The involvement of poly(ADP-ribose) polymerase in the oxidative stress responses in plants

Yehudit Amor[a], Elena Babiychuk[b], Dirk Inzé[b], Alex Levine[a,*]

[a] *Department of Plant Sciences, Institute of Life Sciences, The Hebrew University of Jerusalem, Jerusalem 91904, Israel*
[b] *Laboratory of Genetics, University of Ghent, K.L. Ledeganckstraat 35, 9000 Ghent, Belgium*

Received 13 August 1998; received in revised form 21 October 1998

Abstract **In plants many biotic and abiotic stresses can cause secondary oxidative stress. Earlier work showed that, depending on the severity of the oxidative stress, plants can activate either cell protective genes or programmed cell death (PCD). Poly-(ADP-ribose) polymerase (PARP) has been implicated as one of the enzymes in the apoptotic pathways induced by DNA damaging agents or oxidative stress. We show that in cultured soybean cells, PARP is involved in responses to mild and severe oxidative stresses, by mediating DNA repair and PCD processes, respectively. Addition of PARP inhibitors reduced the degree of cell death triggered by H_2O_2. Two windows of NAD consumption after H_2O_2 treatment were detected. Experiments with transient overexpression of *Arabidopsis* PARP cDNA promoted DNA repair and inhibited cell death caused by mild oxidative stress. However, following severe stress PARP overexpression increased cell death. Expression of antisense PARP produced the opposite effects: an increase in DNA nicks and inhibition of cell death at high, but not mild doses of H_2O_2.**

Key words: Poly(ADP-ribose) polymerase; Oxidative stress; Programmed cell death; Nicotinamide adenine dinucleotide; H_2O_2

1. Introduction

In natural environments plants are subjected to many environmental stresses which in turn can cause secondary oxidative stress [1]. In fact, in many cases the secondary oxidative stress is the major cause of the cellular damage during the altered environmental conditions. This is true for abiotic stresses such as cold stress [2,3], light stress [4], mechanical stress [5] and also for stresses caused by bacterial and fungal pathogens [6,7]. The excessive formation of reactive oxygen species (ROS) in response to the primary, environmental stress activates a signal transduction pathway that may be independent of or additive to the signals induced by the primary stress [8].

Oxidative burst constitutes one of the first plant responses to pathogen attack. We have shown previously that in soybean cells a high dose of H_2O_2 induces a programmed cell death (PCD) response [9]. In plants, PCD has been implicated in differentiation of tracheary elements, hypersensitive response to pathogens, normal root, leaf and embryo development [10]. A number of intrinsic (hormones, position) and extrinsic (pathogens, light) signals can initiate PCD, yet the variety of morphological and biochemical profiles of PCD in plants is so broad that it is not clear whether a single or multiple programs execute plant PCD [10,11].

Several groups have shown that differentiation of tracheary elements could be prevented by 3-aminobenzamide, nicotinamide and 6(5*H*)-phenanthridinone, substances that are considered rather specific inhibitors of poly(ADP-ribosyl)ation [12–14]. Poly(ADP-ribosyl)ation is a unique post-translational modification of nuclear proteins, which is strongly stimulated in cells following environmental insults [15]. NAD^+:protein-(ADP-ribosyl)transferase (polymerase) (ADPRT or PARP; EC 2.4.2.30) catalyzes poly(ADP-ribosyl)ation by covalent attachment of ADP-ribose from nicotinamide adenine dinucleotide (NAD^+) to glutamic acid residues in protein, followed by further transfer of ADP-ribose units onto the initial adduct to form poly(ADP-ribose) [16,17]. Poly(ADP-ribosyl)ation, as a type of secondary protein modification, is widely distributed among eukaryotes including animals, insects, fungi, plants, and dinoflagellates [18]. PARP is a nuclear protein with a bipartite nuclear localization signal [19] that is tightly bound to chromatin or nuclear matrix [18,20]. Low basal PARP activity is stimulated up to 100-fold following PARP binding to nicked DNA [21] and massive synthesis of poly(ADP-ribose) is part of the rapid stress response in mammalian cells toward generation of DNA strand interruptions by DNA damaging agents [15]. In vivo poly(ADP-ribose) is a short-lived polymer with a half-life of 1–2 min, being degraded by poly(ADP-ribose) glycohydrolase [15]. Although poly(ADP-ribosyl)ation of a number of nuclear proteins has been described [22–24], there is no evidence at present that poly-(ADP-ribose) synthesis on nuclear proteins, other than PARP, is of any physiological significance [18]. Many studies on the functional role of PARP, among them using PARP inhibitors, have indicated that it is associated with DNA repair and DNA replication [20,25]. However, PARP-deficient mice seem healthy and fertile, and therefore it was suggested that PARP plays no essential role in cell proliferation, differentiation or development, but controversial results were reported for the capacity of DNA repair and sensitivity to DNA damage in transgenic animals [26,27].

One of the consequences of PARP activation is depletion of NAD. It was hypothesized that a cause and effect relationship exists between PARP activation, loss of NAD, and cell death induced by oxygen radicals [28,29]. Therefore, at least in some animal cell types PARP may play a role in control of cell death through NAD depletion. The association of PARP with PCD is supported by the discovery that PARP is one of the targets of ICE-related proteases [30,31]. The generation of a separate zinc finger domain during apoptosis may be equivalent to the in vivo expression of the dominant-negative amino-terminal fragment of PARP [32,33], and might be ex-

*Corresponding author. Fax: (972) (2) 658 4425.
E-mail: alexl@leonardo.ls.huji.ac.il

PII: S0014-5793(98)01408-2

pected to enhance cell death in conjunction with DNA fragmentation.

The biology of PARP in plants is much less understood. Enzymatic activity was partially purified from maize seedlings [34,35]. The activity detected is most likely associated with the *zap* gene of maize PARP (E. Babiychik and D. Inzé, unpublished results). The ZAP (EMBL accession number AJ222589) protein is highly similar to the animal PARP gene as regards domain organization. A different shorter type of PARP (72–73 kDa) was cloned earlier from *Arabidopsis thaliana*, called APP [36] and recently its homologue was cloned from *Zea mays*, and called NAP (EMBL accession number AJ222588). Thus, in maize plants there are two PARP genes. The APP/NAP proteins do not possess a classical DNA binding domain composed of zinc fingers, yet they are localized in the nucleus and their activity is inducible by binding to nicked DNA, and inhibited by nicotinamide and 3-aminobenzamide (Babiychuk et al., in preparation). It has been suggested that in plants PARP activity may also have a role in the regulation of a major branch in secondary metabolism, the activation of phenylalanine ammonia lyase (PAL) in response to severe oxidative stress [37].

In the present study we have analyzed the possible involvement of PARP proteins in plant PCD, using cultured soybean cells. In this system PCD can be induced directly, through application of an exogenous oxidative stress [9], in a manner recently reported for *Arabidopsis* cell cultures [38]. We show that in soybean cells PCD is preceded by a drop in cellular NAD levels, indicating the activation of PARP. The PCD induced by a high dose of H_2O_2 was inhibited by PARP inhibitors and by ectopic expression of the *app* gene in the antisense orientation as opposed to the sense orientation. On the other hand, overexpression of the *app* gene in the sense orientation improved the survival of soybean cells following mild oxidative stress. The transformation experiments also affected the number of DNA nicks. In summary, our data are in favor of an important role for poly(ADP-ribose)polymerase(s) in the regulation of cell protective and PCD responses in plants.

2. Materials and methods

2.1. Cell cultures

Soybean cells, cultivar William's 82, were maintained in 250 ml flasks in Murashige and Schoog medium (Sigma) at 25°C. Cultures were maintained by transferring 6 ml of a 2 week old culture into 40 ml fresh medium. Two day old cells were used for all experiments. Inhibitors were dissolved in sterile water and added to culture medium. Cell death was assayed as described in [9].

2.2. NAD measurements

Cells were harvested by vacuum filtration and immediately lysed in 200 μl of 2% perchloric acid. Lysed cells were centrifuged at $20\,000\times g$ for 20 min and the supernatants analyzed by a cycling enzymatic reaction which measures NAD content [39]. The concentration was determined by standard curve fitting with NAD (Sigma). To determine the dry weight of cells, the samples were dried overnight at 80°C.

2.3. Plasmid constructs

For expression of the *app* cDNA in plant cells, in either sense or antisense orientation, the full-length *app* cDNA was excised from a pC3 clone [36] as a *Xho*I-*Bam*HI fragment, blunted with Klenow polymerase (NEB) and ligated into the *Sal*I site of pGSCDH35 [40]. In two resulting plasmids pSPA9 and pSPA10, the *app* cDNA was placed under the control of the 35S CaMV promoter in antisense and sense orientations, respectively. To combine the *app* expression cassettes with a cassette expressing the *Escherichia coli uidA* gene, to be used as gene-reported for data normalization, the bacterial GUS gene was inserted as a translational fusion with *app*. Finally, binary vectors were introduced into *Agrobacterium tumefaciens* by the freezing-thawing procedure.

2.4. Soybean cell transformation

Cells of recombinant *A. tumefaciens* were grown in LB for 40 h at 28.5°C. Prior to inoculation the bacteria were washed two times to remove the LB, resuspended in 10 mM $MgCl_2$ and added to 36 h old soybean cells at a concentration of 3×10^8 cells/ml. A second identical dose of agrobacteria was added to the suspension 24 h later. 48 h after the initial inoculation the agrobacteria were removed by extensive washing over Miracloth (CalBiochem) and cells were left quiet for 6 h prior to treatment with H_2O_2. A sample of the cells transformed with GUS was fixed and stained with X-gluc.

2.5. Western analysis

Total protein extracts from soybean culture cells were separated by SDS-PAGE and transferred to nitrocellulose (Millipore). The membrane was blocked for 30 min with 3% low fat milk in TBS and incubated overnight at 4°C in blocking solution containing anti-*Arabidopsis* APP at 1:1000 dilution. The anti-APP antibody was prepared in rabbits immunized with a bacterially produced carboxy-terminal part of the protein (Met^{310}-His^{637}). The second antibody was goat anti-rabbit conjugated to horseradish peroxidase. The PARP protein was detected by the ECL method (New England Biolabs).

2.6. Nucleus preparation and nick translation

Cells from 5 ml cultures were harvested with vacuum and immediately frozen in liquid nitrogen. Nuclei were prepared by grinding the cells with a mortar and pestle in order to keep the nuclei intact, as described in [41]. The frozen powder was transferred to 1 ml of buffer A (20 mM Tris, 0.25 M sucrose, 10 mM EDTA, 200 mM NaCl, 0.5% Triton X-100, 150 μM spermine and 0.5 mM spermidine), filtered through two layers of Miracloth (CalBiochem) and centrifuged for 30 s at 4°C, $1200\times g$. The nuclear pellet was washed three times in buffer A and two more times in buffer B (20 mM Tris, 100 mM NaCl, 1mM EDTA, 15 μM spermine and 50 μM spermidine). Nuclei were resuspended in nick translation buffer (New England Biolabs) with 2.5 μCi of [α-^{32}P]dCTP (3000 Ci/mmol, ICN) and 300 μM of other dNTPs in 50 μl reaction mixture. The reaction was initiated by addition of 1 unit of DNA polymerase (NEB, Klenow fragment). The reaction was stopped by adding 1 ml of cold 10% TCA incubated on ice for 5 min and centrifuged at $20\,000\times g$ for 10 min. The pellet was washed three times with cold 10% TCA to remove unincorporated label. Samples were counted in a Beckman scintillation counter.

3. Results

3.1. Inhibition of soybean PCD by PARP inhibitors

Earlier studies have shown that in cultured soybean cells oxidative stress activates the PCD response, which resembles apoptosis in animals [8]. Most importantly, H_2O_2 induces DNA fragmentation, i.e. double stranded DNA breaks, which were shown to act as activators of the PARP enzyme in animal systems [16,18]. As a first step to test the involvement of poly(ADP-ribose) polymerase in the PCD response we used two PARP inhibitors, 3-aminobenzamide (3-ABA) and nicotinamide [28,42]. Soybean cells were preincubated for 30 min with the inhibitors and then treated with 5 mM H_2O_2, a concentration that induced PCD in about 50% of cells [9]. Both PARP inhibitors blocked cell death induced by the oxidative stress, suggesting that PARP activity may play an important role in soybean PCD (Fig. 1A). The degree of protection depended on the dose of the inhibitor, in agreement with the competitive mode of action of these inhibitors, although additional factors cannot be excluded. The reduction in cell death by the inhibitors also indicates that the given concentration of H_2O_2 was not toxic on its own. These results were

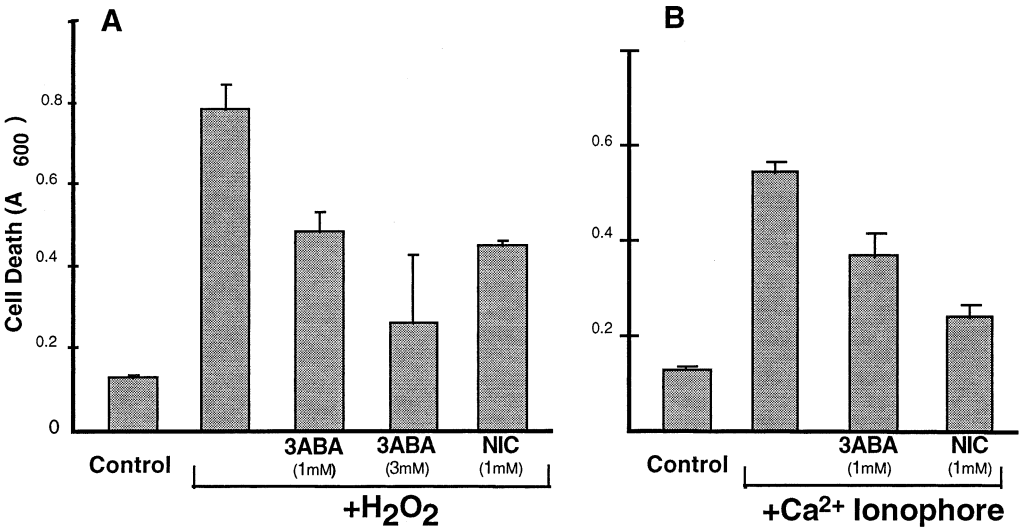

Fig. 1. The effect of PARP inhibitors on H_2O_2 induced cell death in soybean cells. Suspension cultured soybean cells were preincubated with the indicated concentrations of competitive PARP inhibitors and then challenged with 6 mM H_2O_2 (A) or 15 μM A23187 calcium ionophore (B). Cell death was measured 9 h later by staining with Evans blue as described in Section 2. 3ABA, 3-aminobenzamide; NIC, nicotinamide.

further corroborated by viability staining of cells for 1 week following H_2O_2 treatment and by plating the cells on agarose containing plates in a clonogenic assay (data not shown).

One of the earliest events following the addition of H_2O_2 to the cells is an influx of extracellular calcium. In fact, PCD can be induced by calcium ionophores in the absence of oxidative stress [8]. Both inhibitors also reduced the cell death triggered by the calcium ionophore (Fig. 1B), suggesting that PARP acts downstream of the calcium influx.

Previous studies have shown that PCD induced by H_2O_2 is completed within 8 h. To resolve the timing of PARP action we first estimated the critical window for PARP activity following oxidative stress by varying the time of 3-ABA addition. As can be seen in Fig. 2, the critical time for PARP activity is early in the process. Importantly, considerable protection (75%) was achieved when the inhibitor was added after the concentration of H_2O_2 returned to basal levels [9], excluding direct scavenging activity of 3-ABA as the cause of protection against oxidative stress. Assay of the 3-ABA scavenging capacity also did not detect any direct antioxidant activity of the inhibitor.

3.2. H_2O_2 causes depletion of NAD

To substantiate the inhibitor results, and to determine the timing of PARP activation more precisely, we measured the changes in the concentration of intracellular NAD, the substrate that is consumed by PARP activity [29]. Two periods of NAD depletion were seen (Fig. 3), which corresponds with the proposed roles of PARP in DNA repair and in PCD induction [18,28]. 30 min after H_2O_2 treatment NAD levels dropped to 15% of the initial value, returning to normal within 1 h. The second drop was less acute, beginning at 2 h and declining steadily from there on. The two phases are parallel with a rapid DNA damage by ROS [43] and the subsequent appearance of 50 kb DNA fragments several hours after H_2O_2 induced PCD in these cells [8].

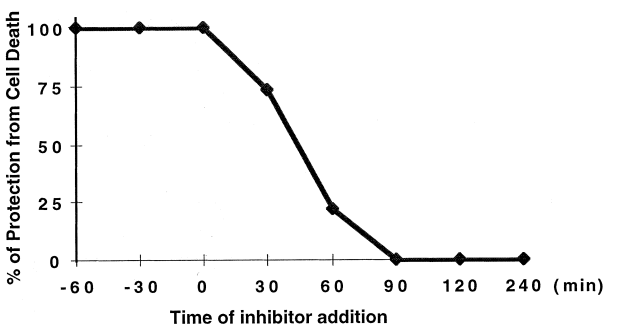

Fig. 2. The effect of the time of 3-ABA addition on soybean cell death. 3 mM 3-ABA was added at the indicated time (negative numbers indicate preincubation period) and the cells challenged with 6 mM H_2O_2. Cell death was measured 9 h later by staining with Evans blue. The results are expressed as percent of maximal protection achieved by 30 min preincubation in 3 mM 3-ABA.

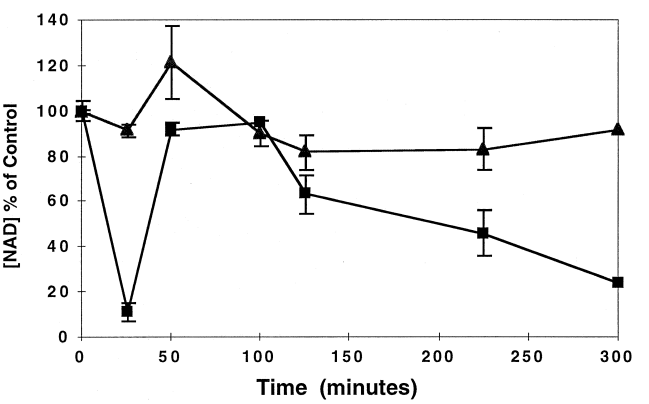

Fig. 3. Changes in NAD concentration in soybean cells after stimulation with H_2O_2. Cells were harvested at the indicated times and the total NAD content was measured in samples of 1 ml soybean cultures. The data are presented as percent of NAD concentration in untreated cells, which was around 100 μM/mg dry weight cells. ▲, untreated control cells; ■, 6 mM H_2O_2.

3.3. The effect of ectopic PARP expression on soybean PCD

In order to establish a more direct and causative link between the activity of PARP and the H_2O_2 induced PCD, we transformed soybean cells with a recombinant PARP gene, isolated from *A. thaliana* [36]. To manipulate the PARP mRNA levels in both directions the *Arabidopsis app* gene was introduced in sense and antisense orientations. The expression of mRNAs in both cases was driven by a constitutively expressed 35S CaMV promoter. Transformation efficiency was tested by introduction of the bacterial GUS gene fused to the *Arabidopsis app* gene. This construct contains 75% of the PARP coding N-terminal region followed by the bacterial GUS and allows detection of transformed cells by X-gluc staining and microscopic examination (Fig. 4A). Transformation was carried out by co-inoculation of the *A. tumefaciens* carrying the appropriate constructs with the cultured soybean cells for 48 h. After the period of co-cultivation the majority of *Agrobacterium* cells were removed by extensive washing and the soybean cells resuspended in the initial volume of fresh medium. The above procedure yielded $>60\%$ transformed cells, as can be observed from the number of blue cells after staining for β-glucuronidase activity of the GUS transformed cells (Fig. 4A). The effectiveness of the overexpression was further confirmed by Western blotting with antibodies raised against the *Arabidopsis* APP which cross-reacted with the soybean enzyme (Fig. 4B). Only traces of APP expression were detected in the removed co-inoculated *Agrobacterium* cells and no reaction was seen with the preimmune serum (not shown).

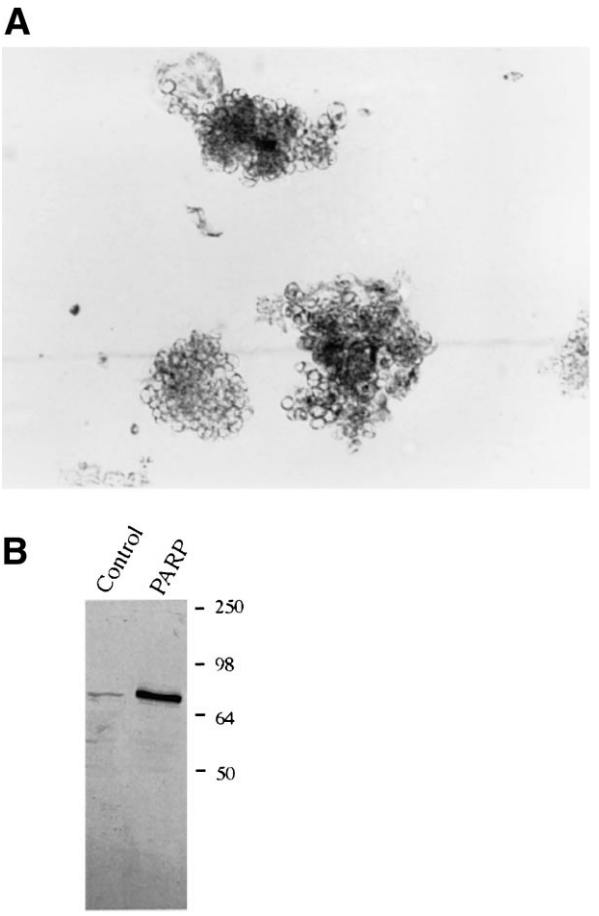

Fig. 4. Expression of *Arabidopsis app* gene in cultured soybean cells. Suspension cultured cells were transformed with *A. tumefaciens* carrying the *app* gene fused to the bacterial GUS gene cloned into a binary vector in the sense orientation. A: Cells were stained with X-gluc to visualize the transformation efficiency. B: Western blot analysis of APP expression in the transformed cells and probed with anti-APP antibodies. Control, untransformed cells; PARP, cells transformed with the sense *app* construct.

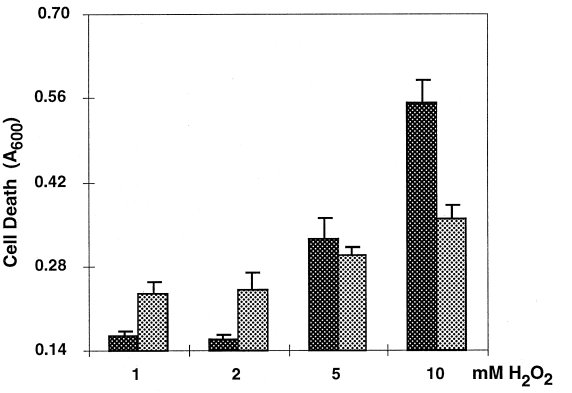

Fig. 5. The effect of PARP expression on H_2O_2 induced cell death in soybean. Suspension cultured soybean cells were transformed with sense or antisense *app* constructs. Following removal of agrobacteria the soybean cultures was challenged with the indicated concentrations of H_2O_2. Cell death was assayed 9 h later by Evans blue staining. Dark bars (on the left) represent cells transformed with the *app* construct inserted into the vector in the sense orientation and the light bars (on the right) represent the antisense *app* transformed cells.

Transformed cells were challenged with increasing concentrations of hydrogen peroxide to produce a mild and severe oxidative stresses. The concentrations of 1 mM and 2 mM H_2O_2 represent a mild oxidative stress that induced the cell protective responses, such as induction of glutathione *S*-transferase and glutathione peroxidase genes, with little cell death; concentrations above 5 mM H_2O_2 induce PCD rather than the cell protective genes [9]. In plants, a high H_2O_2 concentration is generated during the pathogenesis response by the cells in the vicinity of the pathogen [44], and lower levels of oxidative stress are found during chilling and light stress [45].

The effects of manipulations of PARP transcription through overexpression of sense or antisense constructs on the H_2O_2 induced cell death are presented in Fig. 5. Cell death was assayed 9 h after addition of H_2O_2 by staining with Evans blue and, in parallel, with propidium iodide, two different dyes that are excluded from living cells. The degree of cell death in the cultures transformed with the 'sense' construct and then treated with H_2O_2 showed a threshold between 2 and 5 mM H_2O_2. This was not observed in the antisense transformants, which showed a progressive increase in cell death. Most notably, when the 'sense' transformed cells were treated with a mild oxidative stress (1 or 2 mM H_2O_2), the degree of cell death was less than in the 'antisense' transformed cells, while after higher doses of H_2O_2 (5 and 10 mM), the degree of cell death was higher in the 'sense' transformed cells (Fig. 5).

3.4. The effect of ectopic PARP expression on DNA nicks

The mild and severe oxidative stresses that were applied here are expected to produce mild and severe DNA damage respectively. This was confirmed by analyzing the content of

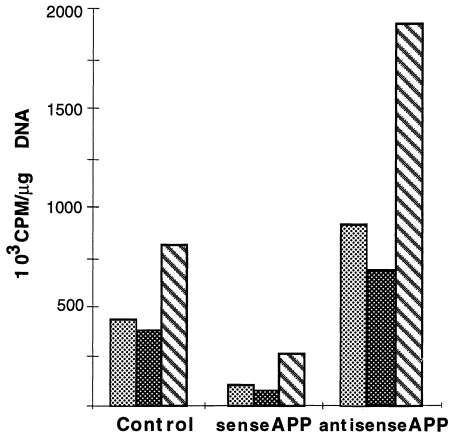

Fig. 6. The effect of PARP expression on DNA nicks. Soybean cells were transformed with sense and antisense *app* constructs. 120 min after stimulation of cells with water (light bars, on the left), with 2 mM H_2O_2 (dark bars, in the center), or with 10 mM H_2O_2 (hatched bars, on the right), cells were harvested and nuclei prepared as described in Section 2. The amount of nicks was estimated by nick translation in isolated nuclei and the incorporated label measured in a scintillation counter. The experiments were performed three times with similar results.

8-hydroxy-2-deoxyguanosine [46], following H_2O_2 treatment (unpublished results). To test the amount of DNA nicks we adapted the protocol of nick translation to perform the procedure in situ, in the nuclei [47] (Fig. 6). In this assay the amount of radioactivity incorporated into DNA is directly proportional to the number of nicks in the DNA [47]. The soybean cells were transformed with the *app* gene constructs as described above and cells were harvested 2 h after treatment with H_2O_2. As expected, the degree of DNA strand breaks was proportional to the severity of oxidative stress as seen in the untransformed (control) cells (Fig. 6). Overexpression of APP decreased the number of nicks in DNA following mild and severe oxidative stress (compare the black and hatched bars in the different transformants), while transformation with the antisense *app* gene increased the amount of DNA nicks. These results implicate plant PARP in DNA repair and PCD responses following oxidative stress.

4. Discussion

Coping with oxidative stress constitutes a substantial part of a plant life cycle. Plants are exposed to environmental stresses, which as a consequence generate secondary oxidative stress. The intensity of the secondary oxidative stress may vary from very mild to extreme, depending on the severity of the primary stress, resulting in a range of symptoms from null to visible tissue oxidation and necrosis. It was shown before that the cellular responses to oxidative stress directly depend on the severity of the stress: during mild stress plants activate the antioxidant responses, more severe stress activates the PCD pathway, while extreme stress causes necrosis [9,48]. In the cultured soybean system, because of the very short H_2O_2 half-life, concentrations of 1–2 mM mainly activate the antioxidant responses and concentrations of >5 mM induce PCD [9]. It should also be noted that Legendre et al. calculated that the concentration of H_2O_2 in this system can reach millimolar levels during the pathogenesis response, comparable to mammalian phagocytes [44].

A major event in the execution of PCD is the activation of endonucleases which in many cell types leads to characteristic 50 kb DNA fragments, followed later by a different set of endonucleases to the production of oligonucleosomal length DNA fragments [49,50]. The first type of cleavage is thought to be the result of the release of chromatin loops and is observed in almost all cases of apoptosis, the subsequent nucleosomal laddering occurs less often and is not essential for apoptosis [51,52]. We showed earlier that oxidative stress produced DNA fragmentation in soybean cell cultures [8]. Thus, DNA damage induced by H_2O_2 dependent formation of DNA adducts results, first, in the formation of nicks and strand breaks, and later as a result of the endonucleases [53], it produces additional DNA ends.

The result of ROS dependent DNA adducts and strand breaks is an immediate response that activates PARP, which attaches to the strand breaks and synthesizes short polymers of poly(ADP-ribose). The rapid synthesis coupled with subsequent degradation of the poly(ADP-ribose) chains creates a window for DNA repair [18]. The involvement of PARP in the early steps of PCD induced by H_2O_2 is supported by our results shown in Figs. 1 and 2. The two phases of NAD depletion (Fig. 3) probably reflect the activation of PARP by damaged DNA. The first drop may reflect the involvement of PARP in the DNA repair machinery which is induced by ROS dependent DNA damage, while the second drop may be the result of DNA fragmentation by PCD induced endonuclease activation [53]. These results further support the connection between H_2O_2 production and the activation of PARP.

Recently, using PARP deficient mice it was found that PARP activity was crucial for ROS dependent cell death in islet cells [29]. Nevertheless, despite extensive research in animal systems, the precise role of PARP in apoptosis is still not known. PARP is a major target of the apoptosis regulating cysteine proteases. The cleavage of PARP at the internal site ($DEVD^{216}$-G^{217}) during apoptosis has been described in a large number of animal systems and is induced by diverse stimuli, including oxidative stress [54]. Western analysis with antibodies raised against the *Arabidopsis* APP or the human protein did not detect cleavage of the soybean PARP or the *Arabidopsis* transgene following treatment with 5–10 mM H_2O_2, conditions which induce 40–70% cell death respectively in this system. To our knowledge cleavage of PARP in plants has not been described. Plants may possess more than one gene for PARP and therefore antibodies raised against the 72 kDa isoform did not recognize the larger PARP. The fact that the homology between the two genes is not high can be deduced from the result that no additional bands were seen on genomic Southern blots of *Arabidopsis* (data not shown).

The transformation experiments with the sense and antisense *app* constructs described here allowed us to probe more directly the role of the plant PARP enzyme during stress. In general, the results of PCD inhibition by antisense PARP expression (Fig. 5) are in agreement with the inhibitor data (Fig. 1), though these treatments reduce the activity of PARP by completely different mechanisms. Contrary to many types of animal cells, where PARP protein comprises one of the major nuclear proteins [18], the abundance of PARP in plant cells is rather limited ([36] and see our own results in Fig. 4). Our results show that the PARP enzyme activity has

the potential to promote repair of DNA nicks that arise from oxidative stress induced DNA damage. This finding is consistent with the role of PARP in the repair of γ-ray or alkylating agent induced DNA damage in mice [18,27,55]. It is particularly efficient in coping with minor DNA damage, which is caused by mild oxidative stress accompanying various environmental stresses, such as salt, drought or chilling [1,3,56]. The fate of oxidatively damaged cells will therefore depend on the severity of the initial stress, and on the efficiency of the repair machinery. Following mild stresses, where damage can be repaired, the DNA nicks recruit PARP for the process of DNA repair, resulting in cell recovery, while after severe stresses, which cause irreparable damage, PARP activity would be directed towards apoptosis, probably through NAD depletion ([29] and our results in Fig. 3). It should be noted that our results do not differentiate between the activities of the specific PARP isozymes. It is possible that in plants the specific isozymes have different expression patterns or function in different signaling pathways. For example, one of the genes may act in the PCD pathway, while the other acts in the regulation of PAL [37].

It has been hypothesized by Berglund and co-workers that nicotinamide, the product of PARP activity, is a stress signaling molecule in the induction of cell protective responses [57]. This hypothesis is based mainly on the observed rise in nicotinamide following exposure to various stresses and on the ability of exogenously supplied nicotinamide to induce a number of general plant stress associated genes, such as PAL and glutathione reductase [37]. Among the stresses that particularly induced the nicotinamide level in plants are UV radiation and oxidative stress, both stresses that can directly damage DNA [57]. In the soybean system addition of H_2O_2 at a concentration that induced PCD did not induce the cell protective genes, such as PAL or GST [9]. This signaling mechanism may, however, be species specific, since in *Arabidopsis* cell cultures the PCD inducing concentrations of H_2O_2 induced both PAL and GST gene expression [38]. Thus, the question whether nicotinamide is the signal or the by-product of stress response to external environmental conditions still remains.

Our results support the role for plant PARP in the regulation of the DNA repair and PCD processes. Interestingly, the same enzyme seems to be involved in regulating these two opposing processes, converting quantitative input (the amount of stress) into qualitative output of life (DNA damage repair) or death (induction of PCD). Minor DNA damage caused by mild oxidative stress would be repaired, while higher levels of DNA damage, resulting from severe environmental stresses or following attack by avirulent pathogens, could super-activate the PARP enzyme, resulting in NAD depletion and activation of PCD [29]. Since PARP activation does not differentiate between the different causes of DNA damage and strand breaks it will translate the internal signal irrespective of the type of stress. Our results provide evidence that the plant PARP has an important role in signal transduction of oxidative stress. Further work will address the mechanisms of how PARP transmits these signals at teh molecular level.

Acknowledgements: We thank M. Chevion from the Hadassah Medical School, Jerusalem for performing the 8-hydroxy-2-deoxyguanosine measurements. This research was supported by a grant from the Binational (US-Israel) Science Foundation.

References

[1] Hippeli, S. and Elstner, E.F. (1996) J. Plant Physiol. 148, 249–257.
[2] O'Kane, D., Gill, V., Boyd, P. and Burdon, R. (1996) Planta 198, 371–377.
[3] Prasad, T.K. (1996) Plant J. 10, 1017–1026.
[4] Foyer, C.H., Lelandais, M. and Kunert, K.J. (1994) Physiol. Plant. 92, 696–717.
[5] Yahraus, T., Chandra, S., Legendre, L. and Low, P.S. (1995) Plant Physiol. 109, 1259–1266.
[6] Hammond-Kosack, K.E. and Jones, J.D.G. (1996) Plant Cell 8, 1773–1791.
[7] Doke, N., Miura, Y., Sanchez, L.M., Park, H.J., Noritake, T., Yoshioka, H. and Kawakita, K. (1996) Gene 179, 45–51.
[8] Levine, A., Pennell, R., Alvarez, M., Palmer, R. and Lamb, C.J. (1996) Curr. Biol. 6, 427–437.
[9] Levine, A., Tenhaken, R., Dixon, R. and Lamb, C. (1994) Cell 79, 583–593.
[10] Jones, A.M. and Dangl, J.L. (1996) Trends Plant Sci. 1, 114–119.
[11] Mittler, R. and Lam, E. (1996) Trends Microbiol. 4, 10–15.
[12] Rankin, P.W., Jacobson, E.L., Benjamin, R.C., Moss, J. and Jacobson, M.K. (1989) J. Biol. Chem. 264, 4312–4317.
[13] Tanaka, Y., Yoshihara, K., Tsuyuki, M. and Kamiya, T. (1994) Exp. Cell Res. 213, 242–252.
[14] Tanaka, Y., Yoshihara, K., Tohno, Y., Kojima, K., Kameoka, M. and Kamiya, T. (1995) Cell Mol. Biol. 41, 771–781.
[15] Alvarez-Gonzalez, R. and Althaus, F.R. (1989) Mutat. Res. 218, 67–74.
[16] de Murcia, G., Schreiber, V., Molinete, M., Saulier, B., Poch, O., Masson, M., Niedergang, C. and Ménissier-de Murcia, J. (1994) Mol. Cell Biochem. 138, 15–24.
[17] Marsischky, G.T., Wilson, B.A. and Collier, R.J. (1995) J. Biol. Chem. 270, 3247–3254.
[18] Lindahl, T., Satoh, M.S., Poirier, G.G. and Klungland, A. (1995) Trends Biochem. Sci. 20, 405–411.
[19] Schreiber, V., Molinete, M., Boeuf, H., de Murcia, G. and Ménissier-de Murcia, J. (1992) EMBO J. 11, 3263–3269.
[20] de Murcia, G. and Ménissier-de Murcia, J. (1994) Trends Biochem. Sci. 19, 172–176.
[21] Ikejima, M., Noguchi, S., Yamashita, R., Ogura, T., Sugimura, T., Gill, D.M. and Miwa, M. (1989) J. Biol. Chem. 28, 2034–2040.
[22] Krupitza, G. and Cerutti, P. (1989) Biochemistry 28, 4054–4060.
[23] Krupitza, G. and Cerutti, P. (1989) Biochemistry 28, 2034–2040.
[24] Scovassi, A.I., Mariani, C., Negroni, M., Negri, C. and Bertazzoni, U. (1993) Exp. Cell Res. 206, 177–181.
[25] Griffin, R.J., Curtin, N.J., Newell, D.R., Golding, B.T., Durkacz, B.W. and Calvert, A.H. (1995) Biochimie 77, 408–422.
[26] Wang, Z.Q., Auer, B., Stingl, L., Berghammer, H., Haidacher, D., Schweiger, M. and Wagner, E.F. (1995) Genes Dev. 9, 509–520.
[27] de Murcia, J.M. et al. (1997) Proc. Natl. Acad. Sci. USA 94, 7303–7307.
[28] Nosseri, C., Coppola, S. and Ghibelli, L. (1994) Exp. Cell Res. 212, 367–373.
[29] Heller, B., Wang, Z.Q., Wagner, E.F., Radons, J., Burkle, A., Fehsel, K., Burkart, V. and Kolb, H. (1995) J. Biol. Chem. 270, 11176–11180.
[30] Lazebnik, Y.A., Kaufmann, S.H., Desnoyers, S., Poirier, G.G. and Earnshaw, W.C. (1994) Nature 371, 346–347.
[31] MacFarlane, M., Cain, K., Sun, X.M., Alnemri, E.S. and Cohen, G.M. (1997) J. Cell Biol. 137, 469–479.
[32] Molinete, M., Vermeulen, W., Burkle, A., Ménissier-de Murcia, J., Kupper, J.H., Hoeijmakers, J.H. and de Murcia, G. (1993) EMBO J. 12, 2109–2117.
[33] Schreiber, V., Hunting, D., Trucco, C., Gowans, B., Grunwald, D., De Murcia, G. and De Murcia, J.M. (1995) Proc. Natl. Acad. Sci. USA 92, 4753–4757.
[34] Chen, Y.M., Shall, S. and O'Farrell, M. (1994) Eur. J. Biochem. 224, 135–142.
[35] O'Farrell, M. (1995) Biochimie 77, 486–491.
[36] Lepiniec, L., Babiychuk, E., Kushnir, S., Van Montagu, M. and Inzé, D. (1995) FEBS Lett. 364, 103–108.

[37] Berglund, T., Kalbin, G., Strid, A., Rydstrom, J. and Ohlsson, A.B. (1996) FEBS Lett. 380, 188–193.
[38] Desikan, R., Reynolds, A., Hancock, J.T. and Neill, S.J. (1998) Biochem. J. 330, 115–120.
[39] Bernofsky, C. and Swan, M. (1973) Anal. Biochem. 53, 452–458.
[40] Babiychuk, E., Kushnir, S., Belles-Boix, E., Van Montagu, M. and Inzé, D. (1995) J. Biol. Chem. 270, 26224–26231.
[41] Guidet, F., Rogowsky, P. and Langridge, P. (1990) Nucleic Acids Res. 18, 4955.
[42] Coppola, S., Nosseri, C., Maresca, V. and Ghibelli, L. (1995) Exp. Cell Res. 221, 462–469.
[43] Halliwell, B. and Gutteridge, J.M.C. (1989) Clarendon Press, Oxford.
[44] Legendre, L., Rueter, S., Heinstein, P.F. and Low, P.S. (1993) Plant Physiol. 102, 233–240.
[45] Prasad, T.K., Anderson, M.D., Martin, B.A. and Stewart, C.R. (1994) Plant Cell 6, 65–74.
[46] Wagner, J.R., Hu, C.C. and Ames, B.N. (1992) Proc. Natl. Acad. Sci. USA 89, 3380–3384.
[47] Wood, R.D., Biggerstaff, M. and Shivji, M.K.K. (1995) Methods 7, 163–175.
[48] Willekens, H., Chamnongpol, S., Davey, M., Schraudner, M., Langebartels, C., Van Montagu, M., Inzé, D. and Van Camp, V. (1997) EMBO J. 16, 4806–4816.
[49] Pandey, S., Walker, P.R. and Sikorska, M. (1994) Biochem. Cell Biol. 72, 625–629.
[50] Walker, P.R., Weaver, V.M., Lach, B., LeBlanc, J. and Sikorska, M. (1994) Exp. Cell Res. 213, 100–106.
[51] Oberhammer, F., Wilson, J.W., Dive, C., Morris, I.D., Hickman, J.A., Wakeling, A.E., Walker, P.R. and Sikorska, M. (1993) EMBO J. 12, 3679–3684.
[52] Oberhammer, F.A., Hochegger, K., Froschl, G., Tiefenbacher, R. and Pavelka, M. (1994) J. Cell Biol. 126, 827–837.
[53] Mittler, R. and Lam, E. (1995) Plant Cell 7, 1951–1962.
[54] McGowan, A.J., Ruiz-Ruiz, M.C., Gorman, A.M., Lopez-Rivas, A. and Cotter, T.G. (1996) FEBS Lett. 392, 299–303.
[55] Wang, Z.Q., Stingl, L., Morrison, C., Jantsch, M., Los, M., Schulze-Osthoff, K. and Wagner, E.F. (1997) Genes Dev. 11, 2347–2358.
[56] Hernandez, J.A., Corpas, F.J., Gomez, M., Del Rio, L.A. and Sevilla, F. (1993) Physiol. Plant. 89, 103–110.
[57] Berglund, T. (1994) FEBS Lett. 351, 145–149.