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EFFECT OF γ-RADIATION ON THE PLASMA AND VACUOLAR MEMBRANES OF CULTURED SPINACH CELLS

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Key Word Index—*Spinacia oleracea*; Chenopodiaceae; cultured cells; membrane damage; γ-radiation; plasma membrane; vacuolar membrane; tonoplast; ^{31}P NMR; inorganic phosphate.

Abstract—By the use of ^{31}P NMR spectroscopy, a higher radiosensitivity of the vacuolar membrane (tonoplast) than the plasma membrane was inferred for cultured spinach cells irradiated with 0–7.5 kGy and immediately transferred to fresh medium. In non-irradiated cells, inorganic phosphate (Pi) was rapidly taken up from the medium and transported into the vacuole; the content of cytoplasmic Pi was maintained at a constant level throughout the incubation period (7 days), whilst the Pi in the vacuole was exhausted. In irradiated cells, Pi uptake was not greatly altered up to 5 kGy, and fluorescein diacetate, a substance used to determine the integrity of the plasma membrane, was readily absorbed. However, movement of the Pi taken up into the vacuole across the tonoplast was markedly reduced with increasing dose. This membrane impairment was not amplified during subsequent incubation. A crucial question as to the fate of the Pi is critically discussed.

INTRODUCTION

Radiation induces various functional impairments to cellular membrane systems through alterations to their physicochemical properties such as viscosity, surface charge, chemical composition and permeability leading to the loss of normal physiological processes [1–4]. Many investigations of the effects of radiation on animals and microorganisms have been carried out, and during the past 10 years, these have been extended to plant materials [5–13], including the plant cell plasma membrane and the tonoplast (vacuolar membrane).

The plasma membrane is the boundary of all living cells and plays an important role in the process of penetration of substances into the cell. The tonoplast is unique to plants and constitutes an intracellular membrane system of mature plant cells that plays a part in maintaining the intracellular homeostasis through controlling the flux of substances into and out of the vacuole [14, 15] e.g. helping to maintain the concentration of inorganic phosphate [16–21] and Ca^{2+} [22] within certain prescribed limits. When these membranes are damaged by various agents, the physiology of the plant cell will deviate from that of the normal state. Radiation can serve as one of these agents that perturb cellular systems *in situ*. If the resulting *in vivo* changes in cells are monitored by a non-invasive method, e.g. by NMR spectroscopy [23], this should produce a lot of valuable information on the permeability properties of membranes. Studies have been carried out on the metabolism of phosphorus compounds in animals [24] and microorganisms [25], but only one such study has been undertaken with plants. Since biomembranes are known to be one of the most radiosensitive sites in cells [26], we have investigated the effects of radiation on plant membranes, especially on the tonoplast. These studies have demonstrated that exposure to radiation increases the permeability of *Chelidonium majus* leaf tonoplast [8] and leads to a deterioration of the enzymes, proteins and lipids of the vacuolar membrane in cultured spinach cells [13].

In the present study the effects of radiation on the permeability of the plasma membrane and tonoplast of cultured spinach cells was investigated by measuring the uptake of inorganic phosphate by the cells from cultured medium and its intracellular movement.

RESULTS

Time course changes in the ^{31}P NMR spectra of cultured spinach cells

These were measured over one incubation cycle (7 days) after the 7-day-old cultured cells had been trans-

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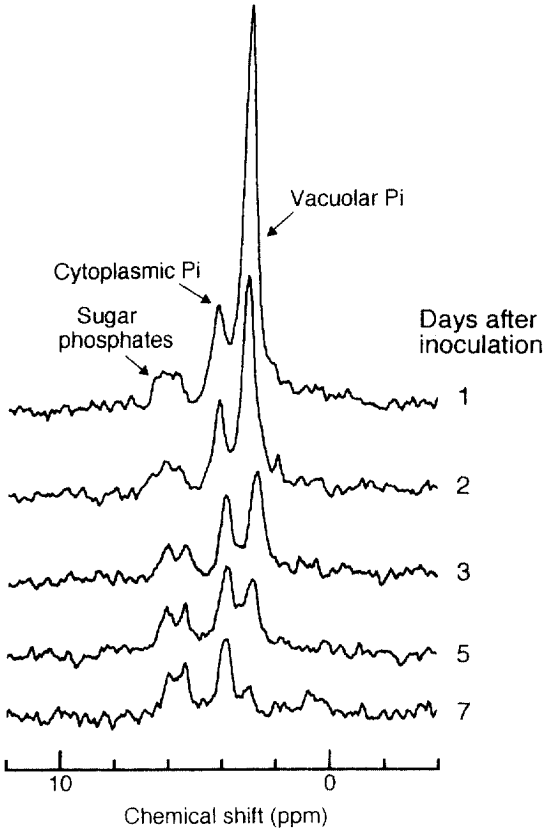

Fig. 1. Time course change of ^{31}P NMR spectra of cultured spinach cells with time. Seven-day-old cells in which the vacuolar Pi was exhausted were transferred to fresh medium A, and incubated for 1, 2, 3, 5 and 7 days under the standard culture conditions and then subjected to NMR analysis. Measurements were taken several times; the trend being essentially the same.

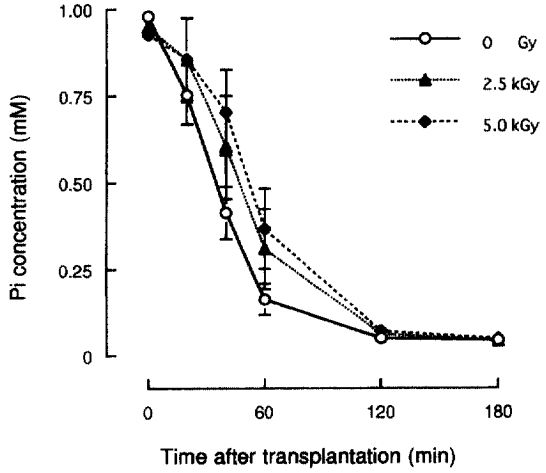

Fig. 2. Time-dependent change of Pi uptake by cells pre-irradiated with doses up to 5.0 kGy and inoculated into fresh medium, whose original Pi concentration was 1.25 mM. Bar: standard error of three experiments.

ferred to fresh medium (medium A) (Fig. 1). The vacuolar and cytoplasmic pools of inorganic phosphate (Pi) were readily identified from their chemical shift values, and several peaks tentatively assigned to sugar phosphates were also present. However, no clear signals for nucleotide compounds such as ATP and NADP could be detected at higher magnetic fields (in view of this, ppm values less than 2.0 are not given in Fig. 1). The cytoplasmic Pi was maintained at an almost constant level, whilst that of vacuolar Pi was at its highest level on day 1, and thereafter progressively decreased with time until it was almost totally absent by day 7.

Effects of radiation on plasma membranes (PM)

Two experiments were carried out.

In the first experiment 7-day-old cells were exposed to radiation up to 5.0 kGy and instantly transferred to fresh culture medium (medium A). Thereafter, the decrease in Pi concentration in the medium was monitored for 3 h (Fig. 2). Non-irradiated cells took up most of the Pi from the medium within 2 h. Pi uptake by irradiated cells was reduced over the first hour in a dose-dependent manner; however, exhaustion of medium Pi was always observed after 2 h even with a dose as high as 5.0 kGy.

In the second experiment, irradiated and non-irradiated cells were incubated with fluoroscein diacetate (FDA) [27] and then examined by light and fluorescence microscopy. This substance is only permeable to the plasma membrane of living cells and after entering cells, undergoes hydrolysis by esterase to yield a fluorescent substance, fluorescein, which is not readily permeable to the plasma membrane. Therefore, the ratio of fluorescent cells to non-fluorescent cells should not be reduced, unless the plasma membrane was significantly damaged by radiation. Seven-day-old cells were irradiated with 7.5 kGy, and then incubated in fresh medium for 3 h. An aliquot from the culture suspension was then treated with FDA and after standing for several min, the cells were photographed first under a light microscope and then under a fluorescence microscope. Fluorescence was observed in most of the cells. Similar results were obtained for the control cells. In non-irradiated 15-day-old cells (coloured brown) which no longer have the ability to divide on transfer to fresh medium, only a small number of cells fluoresced.

Effects of radiation on intracellular Pi movement

Seven-day-old cells were exposed to 5 kGy, transferred to fresh culture medium and incubated for different periods of time. The cytoplasmic and vacuolar Pi contents were monitored by ^{31}P NMR every 2

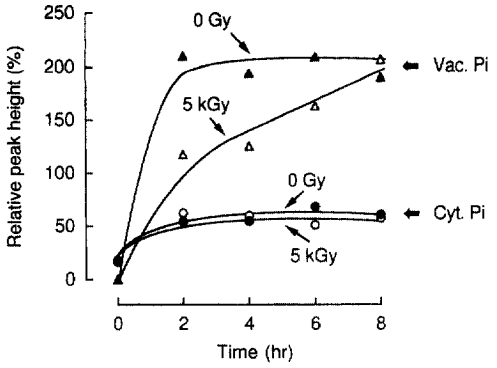

Fig. 3. Time course change in cytoplasmic and vacuolar Pi content in irradiated cells. Seven-day-old cells were irradiated at 5 kGy and then transferred to fresh medium A. After incubation for the specified times under the standard culture conditions, the cells were subjected to NMR analysis. The results are typical of those obtained in four experiments using different samples; statistical analysis is not presented, because the peak heights differed greatly between samples.

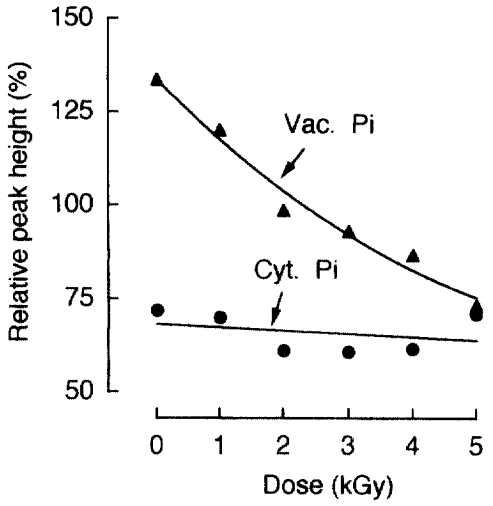

Fig. 4. Dose-dependency of radiation effects on Pi contents in cytoplasm and vacuole. NMR measurements were carried out 2 h after inoculation. The experiment was carried out twice. Essentially the same results were obtained on both occasions.

h over an 8 h period (Fig. 3). In both non-irradiated (0 Gy) and irradiated cells, the cytoplasmic Pi content increased over the first 2 h period and then reached a plateau. However, the vacuolar Pi content increase in irradiated cells was only approximately 50% of the control cells over the first 2 h period, but thereafter increased, although at a slower rate, to reach the level of the control cells (Fig. 3).

Dose-dependency of radiation effects on Pi contents in cytoplasm and vacuole

Seven-day-old cells were exposed to different doses of radiation up to 5 kGy, transferred to fresh culture medium and incubated for 2 h; 2 h was chosen because this was the time when the cytoplasmic and vacuolar Pi contents in non-irradiated cells plateaud (Fig. 3). The results (Fig. 4) showed that, in contrast to the cytoplasmic Pi content which was maintained at an almost constant level irrespective of radiation dose, the content of Pi in the vacuole was dose-dependent; thus with increasing radiation energy the Pi content was reduced almost linearly, i.e., radiation damage increased. This reduction was ca 40% in cells irradiated with 5 kGy.

DISCUSSION

NMR spectroscopy is a useful non-invasive technique for the measurement of intracellular levels and locations of various metabolites. Treatment of biological tissues with medium doses of radiation could bring about stress, akin to that induced by unfavourable external factors like drought, low temperature, anoxia, high salt etc. [28], and, if these *in vivo* alterations are analyzed by non-invasive NMR techniques, it should be possible to elucidate the normal physiological processes on the basis of the information obtained following radiation-induced perturbations. These methods have been applied to the study of phosphorus metabolism in rat brain [24] and yeast [25]. So far as plant materials are concerned, however, only one brief communication is available at present. This described the change of Pi state in irradiated maize root tips [29].

Characteristics of Pi metabolism in non-irradiated cultured cells of spinach

Cultured spinach cells are characterized by their rapid uptake of Pi from the medium. The results in Fig. 2 demonstrated that under the present conditions, Pi in the culture medium was completely exhausted within 2 h of the start of the incubation period. This is accompanied by a rapid migration of the Pi taken up into the vacuole; vacuolar Pi increased progressively and attained a plateau after 2 h (Fig. 3). Unlike other cultured cells [16, 19, 21, 30], no nucleotides such as ATP and NADP were detected. The possibility that this was induced by anaerobiosis of the cells was ruled out by an experiment where the time-course change in cytoplasmic and vacuolar pH of irradiated and non-irradiated cells were measured by NMR (see Experimental) over a 24 h period. The pH was maintained in the range of 7.0–7.2 in the cytoplasm and 6.2–6.4 in the vacuole (data not shown), indicating that the cells were adequately oxygenated [31].

Probable difference of radiosensitivity between the plasma membrane and the tonoplast

Several reports have been published on the effects that ionizing radiations exert on the physiology and

biochemistry of plant membrane systems e.g. changes in transport [32] and ATPase activity [10, 12] of the plasma membrane, electron transfer and lipid peroxidation of mitrochondrial membranes [33], and lipid synthesis by microsome fractions [5, 6]. In the case of the vacuolar membrane, the effects on ATPase activity [11, 13], transport [7, 8, 13] and its components [7, 8, 13] have been investigated. However, no attempts have been made to investigate differences in the radiosensitivity of these membranes. The results of the present investigations suggest a differential response to radiation of the plasma membrane and the tonoplast. Indications of the probable lower radiosensitivity of the plasma membrane are : (1) Pi transport across the plasma membrane from medium into cells was not greatly affected with doses up to 5.0 kGy (Fig. 2); (2) even with 7.5 kGy, the plasma membrane of irradiated cells preserved its integrity with regard to permeability as measured by the fluorescein diacetate technique. The probable lower radioresistivity (higher radiosensitivity) of the tonoplast is inferred from the results in Fig. 3 indicating that, compared with non-irradiated control cells, the content of Pi in the vacuole of irradiated cells was reduced markedly with 5 kGy which did not greatly affect Pi uptake through the plasma membrane (Fig. 2). Furthermore, Pi transport into the vacuole in the cells irradiated with 5.0 kGy was reduced by ca 40% of the control after 2 h (Fig. 4). These results do not contradict the findings of Kondoh *et al.* [13] who showed that, in the same cultured spinach cells irradiated with 1 and 5 kGy under *in vivo* conditions, radiation induced a decrease in the activities of membrane-bound ATPase and pyrophosphatase, a deterioration of membrane lipids and the disappearance of some protein species. It is, therefore, not unreasonable to consider that radiation brought about a greater impairment to the tonoplast than to plasma membrane.

Radiation-induced impairment of the tonoplast

In non-irradiated control cells, the content of vacuolar Pi reached a plateau after 2 h, whilst in irradiated cells this continued to increase linearly for 8 h to attain the level of the control, although with a decreased rate (Fig. 3). These observations suggest that the initial radiation damage to the tonoplast was successively repaired with time by the action of certain intracellular mechanism(s). Lester and Whitaker [12] found that for the plasma membrane of muskmelon fruit. ten days storage after 1 kGy irradiation resulted in a greater retention of total protein, a diminished decline in total phospholipids and in the phospholipids/protein ratio, and a maintenance of greater overall H^+-ATPase activity. However, these retention processes were unlikely to occur in cultured spinach cells during more short-span of 8 h used in the present experiment. If this is the case, the increase of Pi content should proceed exponentially. This occurred however only linearly. It is reasonable to consider that the damage to the tonoplast was not further amplified. Little is known about the repair mechanism(s) of plant membrane systems, and their elucidation must await further investigations.

Fate of Pi in cultured cells

The results in Fig. 1 show that Pi appeared in the vacuole in large amounts at the primary stage of culture, and thereafter decreased steadily without an increase of other phosphorus compounds. Where did this vacuolar Pi go? In cultured *Catharanthus roseus* cells, Brodelius and Vogel [19] reported that the content of vacuolar Pi was continuously decreased with time, and it was suggested that this anion was transported out of the vacuole into the cytoplasm. One possibility may be that Pi which occurred in the cytoplasm in an excess amount is, in order to alleviate its toxicity [34], sequestered in certain intracellular compartment(s) in the form of NMR-invisible compounds (e.g. calcium phosphate in mitochondria [19]). A similar question is posed by the results shown in Fig. 3, i.e. why did the vacuolar Pi content in irradiated cells continue to increase without a corresponding decrease in the cytoplasmic Pi content even under the conditions where Pi in the medium was completely taken up by the cells? Because the NMR spectra revealed neither the presence of other phosphorus compounds such as ATP and NADP which might serve as Pi sources, nor changes in the content of sugar phosphates (data not shown), other sources must be responsible for this increase of vacuolar Pi.

EXPERIMENTAL

Plant material

The cultured cells used in this study were a green strain of *Spinacia oleracea* cv. Hôyô which was established from the central region of the plumule in the hypocotyl [35]. The cells were cultured at 25° on a rotary shaker (130 rpm) in 100 ml Erlenmeyer flasks each containing 40 ml MS medium (pH 6.5) supplemented with 0.5 ppm NAA, 1.0 ppm 6-benzyladenine and 2% sucrose (medium A). They were continuously illuminated with white fluorescent light giving approximately 6000 lux. Transfers were carried out every seven days.

Examination of time course changes in phosphorus compounds

In experiment described in Fig. 2, 7-day-old cells in which the vacuolar Pi was exhausted were transferred to fresh medium A, and incubated under the culture conditions until taken for NMR analysis.

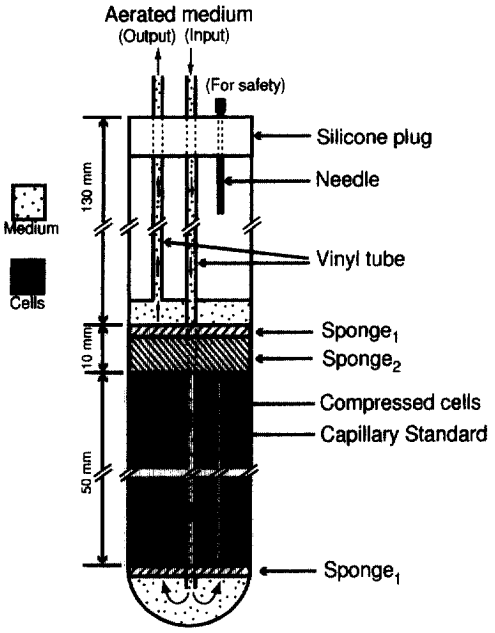

Fig. 5. Design of the continuous perfusion apparatus used for NMR measurements. Details: see Experimental.

Irradiation and subsequent treatments

Seven-day-old cells were irradiated in incubation flasks *in situ* at an average dose rate of 1.5 Gy s^{-1} for the specified time needed to give the desired total dose. The source of radiation was a Gamma cell 220 equipped with a ^{60}Co γ-ray source. Thereafter, the irradiated cells were instantly transferred to fresh medium A. In the experiments reported in Fig. 3, where time-dependent radiation effects were monitored, the irradiated cells were incubated under the culture conditions for the specified times.

^{31}P nuclear magnetic resonance

^{31}P NMR: JOEL EX 400 and Lambda 400 NMR spectrometers operating at 161.7 MHz. The spectra were measured at 2000 scans over 33 min using a 45° pulse angle at 25.0±0.1°. Measurements were carried out in the NMR sample tube illustrated in Fig. 5. The cells in one incubation flask were collected on a glass fibre pad (GC 90, $\phi = 4.5$ cm, Advantec, Tokyo) under weakly reduced pressure and the resultant slurry (vol. ca 4 ml) was quickly washed with 20 ml of medium B pH adjusted to 6.5 with MOPS (3-(*N*-morpholino)propanesulphonic acid) instead of KH_2PO_4 as in medium A [19, 36]. The cells were finally washed with 2 ml of medium C (medium B in 30% D_2O), resuspended in 10 ml of the same medium and transferred to a 10 mm-ϕ NMR tube. After standing for 2 min, the sedimented cells were loosely compressed between two sponge sheets to adjust the height of the cell mass cylinder to 5 cm ($sponge_1$ is hard and porous compared with $sponge_2$). These procedures were completed within 10 min. A glass capillary with 6% hexamethylphosphoric triamide (HMPT) [37] in D_2O was inserted into the cell mass as an ext. standard, and through the upper silicone plug, a needle was introduced in order to equilibrate the pressures between the upper air inside the tube to that of the atmosphere. During the measurements, medium C (200 ml) was circulated to the cells at a constant flow rate of 2.85 ml min^{-1} through input and output vinyl tubes (int. diameter = 1 mm) connected with a perfusion apparatus using a Peristac pump that was set outside the NMR. It was confirmed that perfusion at higher rates, e.g. 5.0 ml min^{-1}, had no effect on the spectra. The medium in this apparatus was constantly aerated at a rate of 40 ml s^{-1} using a second pump to maintain oxygen-saturation. Measurements were started 30 min after the medium had begun to flow. The chemical shift values (ppm) of NMR signals of various phosphorus compounds were referenced to that of 85% phosphoric acid (ppm = 0.0). The amounts of Pi in the cytoplasm and the vacuole were tentatively expressed as percent of the peak height to that of 6% HMPT as ext. standard; peak areas of these Pi were not accurately determined because of the overlapping of their signals.

Assay of Pi in the medium

An aliquot (100 μl) was taken from 40 ml of the medium at specified time after inoculation into fresh medium A, and added to a mixture consisting of 100 μl 2% sodium molybdate, 100 μl 1.5 N sulphuric acid and 400 μl isobutanol. After vigorous shaking followed by standing, 200 μl of the isobutanol layer was withdrawn and mixed with 200 μl 0.5% ascorbate in 0.05% $KHSO_4$ soln and 100 μl EtOH. The mixture was incubated at 37° for 1 h and Pi was assayed at 720 nm at room temp.

Examination of plasma membrane integrity

The integrity of the plasma membranes of cultured cells were examined with fluorescein diacetate (FDA) [27]. After the 7-day-old cells had been irradiated with 7.5 kGy and subsequently incubated in fresh medium A for 3 h, 1 ml of the culture suspension was added to 50 μl freshly prepared 0.5% Me_2CO soln of FDA. After standing for 5 min at room temp, the sample was photographed under a light microscope and the same field was then observed under a fluorescent microscope (Optiphoto-2, Nikon, Tokyo) using excitation and emission filters EX 450–490 and BA 520.

Chemicals

D_2O (purity 99.8%) was purchased from Merck, HMPT (hexamethylphosphoric triamide; purity 95%) from Wako and FDA (fluorescein diacetate; purity 98%) from Aldrich. All other reagents were of special grade.

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