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Is *arcA3* a possible mediator in the signal transduction pathway during agonist cell cycle arrest by salicylic acid and UV irradiation?

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SUMMARY

Progression of BY-2 tobacco cells through the cell cycle was followed after treatments with ultra violet (UV) and salicylic acid (SA) used as a potent inhibitor of the octadecanoid pathway which can mediate response to UV irradiation. Cells in S phase were more sensitive than G_0/G_1 or G_2 cells to UV irradiation. Although SA efficiently blocked cells in G_0/G_1 or G_2, it did not block S phase synchronized cells. UV and SA applied simultaneously to cells in G_0/G_1 delayed the cell cycle progression more than each one separately. Therefore UV irradiation and SA act as agonists to arrest BY-2 cells at cell cycle entry.

To further investigate the signalling pathway mediating UV response, we complemented a UV-sensitive *Escherichia coli* strain with a *Nicotiana xanthi* cDNA expression library. A cDNA (*arcA*3) whose coding sequence is identical to the 2,4-D induced *arcA* cDNA cloned by Ishida et al. (1993) was isolated. We show that *arcA*3 transcription is induced at cell cycle entry but not directly by the 2,4-D treatment. Moreover, *arcA*3 transcription is induced prior to the restriction point as shown with the CDK inhibitor roscovitine. The *arcA*3 transcription level is increased by UV irradiation but prevented by SA. Indeed, addition of SA prior to UV irradiation blocks the induction of *arcA*3 transcription. This suggests that *arcA*3 gene is modulated in both UV and SA responses, the SA effect preceding the UV step. Since *arcA*3 is 67% similar to *RACK1* (functional homology), a rat intracellular receptor for protein kinase C, and possesses identical PKC fixation motifs, it is hypothesised that the *arcA*3 gene is involved in UV and SA cell cycle arrest.

Key words: *arcA*, Tobacco BY-2, Cell Cycle, *RACK1* homologue, Salicylic Acid, Ultraviolet irradiation

INTRODUCTION

The integrity of the genomes of all organisms is constantly at risk from various agents. Throughout evolution, ultraviolet light (UV) has had a profound influence on living organisms, and the repair of UV-induced DNA damage has been of vital importance (Friedberg et al., 1995). The most important toxic and mutagenic UV-photoproducts are the cyclobuthane pyrimidine dimers (CPDs) and (6-4)photoproducts (Friedberg et al., 1995). The response to UV light has been well studied in bacteria, yeast and in animal cell lines. Several strategies like photoreactivation, nucleotide excision repair and recombination repair have been identified in *E. coli* for removing these photoproducts (Yajima et al., 1995). Because most DNA replication in plants occurs in apical or secondary meristems which are usually shielded from the sun by many layers of tissues, it was supposed that recombinational repair may not be important with respect to UV radiation damage in plants (Stapleton, 1992). However, DNA repair is crucial for species survival in isolated plant cells directly exposed to UV, such as plant pollen (Jackson, 1987). Isolation of an *Arabidopsis* photolyase mutant hypersensitive to UV radiation demonstrates that CPD-removal is critical for survival in plants (Landry et al., 1997). Therefore, studies of UV irradiation-induced delay during cell cycle progression to allow DNA repair are pertinent.

After UV-irradiation two pathways for gene activation should be considered, one response which is initiated at or near the plasma membrane and another pathway elicited by damage induced in DNA. In the first pathway, the earliest detectable step is the activation of Src tyrosine kinases, followed by activation of Ha-Ras and Raf-1 in mammalian cells (Dewary et al., 1992; Bender et al., 1997) or the activation of lipases to cause the release of linoleic acid which engages the intracellular octadecanoid signal-transduction pathway in plants (Conconi et al., 1996). In the second pathway, the DNA-damage checkpoint is the mechanism that detects damaged DNA and generates a signal that arrests cells in the G_1 phase of the cell cycle, slows down DNA synthesis, arrests cells in the G_2 phase, and induces the transcription of repair genes. The position of arrest within the cell cycle varies depending upon the phase in which the damage is sensed as has been demonstrated in yeast (Elledge, 1996). UV irradiations are known to damage various plant processes (reviewed by

Stapleton, 1992) but until now plant cell cycle progression after UV irradiation has been little investigated.

Irradiation of tomato leaves with UV light induces the expression of several defense genes that are normally activated through the octadecanoid pathway after wounding. Salicylic acid (SA) which abolished wound and UV induction of proteinase inhibitor I and II (Conconi et al., 1996) is a potent inhibitor of the pathway (Pena-Cortez et al., 1993). In animals, aspirin and aspirin-like compounds have also been found to perturb cell-cell communication, such as platelet aggregation and neutrophil activation. This perturbation may result from interference with G protein-mediated signal transduction (for review see Weissman, 1991). Moreover, spontaneous background production of H_2O_2 is enhanced by SA treatment of parsley cells (Kauss and Jeblick, 1995). SA was considered to be an inhibitor for the metabolism of H_2O_2 by blocking catalase (Chen et al., 1995) and ascorbate peroxidase (APX) (Durner et al., 1997). However, it was then reported that SA did not inhibit catalase or APX in soybean (Tenhaken and Rübel, 1997). It is known that the SA concentration required for particular responses is much higher in a hypersensitive reaction and conditioning than in systemic acquired resistance (Tenhaken and Rübel, 1997). Yet the molecular basis for the function of SA is largely unknown and although its antiproliferative properties on tumor cell lines have been defined (Rüschoff et al., 1998), it is little documented in plant cells.

In this report we study the cell cycle progression of a tobacco BY-2 cell suspension following UV irradiation. This cell suspension, obtained by Nagata et al. (1992), is mainly composed of cells with a 2C DNA content in stationary phase, is easily synchronized (Planchais et al., 1997), has few clumps, and represents an ideal tool for homogeneous UV irradiation. Arguments for using UV-C are based on the assumption that UV-C causes the accumulation in the DNA of the same photoproducts as UV-B but at a higher frequency (Conconi et al., 1996). At 254 nm, the UV-C range (below 280 nm) activates a set of genes that are also induced by UV-A (above 315 nm) and UV-B (310-315 nm) irradiations (reviewed by Cerutti, 1985). Then, to determine if the inhibitor of the octadecanoid pathway induced by UV could interfere in cell cycle progression, the cells were treated with SA. By complementation of a photoreactivation, nucleotide excision repair and recombination repair deficient *E. coli* strain with a tobacco expression cDNA library, a cDNA highly similar to *arcA* was isolated. The *arcA* cDNA was initially identified as an auxin-regulated gene from the tobacco BY-2 cell line (Ishida et al., 1993). Structural analysis revealed that the *arcA*(s) gene product(s) are members of an extended family of proteins with WD-repeats, typified by the β subunit of heterotrimeric G proteins homologous to the rat RACK1 intracellular receptor for protein kinase C (Kwak et al., 1997). Since recent results suggest that protein kinase C may be a conserved regulator of cell cycle events that links signal transduction pathways and cell-cycle machinery (Livneh and Fishman, 1997), we studied *arcA3* transcription levels under UV irradiation and SA treatment.

MATERIALS AND METHODS

BY-2 cell culture and its handling

The tobacco BY-2 cell suspension was grown according to the method of Nagata et al. (1992). 10 ml refreshed cells (diluted 1:40 from the 8 day-old cells at stationary phase) were immediately irradiated for different doses under a 254 nm UV-C lamp in uncovered 10 cm Petri dishes (depth of culture: 2 mm) in culture medium. Cells, strictly kept in the dark, were poured into 50 ml Erlenmeyer flasks for further growth. For S cell synchronization, 4 ml of stationary phase cells were transferred to 40 ml fresh medium composed of 4.33 g/l Murashige & Skoog (M-5524 Sigma), 3% sucrose, 200 mg/l KH_2PO_4, 1 mg/l thiamine, 100 mg/l myo-inositol, supplemented with 1 μM 2,4-D (Sigma) and containing aphidicolin 2 μg/ml (Sigma) prepared in pure dimethyl sulfoxide. The inhibitor was removed from the culture by centrifugation (5 minutes at 2000 rpm (Jouan B3.11) without brake during the deceleration step). The pelleted cells were washed twice in the same volume of culture medium. Finally, the cells were resuspended in the same culture volume of fresh medium. G_2 synchronized cells were obtained 6 hours after S synchronization without a second inhibitor.

Chemicals

Aliquots from a 1 M stock solution of SA were added for conditioning of the cultures immediately after cells were refreshed. Cell treatments were routinely performed with 50 ml batches.

Roscovitine (2-(1-ethyl, 2-hydroxyethylamino)-6-benzylamino-9-isopropylpurine, provided by L. Meijer, Roscoff, France) at 50 μM final concentration and 2 μM staurosporin (Sigma, stocks in dry DMSO) were administered for 24 hours to cell cultures.

Nuclei isolation and cytometric analysis

Nuclei were released from the cells according to the method of Planchais et al. (1997). The samples were successively filtered through nylon membranes of pore size, 25 μm and then 10 μm. Cytometric analysis was performed on 2×10^4 nuclei with a Vantage flow cytometer (Becton Dickinson). The BrdU/HO/PI biparametric method (Ormerod and Kubbies, 1992) was used to follow the S phase engagement of BY-2 cells after different UV doses. Freshly prepared bromodeoxyuridine (BrdU, Sigma, France) was added to 30 μM final concentration for 24 hours immediately after UV treatment of refreshed cells. To detect BrdU incorporation, the nuclei were stained for 15 minutes with 1 μg/ml Hoechst 33258 (HO, Sigma) after 10 μg/ml RNase treatment. Finally, 3 μg/ml propidium iodide (PI, Sigma) was added for a further 15 minutes. In the biparametric analysis described by Glab et al. (1994), nuclei were excited with UV light (351-364 nm) and a bivariate cytogram of red (PI >610 nm) vs blue (408 nm <HO <500 nm) fluorescence was recorded. Care was taken to eliminate both debris and doublets through light scatter and pulse shape analysis.

Northern blot analysis

Total RNA was extracted by grinding the cells in liquid nitrogen in the presence of TRIzol Reagent (Gibco/BRL) according to the manufacturer's instructions.

After electrophoresis in a 1% agarose gel and blotting onto Hybond N^+ membrane (Appligene), hybridization was carried out with ^{32}P-labelled probes corresponding to the coding regions of the *arcA3*, *Arabidopsis* histone *H4* (Chabouté et al, 1987), *Nicta;CycB1;1* (Qin et al., 1996) and elongation factor EF-1 alpha (Axelos et al., 1989) genes at 63°C in a buffer described by Church and Gilbert (1984).

Complementation of the UV-sensitive *E. coli* strain SY2

We followed the previously reported method for isolation of a photolyase gene by complementation in SY2 *E. coli* cells (Yasuhira and Yasui, 1992) with modifications. The pBluescript II phagemids obtained from the cDNA library of *Nicotiana tabacum L. cv. Xanthi* (Galvez et al., 1996) were mixed with XL1-Blue host cells and plated to recover the complete cDNA library in the pSK^- plasmids. The cDNA library was introduced by electroporation into *E. coli* SY2 cells (JM 107 Δ*phr::Cm*^{r} Δ*uvrA::Km*^{r} Δ*recA:: Tet*^{r}). 100 μl of an overnight culture of the transformed bacteria was plated on LB supplemented

with ampicillin (50 µg/ml), kanamycin (50 µg/ml), chloramphenicol (10 µg/ml) and tetracyclin (12.5 µg/ml) prior to UV-C irradiation (0.2 J/m^2 254 nm) followed by illumination with visible light for 5 minutes. 24 hours later, living cells were collected in LB plated again on LB plus antibiotics and subjected to a further round of UV-C irradiation. After three rounds of selection, 28 colonies remained and were tested for their UV-C resistance. Isolated cDNAs were sequenced automatically using an Applied Biosystems kit for dye primer cycle sequencing.

RESULTS

Sensitivity of BY-2 cells to UV

An 8-day cell suspension was refreshed (1 ml/40 ml), immediately submitted to different doses of UV-C radiation (152 J/m^2 to 1824 J/m^2) in 10 ml Petri dishes and then cultivated in the dark, according to the method of Nagata et al. (1992). After 8 days 5 ml of cell suspension were centrifuged

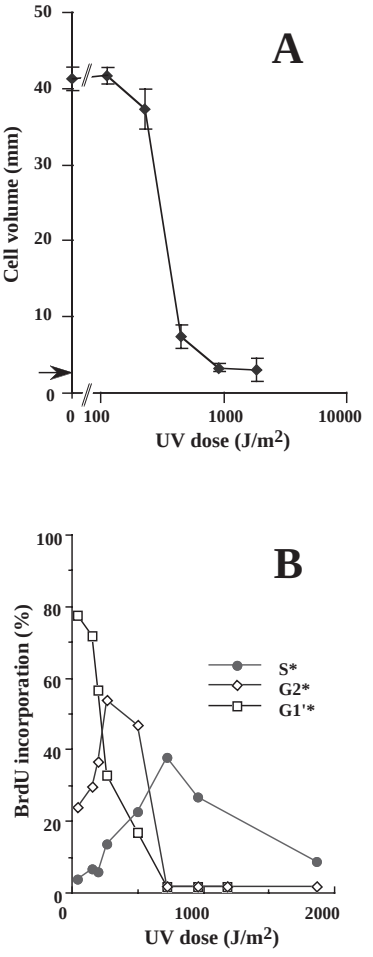

Fig. 1. BY-2 cell suspension growth and cell cycle entry under UV irradiation. (A) UV effect on the cell suspension growth. Increasing UV doses were applied to a 7-day cell suspension immediately after it was refreshed. The volume of the cells was measured after 7 days from centrifugation in 5 ml tubes. Values corresponded to the height (mm) of the pellet of cells. Arrow corresponds to the volume of the cells at 0 hours. X axis: log scale. (B) Progression of UV irradiated cells in the different cycle phases. A stationary suspension was UV irradiated immediately after it was refreshed. BrdU was added for 24 hours. HO/PI staining of nuclei and cytometric analysis allow to separate the resting cells G_1 and G_2 from the cells which have incorporated BrdU: S*; G_2* and from the cells of the next generation G_1'*.

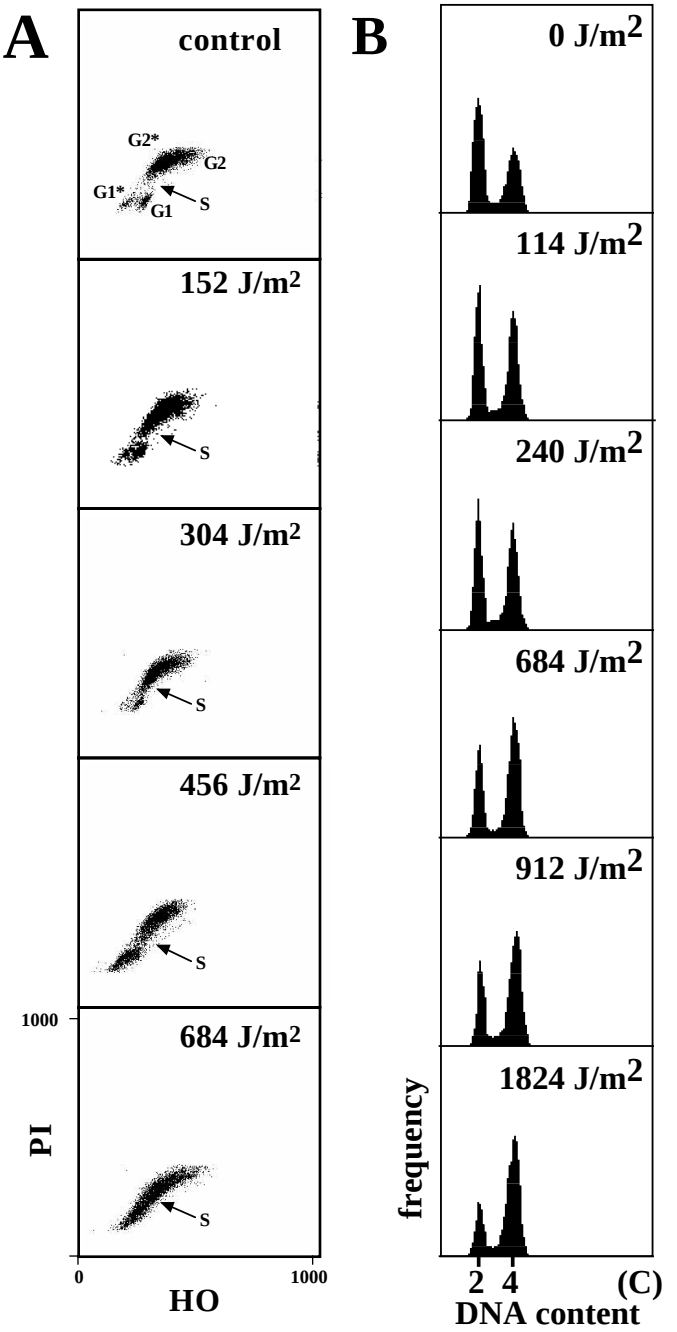

Fig. 2. Cell cycle progression after UV irradiation of cells synchronized at S or G_2 phases. (A) HO/PI cytograms of BY-2 cells UV irradiated after synchronisation of cells at S phase. Cells, aphidicolin synchronized, were washed twice, resuspended in fresh medium and UV irradiated before BrdU addition during 16 hours. (B) DNA content of BY-2 cells UV irradiated at G_2 phase. Cells, aphidicolin synchronized, were twice washed, cultivated for 8 hours before UV irradiation at different doses. BrdU was added for 16 hours before HO/PI staining of the nuclei. Nuclear DNA distribution is represented by the projection of HO values on the *x*-axis.

and the packed cell volume measured (Fig. 1A). For doses above 1 kJ/m^2 the cell volume barely increased, indicating that growth was arrested.

The BrdU/HO/PI technique is an efficient tool to study the time course of cell division following mitogenic stimulation within the time interval between the beginning of BrdU incorporation and cell harvest (Ormerod and Kubbies, 1992) since the activity/inactivity of subpopulations may be identified. 24 hours after UV irradiation plus BrdU treatment, the amount of cells able to reach the G_1 phase of the next generation G_1'* (the asterisk indicating BrdU incorporation) decreased for cells exposed at or above 152 J/m^2 (Fig. 1B). Simultaneously, the amount of G_2* BrdU labelled cells increased until the UV dose of 456 J/m^2. For doses higher than 456 J/m^2, G_1'* and G_2* were not observed while S* increased until the dose of 684 J/m^2. Therefore, when cells in stationary phase were irradiated, doses higher than 912 J/m^2 were required to block S phase entry.

To measure the sensitivity of the different cell cycle phases, BY-2 cells were synchronized with aphidicolin to obtain S phase cells as previously described (Planchais et al., 1997), washed and then irradiated prior to BrdU addition. After 20 hours and HO/PI staining, the cell cycle progression of the cells was analysed (Fig. 2A). During that period, while control cells that were not UV irradiated had reached the G_2* phase, a 684 J/m^2 UV dose prevented the progression of S cells towards G_2. To obtain G_2 cells, aphidicolin synchronized cells were washed and cultivated for 8 hours as previously described (Tréhin et al., 1997). BrdU was then added to the medium just after irradiation. In this experiment, cells went back to G_1' before they incorporated BrdU. After 16 hours, few S* cells were observed in the cultures up to the UV dose of 114 J/m^2. Therefore the progression through G_2 to G_1' was analysed from monoparametric DNA histograms. Higher UV doses blocked the passage of cells from G_2 to the G_1' phase of the next generation. However, the highest 1824 J/m^2 UV dose did not block all the cells in the G_2 phase (Fig. 2B).

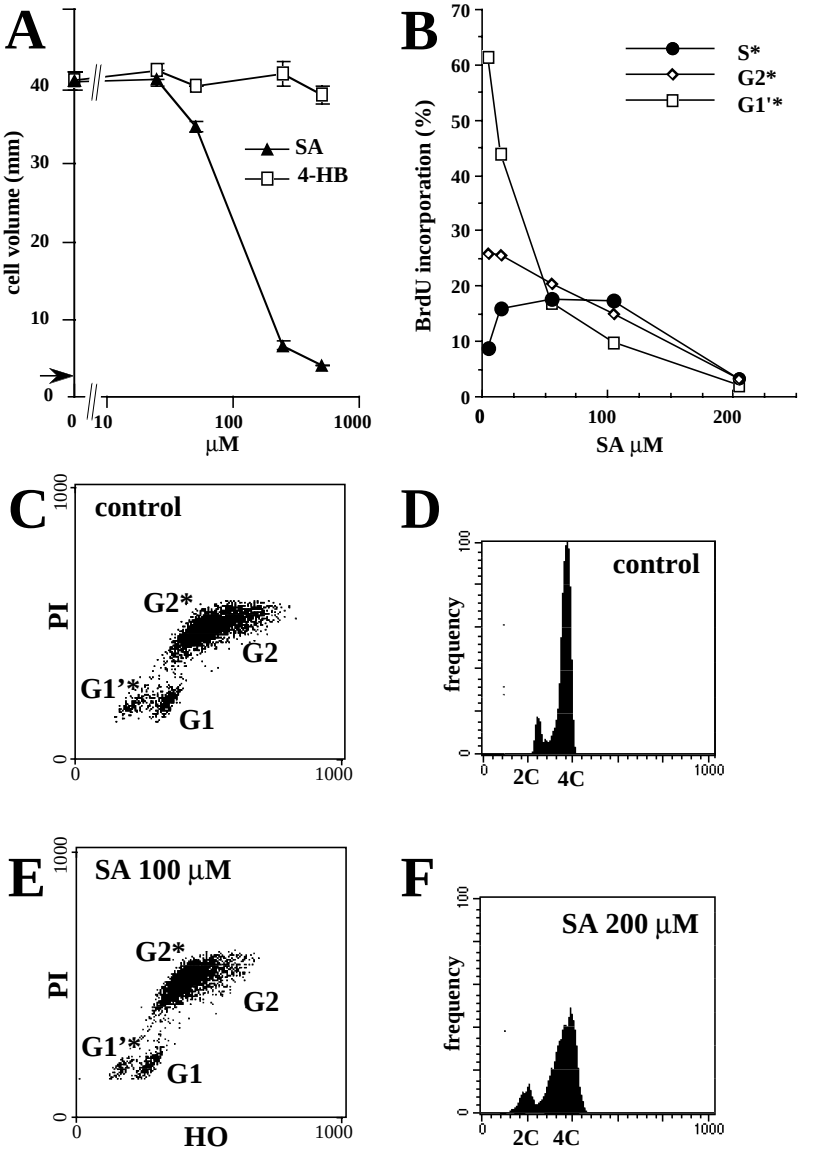

Fig. 3. Sensitivity of BY-2 cell supension to salycilic acid treatments. (A) Different SA and 4-hydroxybenzoic acid (4-HB) concentrations were applied for 8 days to refreshed cells. The curves correspond to two different experiments. The volume of the cells was measured after 7 days from centrifugation of cells in 5 ml tubes. Arrow corresponds to the volume of the cells at 0 hours. *x*-axis (log scale) SA and 4-HB in μM. (B,C,D,E,F) Cell cycle progression of cells treated with SA, respectively, from cell suspension at stationary phase (BrdU was added for 24 hours), S phase cells synchronized with aphidicolin (BrdU was added for 16 hours) and G_2 cell, synchronized with aphidicolin, washed, then cultivated for 8 hours before BrdU was added for 16 hours (G_2).

BY-2 cell cycle progression is stopped by salicylic acid

An 8-day cell suspension was refreshed (1 ml/40 ml) and then aliquots were cultivated in the presence of different concentrations of salicylic acid or an inactive analogue, 4-hydroxybenzoic acid (4-HB), for a further 8 day period. Increasing SA decreased growth; 200 μM SA virtually blocked growth (Fig. 3A). At 1 mM the cell growth was totally inhibited (results not shown). The 4-HB analogue had no effect in the same concentration range. At 1 mM, however, a slight decrease of the cell volume was observed.

To determine precisely the sensitivity of the different cell cycle phases to salicylic acid, cell cycle progression was checked in the presence of increasing doses of SA after 24 hours BrdU incorporation (Fig. 3B). 20 μM SA clearly decreased the G_1'* population. At 100 μM, few cells were observed that had reached the next generation. However, at this SA concentration, cells were always engaged in S* and G_2* phases. 200 μM SA prevented the cells from entering S phase.

When an aphidicolin synchronized cell suspension was supplemented either with 100 μM or 200 μM SA, cells were able to reach the G_2 phase and, moreover, some cells reach the G_1'* phase (Fig. 3E). At these concentrations, SA did not prevent the progression of S phase synchronized cells into G_2 phase. On the contrary, when 200 μM SA was applied for 16 hours to G_2 cells prepared as above, the cells remained at the 4C DNA stage and very few reached the G_1'* phase (Fig. 3F).

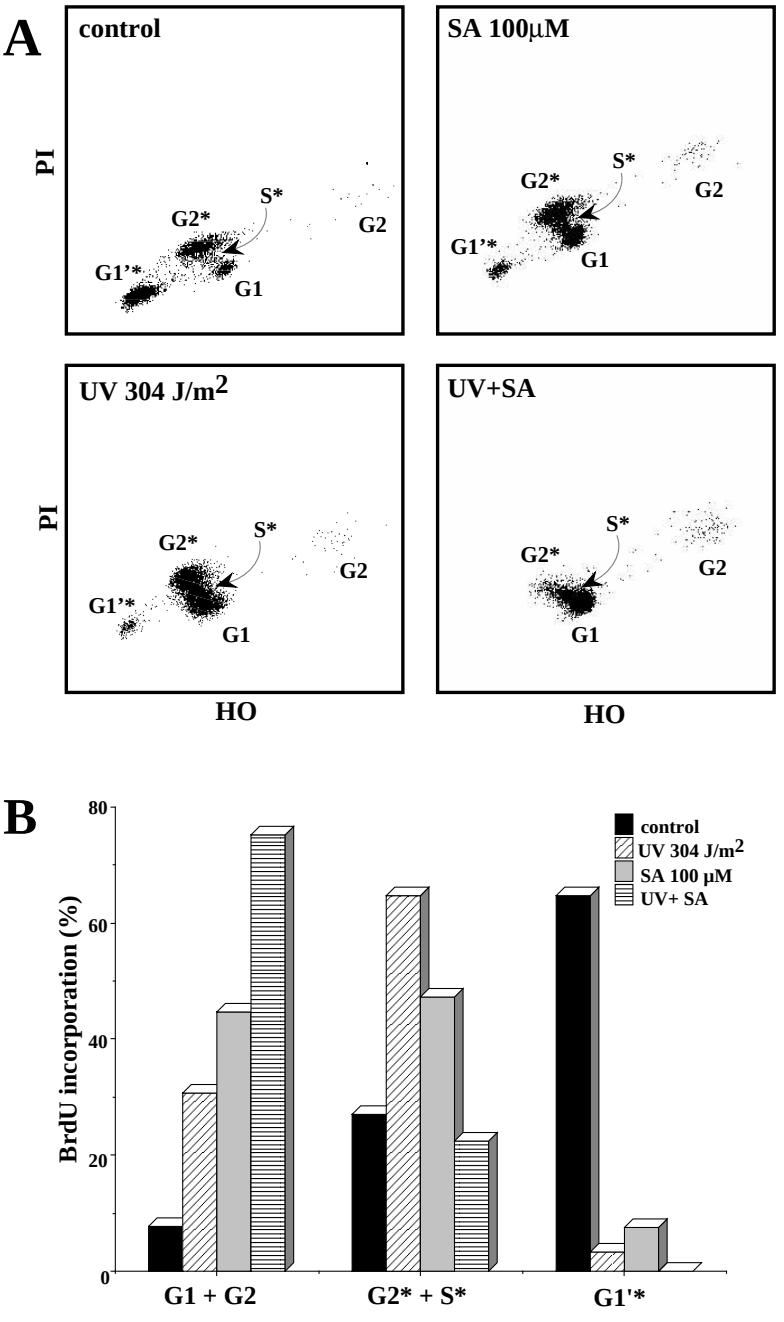

Fig. 4. SA potentiates the UV effect upon cell cycle delay. (A) HO/PI cytogrammes. UV irradiation and/or SA were directly administered after 8 days cultured cells were refreshed. BrdU was incorporated for 24 hours. Nuclei released from the control; SA 100 μM; UV (304 J/m^2); and UV 304 J/m^2 plus SA 100 μM were stained with HO and then with PI.
(B) Percentages of resting cells (G_1 + G_2) and percentages of cycling cells which incorporated BrdU (S*, G_2* and G_1'* phase of the next cell cycle).

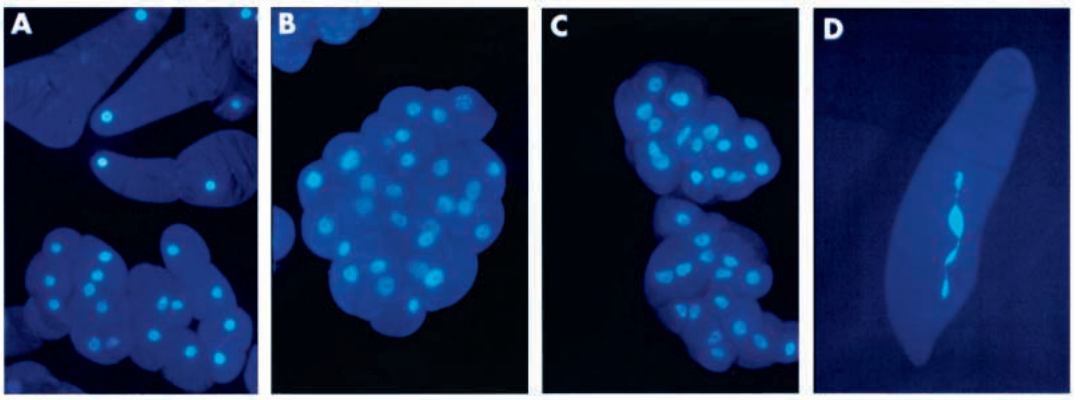

Fig. 5. Morphology of the BY-2 cell suspensions cultivated for 8 days after UV irradiation and SA treatment. (A) Isolated cells and clump in the control. (B and C) Clumps of small cells in SA 200 μM, and UV irradiated cells (1140 J/m^2), (D) Giant cell after 8 days and a 1840 J/m^2 UV dose. After 20 minutes fixation in 2% paraformaldehyde dissolved in phosphate buffer pH 7, cells were stained in nuclei isolation buffer with Hoechst 33342 1 μg/ml^{-1}. magnification ×400.

To test if the SA block was reversible, SA (10, 50, 100 and 200 μM) was added for 24 hours to freshly subcultured cells, then the cells were washed and cultivated for another 24 hours in presence of BrdU. After flow cytometry analysis, BrdU incorporation showed the same pattern as in cells cultivated without SA (results not shown). Therefore, for this concentration range, the cell cycle arrest induced by SA was reversible.

SA blocked cell cycle progression in synergy with UV

To study the UV-SA interaction upon cell cycle progression, a UV dose and SA concentration was chosen whose effects were not too drastic. With respect to the decrease of G_1'* cells after 24 hours, treatments of 304 J/m^2 UV and 100 μM SA were retained (Figs 1B and 3B). Four treatments were applied to freshly subcultured cells: Control (no treatments); UV irradiation at 304 J/m^2; 100 μM SA; simultaneous irradiation and SA treatment. BrdU was added for 24 hours to each sample (Fig. 4A,B). The rate of nuclei reaching the G_1'* phase was the lowest when the two treatments were done simultaneously. Thus, addition of SA plus UV irradiation clearly blocked more efficiently the cell cycle progression than did UV or SA separately. This experiment revealed a synergy of SA with UV such that SA potentiated the arrest of the cell cycle progression.

After 8 days of culture, untreated cell suspensions reached lag phase and most of the cells were big and isolated, with a few clumps comprising only a few cells as observed at the microscopic level (Fig. 5A). 8 days after the treatment with SA (100 μM) or UV (1140 J/m^2), the cell suspensions were composed of clumps of numerous small cells (Fig. 5B,C). A majority of very big cells was observed in UV (1840 J/m^2) irradiated cells (Fig. 5D), but not in SA (200 μM) treated cells. Despite some viable cells remaining, the cell suspension was unable to grow.

arcA3 transcription is induced prior to the restriction point

Many eukaryotic organisms, including humans, remove ultraviolet damage from their genomes by the nucleotide excision repair pathway, which requires more than 10 separate protein factors. By complementing UV-sensitive *E.coli* strain (JM 107 Δ*phr::Cm*^{r} Δ*uvrA::Km*^{r} Δ*recA:: Tet*^{r}), Yajima et al. (1995) cloned the *mus-18 Neurospora crassa* gene encoding an endonuclease that specifically repairs DNA damaged by UV light. By complementation of the same *E. coli* strain with a *Nicotiana tabacum* cDNA library we obtained a cDNA whose coding sequence showed 89% identity to the plant *arcA* obtained by Ishida et al. (1996). The 3′ non-coding region differs and presents a poly(A) tail. Different experiments done to complement again the *E. coli* UV sensitive strain with the *arcA3* purified clone have not been successful.

arcA was previously shown to be induced in cell suspension in response to cell proliferation after treatment by the synthetic growth factor 2,4-D (Ishida et al., 1993). To further investigate the timing of the induction at cell cycle entry, 8 day-old BY-2 cells were refreshed in the presence of roscovitine (50 μM) or staurosporin (2 μM). Roscovitine is an efficient cyclin-dependent kinase inhibitor which blocks the BY-2 cells at restriction point (Planchais et al., 1997) while staurosporin is a general kinase inhibitor. Cells were cultivated for 24 hours before total RNA was extracted, blotted onto nitrocellulose and probed with the full length *arcA*3 cDNA (Fig. 6). The *arcA*3 RNA transcription level had increased in untreated cells compared to the resting cells. Although being slightly lower, the transcription level had also increased in roscovitine or staurosporin treated cells. RNAs were also extracted from cells treated with 200 μM SA for 24 hours. As mentioned above, 200 μM SA applied for 24 hours stopped cell cycle entry before S phase was engaged but this effect was reversible. In these SA treated cells, despite the presence of 2,4-D, the *arcA3*

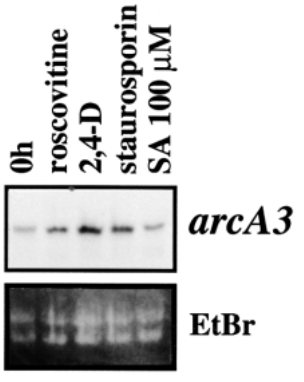

Fig. 6. *arcA3* transcription level increases at cell cycle entry. *arcA3* transcription level in 8-day starving cells (L1) and after cells were cultivated 24 hours in complete medium (L3); cell refreshed for 24 hours plus roscovitine (L2); staurosporin (L4) or SA (L5). In order to control the quantity of RNA loaded (total RNA 50 μg), gels were stained with ethidium bromide (EtBr).

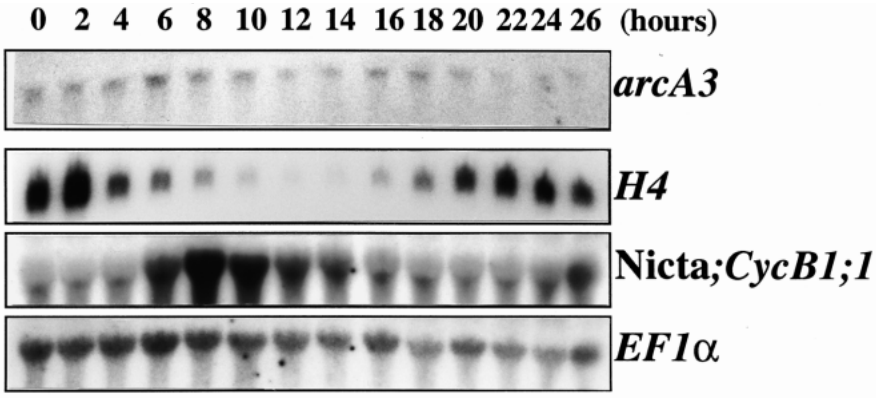

Fig. 7. *arcA 3* transcription level is constant during the cell cycle. Cells were synchronized with aphidicolin (2 µg/ml) then washed twice. RNA was prepared from samples taken immediately before 0 hours and up to 26 hours after release. An RNA blot was sequentially hybridized with *arcA*, histone H4, NtCycB1;1 and Ef1α probes.

transcription level was not different from that observed in resting cells (Fig. 6).

Ishida et al. (1993) reported that the *arcA* transcription level increased when stationary cells became cycling cells but then remained constant. Using synchronized cells we followed the level of *arcA3* transcription from two successive cell cycles. BY-2 cells at stationary phase were synchronized with aphidicolin for 24 hours. After release, cells were sampled every two hours for 26 hours. Histone H4 and *Nicta*;*Cyc*B1;1 transcription levels increased successively indicating the S and G_2 phases. When the second cell cycle was engaged (after 16 hours) we did not observe a modification of the *arcA*3 transcription level (Fig. 7). Therefore the transcription is induced at the transition from stationary phase to cycling phase and then remains constant throughout cell cycle progression.

arcA3 expression after UV irradiation and SA treatments

A possible involvement of *arcA3* in the UV response was examined. An 8-day BY-2 cell suspension was refreshed and UV irradiated at differents doses, then further cultivated for 4 hours. At 114 J/m^2 UV dose, the *arcA3* transcription level was 4-fold higher (Fig. 8) than in the non-irradiated proliferating control.

Since SA treatment blocked the increase in transcription (Fig. 6), we tested whether UV irradiation induced *arcA*3 transcription in the presence of SA. Seven day refreshed cells pretreated or not for one hour with SA 100 µM were UV irradiated (Fig. 9). Four hours later, the steady state levels of *arcA*3 transcripts were compared. Although an increase in the transcription level was observed after treatment with the lowest UV dose, the transcription level remained low in cells cultivated in the presence of SA. Therefore, despite UV irradiation, SA blocked the induction of the *arcA*3 transcription. This may suggest that in the transcription induction, SA control was upstream to that of UV irradiation. Moreover, the presence or absence of the synthetic growth factor 2,4-D during 24 hours, in the culture medium, did not influence the transcription level.

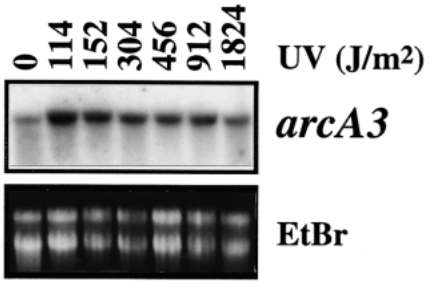

Fig. 8. *arcA3* is induced by UV. An 8-day suspension culture was refreshed and immediately UV irradiated at different doses then kept in the dark for 4 hours before RNA was extracted. Total RNAs 50 µg/lane.

DISCUSSION

UV irradiation is known to damage various plant processes (reviewed by Stapleton, 1992). Until now, plant cell cycle progression after UV irradiation has been seldom documented partly because of the difficulty to obtain synchronized plant cell suspensions. For example the simultaneous presence of histone H3 and mitotic type cyclin transcripts showed that parsley suspensions contain both S and G_2 cells before irradiation (Logemann et al., 1995), making them unsuitable to follow UV cell cycle phase sensitivity. The tobacco BY-2 cell suspension has been shown to be highly synchronizable (Nagata et al., 1992; Planchais et al., 1997). We first determined that the threshold of UV sensitivity was lower than 2 kJ/m^2 which is one thousandfold higher than the dose which prevents *E. coli* cell growth. Furthermore, our results suggest that S phase BY-2 cells are more sensitive to UV-C irradiation than G_0/G_1 cells. While the G_2 phase cells are also affected by UV irradiation, the S phase cells remain the more sensitive.

UV light induces the expression of several plant defensive genes that are normally activated through the octadecanoid signalling (Conconi et al., 1996) of which SA is a potent inhibitor (Pena-Cortez et al., 1993). Therefore, SA inhibition of the pathway could increase sensitivity to UV. We first examined if SA could, alone, affect cell division. Although SA blocks the cell cycle progression as does UV for G_1 and G_2 cells, SA might not be active upon S phase cells. In a soybean cell suspension, it was shown that SA (at concentrations from 10 µM to 1 mM) did not cause appreciable cell death (Shirasu et al., 1997). Similarly, we observed that the SA treatment for 24 hours followed by a 24 hour release in SA-free medium (data not shown) did not

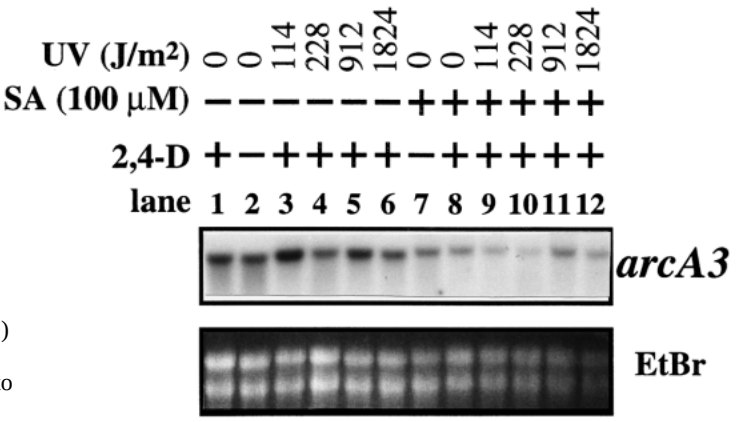

Fig. 9. SA treatment of cells prevents *arcA3* transcription increase upon UV irradiation. 7-day cells refreshed in medium plus 2,4-D (+) or without 2,4-D (–) were cultivated for 4 hours after differents UV doses without SA (L1 to L6) or in the presence of SA 100 μM (L7 to L12). 50 μg total RNA were loaded.

modify progression of the cells through S and G_2 towards G_1'. Therefore the effect of SA, in the range of concentrations applied which can block cell cycle engagement, is reversible when only added for 24 hours.

A different picture emerges when UV and SA are applied simultaneously to the cells. We observe that SA potentiates the blockage of cell cycle progression in UV-C treated cells. UV has been shown to induce oxidative stress through H_2O_2 production. This was compared to the global reponse of bacteria to oxidants (Demple and Amabile-Cuevas, 1991). H_2O_2 was shown to stimulate SA biosynthesis in tobacco leaves (Leon et al., 1995). From this observation we could have explained the synergistic inhibitory effect of UV irradiation and SA on cell cycle progression. Indeed, exogenous application of SA would increase the SA pool, synthesized after H_2O_2 accumulation via UV irradiation. But there are other data that contradict this interpretation. SA was alternatively shown to potentiate pathogen-induced cell death with a rapid accumulation of H_2O_2 (Shirasu et al., 1997). However, SA had no effect on the induction of cell death by exogenous H_2O_2, and the catalase inhibitor 3-aminotriazole (Chen et al., 1995) failed to mimic H_2O_2 effects on the response to bacteria, placing SA action upstream of H_2O_2 accumulation (Shirasu et al., 1997; Tenhaken and Rübel, 1997). Thus, SA could not originate from H_2O_2. Furthermore, plant defense response to UV irradiation activates the octadecanoid defense signalling pathway (Conconi et al., 1996). Since SA was shown to block this pathway (Pena-Cortez et al., 1993) and since we show that SA is synergistic with irradiation by increasing UV irradiation sensitivity, we suggest that the octadecanoid pathway is involved in protecting the BY-2 cells against UV irradiation.

To elucidate the signalling pathway mediating UV response we focused our attention on the complementation of *E. coli* (Δ*phr::Cm*^{r} Δ*uvrA::Km*^{r} Δ*recA:: Tet*^{r}) cells with an expression cDNA library of *Nicotiana tabacum* (Yasuhira and Yasui, 1992). UV induces cell cycle arrest in animal cells and in plant cells (Logemann et al., 1995). A screen based on isolating cDNAs which complement an *E. coli* UV-sensitive strain may simply isolate clones which act as UV protectant. However, a UV protectant might be included in a signalling pathway that induces cell cycle arrest in response to UV irradiation. We isolated a cDNA (*arcA3*) whose coding sequence is similar to the 2,4-D inducible *arcA* gene (Ishida et al., 1993). Likewise, we observed that the *arcA*3 transcription level increases after 24 hours in stationary cells refreshed in the presence of the growth factor, 2,4-D. Moreover, we showed that the *arcA3* transcription level increased after low doses of UV irradiation. A discrepancy appears: UV irradiation blocks the cell cycle progression but induces transcription of *arcA3*, a gene which is stimulated during the transition from stationary phase to cycling S phase. In mammalia, it has been shown that the UV response encompasses two distinct pathways, one triggered by oxidative stress and the other by DNA damage. The response to oxidative stress is similar to the growth stimulatory response (Cerutti, 1985) and it was recognized that several of the UV-induced mRNAs were also induced upon treatment of cells with growth factors (Karin and Herrlich, 1989; Bender et al., 1997). A common protective measure against DNA damage is to arrest cell division until the damage is repaired (Friedberg et al., 1995). Hence, the activation of a signalling pathway similar to the one involved in the growth response may seem paradoxical. However, it is likely that, under many circumstances, DNA repair is highly efficient, and therefore most cells escape moderate DNA damage with an intact genome. Yet, by causing oxidative stress, most DNA-damaging agents also damage other cellular constituants. Most likely, induction of a response similar to the growth response has an important role in replacing damaged cellular constituants with newly synthesized counterparts. Only when the amount of DNA damage exceeds the capacity of the repair system is cell division blocked via the activation of a yet unknown pathway. Some of these responses resemble those of growth factors in that, at least in part, identical immediate response genes are activated (reviewed by Bender et al., 1997). We show that in the same conditions, addition of SA (in the presence of 2,4-D) prevents *arcA*3 transcription although SA does not block general gene transcription (Qin et al., 1994) but prevents cell cycle entry. This is consistent with the absence of an as-1 like element, reported to respond to SA in the *arcA* promoter (Qin et al., 1994; Ishida et al., 1996). The kinase inhibitor roscovitine, which has been shown to block the BY-2 cell cycle entry at the restriction point before S phase engagement (Planchais et al., 1997), did not inhibit *arcA*3 transcription. This suggests that *arcA*3 transcription increased prior to the restriction point and therefore SA inhibition was also effective before this restriction point. Yet with or without UV treatment, in the presence of SA, the *arcA*3 transcription

level did not increase, suggesting that the SA effect was upstream from that of UV irradiation in the *arcA3* signalling pathway(s).

The effects of UV irradiation on plants include the destruction of plasma membrane-associated ATPases (Imbrie and Murphy, 1984). Signal transduction mediated by heterotrimeric guanine-nucleotide-binding regulatory proteins (G proteins) is one of the most widespread mechanisms for relaying information across the plasma membrane in eukaryotes. Gα-GTP and Gβγ are now thought to regulate effector molecules such as adenyl cyclases, phospholipases and ion channels, either independently or synergically (reviewed by Iniguez-Lluhi, 1993). G-proteins play a major role in the regulation of phospholipase by a variety of agonists that activate receptors with seven membrane-spanning domains (reviewed by Exton, 1997). As stated above, *arcA* genes are members of the extended family of proteins with WD-40 repeats, of which a representative is the β-subunit of heterotrimeric G proteins (for review, see Durner et al., 1997). In plants, characterization of WD-40 repeat proteins is recent. *Arabidopsis* AGB1 and maize ZGB1 are closely related to the mammalian Gβ subunit proteins (Weiss et al., 1994). Like *Medicago sativa Msgb* (McKhann et al., 1997), rice RWD (Iwasaki et al., 1995) and *Brassica napus* BGB1 (Kwak et al., 1997) *arcA3* is most closely related to the rat RACK1 (Receptor for activated C kinase) protein. This protein provides an anchoring site in close proximity to the protein kinase C (PKC) specific substrates. *arcA*3 contains homologous sequences between the rat RACK1 and PKC isozymes (Ron et al., 1994). Moreover the RACK1 sequence DVLSVAF homologous to KCIP-1 inhibitor is present. RACKs are proteins that binds to protein kinase C and also to phospholipase C-gamma. Membrane anchoring of components in signal transduction pathways by virtue of anchoring proteins now appears to be one of the general mechanism of these pathways in mammals (Ron et al., 1994). PKCs are a family of phospholipid-dependent serine threonine kinases that regulate cell growth and differentiation. However, so far, this type of component has not been found in plant signal transduction pathways but structural similarity between *arcA* and RACK1 homologue protein, which contains three highly conserved motifs of PKC (Ron et al., 1994) plus the PKC motif for the fixation of the inhibitor KCIP1 and the annexin motif, suggests that a mechanism similar to RACKs-mediated signal transduction may be operating in plants. PKC enzymes appear to operate as regulators of the cell cycle at two sites, during G_1 progression and G_2/M transition (reviewed by Livneh and Fishman, 1997). Examination of whether *arcA* binds other components in plant signal transduction pathways by functioning as a membrane anchoring protein would provide insight into cell cycle blocks by UV and SA and also the pathogen defense mechanisms.

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