receptacles (85 cm^3 capacity and 11 cm in height), using filtered tap water (*Milli RO-4* from *Millipore*, Bedford, USA). Water was renewed every 24 h and aerated continuously by bubbling air using an aquarius pump (*René Super*, Lyon, France), through a flask containing distilled water, so that the air reached each of the culture tubes saturated with humidity. The rate of air bubbles was kept so that a 15 to 20 cm^3 min^{-1} oxygen was supplied to the cultures. The length of the 6 to 10 longest roots was measured daily. All the experiments were repeated twice.

Under the first protocol, the irradiation was carried out on bulbs after taking off their dry cover layers. They were placed in a perspex supporter inside the X-ray equipment (*Philips M6-102* model, Eindhoven, The Netherlands). The largest exposure window was used. There were 23 cm between the source and the dry crown where primordia are located. Irradiation took place at 80 kV and 15 mA. Irradiation times (1.5, 3, 6 and 12 min) provided 2.5, 5, 10 and 20 Gy of X-ray exposure, as estimated through the calibration curves obtained from the Radiological Protection Service of the Centro de Investigaciones Biológicas. The unirradiated bulbs stayed at room temperature for half an hour. After irradiation, control and irradiated bulbs were returned to the incubators.

Under the second protocol, irradiation of the growing roots was carried out 72 h (3 d) after imbibition. Five bulbs with their roots growing immersed in the culture tubes were exposed simultaneously. After irradiated, they were re-located in the 25 °C incubators with air bubbling.

The extracted root primordia and the elongating roots were fixed for about one week in a 3:1 mixture (v/v) of ethanol and acetic acid. Root fixations were carried out, every 24 h, for 4 d after irradiation. The nuclei were stained with 1 M HCl-acetic orcein. Squashes were prepared from whole primordia extracted from the bulbs or from the apical 2 mm of the growing roots, after their staining. Coverslips were removed by the dry ice method (Conger and Fairchild 1953). For recording mitotic index, at least four root meristems from five different bulbs were studied at each fixation time. About 2000 cells per root were sampled to estimate the mitotic index as well as the frequency of aberrant mitosis and of interphasic cells with micronuclei.

To assess the statistical significance of the data, the univariate analysis was carried out by one or two way *ANOVA*, using the *SPSS* statistical program for *Windows*. Pairwise comparisons were done by variance and Bonferroni adjustments. For multivariate comparisons, the Stepwise Discrimant Analysis was carried out by the *BMDP* program for *VMS* in *ALPHA 200*. The probability to reject the null hypothesis was fixed at $P < 0.05$. An F value greater than 4.0 in this last test will represent a significant difference.

Results and discussion

The control (unirradiated) proliferating cells lengthened 49 mm in four days, from the 3rd to the 7th day after imbibition of the bulb crown where dormant primordia are located. However, roots sprouted and grew as much as 77 mm in the first four days following imbibition of the dormant primordia (Table 1). The estimated rates of root lengthening in control cells (Fig. 1) increased up to a peak that took place 2 d after water imbibition of the dormant primordia. On the other hand, the rates of root lengthening in the control roots decreased from the 3+1 d after imbibition (1 d in the left upper panel of Fig. 1). The radiosensitivity of the already proliferating cells is similar to that displayed by *Scilla* cultured cells when challenged by γ-irradiation (Chakravarty and Sen 2001).

Table 1. Mean length of the roots on the day 4 after irradiation of proliferating cells (X-rays in cycle) or of dormant cells (X-rays in G_0). * - values different from controls (0 Gy) at $P < 0.05$.

Doses [Gy]	Proliferating cells length [mm]	[%]	Dormant cells length [mm]	[%]
0	49	100	77	100
2.5	51	104	75	97
5	43*	88	64*	83
10	37*	75	50*	65
20	29*	59	42*	56

In the unirradiated onion controls, the highest intrinsic rate of root lengthening took place during the first three days after imbibition. This explains the apparent paradox of a higher X-ray depressing effect on root growth when the already proliferating meristems were irradiated than when they were challenged in G_0.

Under both protocols, however, irradiation decreased the rates of root lengthening to similar extents at different X-ray doses (Table 1).

The mitotic index and the frequency of aberrant mitosis (metaphases with chromatid/chromosomal breaks or DNA fragments, and anaphases with chromosomal bridges) were recorded under both irradiation protocols. The mitotic index declined in the unirradiated meristems

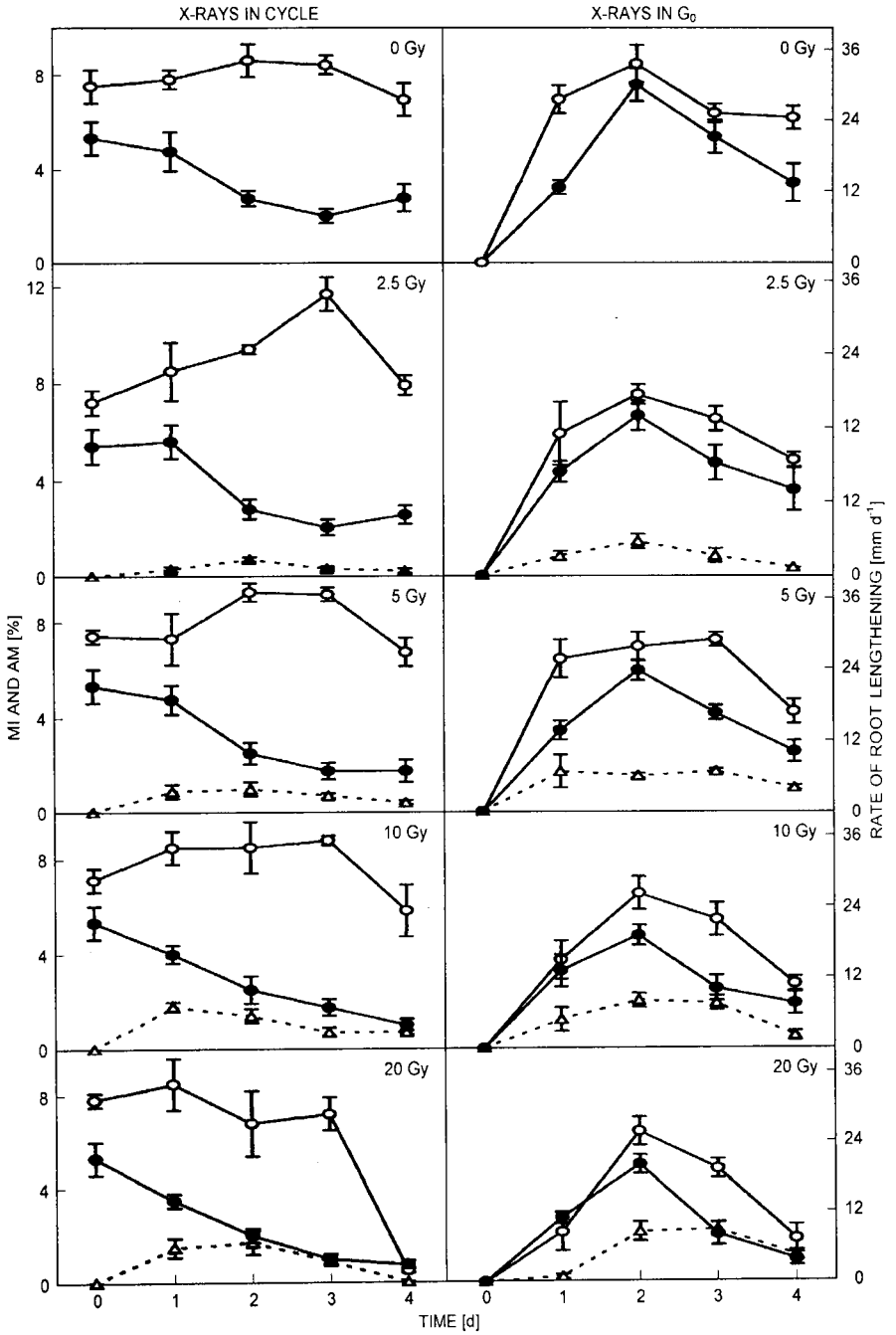

Fig. 1. Changes in mitotic index (*open circles*), aberrant mitosis (*open triangles*) and rate of root lengthening (*closed circles*), from one to four days after different doses of X-rays. Irradiation was applied to the already proliferating meristems at the 3rd day after their imbibition so that, in the left column, the time of irradiation (0 time) corresponds to the 3rd day after imbibition. On the other hand, in the right column, irradiation was accomplished to the dormant primordia containing cells in G_0 at 0 d, when both irradiation and imbibition started.

from the day 3 onwards, *i.e.* from the 3 + 3 d after water imbibition (Fig. 1). The most striking feature of the response to radiation was some increase in mitotic index. This was only observed when the proliferating meristems were irradiated.

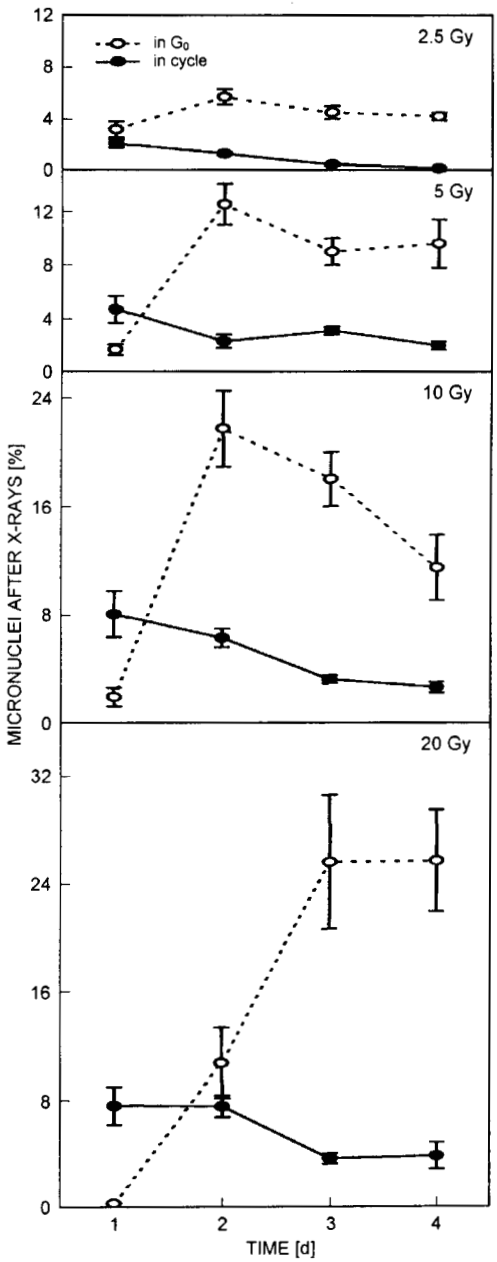

Fig. 2. Frequency of interphases with micronuclei after irradiation of the already proliferating cells (*closed circles*), or in the cells irradiated when still in G_0 (*open circles*). Times in abscissa, as in Fig. 1.

The frequency of aberrant mitoses rose with dose of radiation (Fig. 1). The population of aberrant mitosis (evaluated by the area below the curves) was 2-fold larger when irradiation took place in the dormant primordia than in the proliferating meristems, at 2.5 and 5 Gy. However, at 10 and 20 Gy, irradiation only delayed one day the peaks in the frequency of aberrant mitoses, without modifying the size of this sub-population in comparison to that observed in cells irradiated when proliferating. Differences in the frequency of aberrant mitoses, however, did not allow the discrimination between both protocols of irradiation ($P < 0.05$ for the $F < 4.0$ estimated by the stepwise discriminant analysis).

The frequency of interphases with micronuclei was higher when irradiation was anticipated to the dormant primordia than when provided during proliferation (Fig. 2). It should be noticed that the frequency of cells with micronuclei displayed no cumulative frequency in plant root meristems. This is because as many cells as those forming the whole meristem leave it after each cycle time.

Three features distinguished both protocols. First of all, onset of proliferation was unaffected during the first two days after irradiation of the dormant primordia mostly formed by G_0 cells. Thus, stimulation of proliferation (dormancy breakage) was independent from any DNA damage produced by ionizing radiation, at least up to 20 Gy of X-rays. The present data supports the prevalence of the activation of the machinery that stimulates cycle progression over that which prevents entry into proliferation or progression through the cycle when the DNA has not a proper structure (Murray and Hunt 1993, O'Connell *et al.* 2000). This is the case for the attenuated cell dormancy produced in the onion primordia, whose dormancy is limited to one year.

Second, only the cells that were irradiated when still dormant did not display the rise in mitotic index observed in the proliferating meristems. On the other hand, in the range of radiation doses here tested, the frequency of aberrant mitoses was not different from that recorded in cells irradiated when proliferating ($P < 0.05$).

Third, the frequency of interphasic cells with micronuclei was higher when irradiation took place before breakage of dormancy than when performed in proliferating cells.

Checkpoint pathways induced by the presence of DNA lesions (Pelayo *et al.* 2001), incomplete replication (Del Campo *et al.* 1997) or unfunctional DNA topoisomerase II (Giménez-Abián *et al.* 2002) are conserved in the onion proliferating cells. In them, checkpoints delay cycle progression to different extents, before they license the entry into subsequent cycle phases (Hartwell and Weinert 1989, Zhou and Elledge 2000). The lack of delays during their first cycles in cells challenged by X-rays when still in G_0 suggests that checkpoint mechanisms are not yet re-established. It is worthy to compare this situation with that in *Arabidopsis*, where abscisic acid delays seed germination. In addition, abscisic acid reversibly blocks further growth during a narrow developmental time interval, after germination, if the osmotic state becomes potentially lethal (López-Molina *et al.* 2001).

As far as we know, the present report is the first that describes the functional lack of checkpoints during the physiological breakage of dormancy in plant somatic cells previously challenged by X-rays.

References

Chakravarty, B., Sen, S.: Enhancement of regeneration potential and variability by gamma-irradiation in cultured cells of *Scilla indica*. - Biol. Plant. **44**: 189-193, 2001.

Cheah, K.S.E., Osborne, D.J.: DNA lesions occur with loss of viability in embryos of ageing rye seed. - Nature **272**: 593-599, 1978.

Conger, A.D., Fairchild, L.M.: A quick-freeze method for making smear slides permanent. - Stain Technol. **28**: 281-283, 1953.

Del Campo, A., Giménez-Martín, G., López-Sáez, J.F., De la Torre, C.: Frailty of two cell cycle checkpoints which prevent entry into mitosis and progression through early mitotic stages in higher plant cells. - Eur. J. Cell Biol. **74**: 289-293, 1997.

Elder, R.H., Osborne, D.J.: Function of DNA synthesis and DNA repair in the survival of embryos during early germination and in dormancy. - Seed Sci. Res. **3**: 43-53, 1993.

Giménez-Abián, J.F., Weingartner, M., Binarová, P., Clarke, D.J., Anthony, R.G., Calderini, O., Heberle-Bors, E., Moreno Díaz de la Espina, S., Bögre, L., De la Torre, C.: A topoisomerase II-dependent checkpoint in G_2-phase plant cells can be bypassed by ectopic expression of mitotic cyclin B2 expression. - Cell Cycle **1**: 187-192, 2002.

Hartwell, L.H., Weinert, T.A.: Checkpoints: controls that ensure the order of cell cycle events. - Science **246**: 629-634, 1989.

López-Molina, L., Mongrad, S., Chua, N.-H.: A postgermination developmental arrest checkpoint is mediated by abscisic acid and requires the ABI5 transcription factor in *Arabidopsis*. - Proc. nat. Acad. Sci. USA **98**: 4782-4787, 2001.

Murray, A., Hunt, T.: The Cell Cycle. An Introduction. - Oxford University Press, New York - Oxford 1993.

O'Connell, M.J., Walworth, N.C., Carr, A.M.: The G_2-phase DNA-damage checkpoint. - Trends Cell Biol. **10**: 296-303, 2000.

Paulovich, A.G., Toczyski, D.P., Hartwell, L.H.: When checkpoints fail. - Cell **88**: 315-321, 1997.

Pelayo, H.R., Lastres, P., De la Torre, C.: Replication and G_2 checkpoints: their response to caffeine. - Planta **212**: 444-453, 2001.

Sans, J., De la Torre, C.: G_0 and G_2 cells identified by their chromatin pattern in dormant and proliferating meristems. - Eur. J. Cell Biol. **19**: 294-298, 1979.

Zhou, B.B.S., Elledge, S.J.: The DNA damage response: putting checkpoints in perspective. - Nature **408**: 433-439, 2000.