Antisense down regulation of NtBI-1 in tobacco BY-2 cells induces accelerated cell death upon carbon starvation

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Abstract Bax inhibitor-1 (BI-1) protein is proposed to be a conserved programmed cell death suppressor. In this report, we investigate the anti-apoptotic function of plant BI-1 by antisense (AS) down regulation of *NtBI-1* in *Nicotiana tabacum* cv. BY-2 cells. We observed that AS cell lines were more susceptible to autophagy, internucleosomal DNA fragmentation and death than control cells when subjected to sucrose starvation and hypo-osmotic shock, in agreement with a role of BI-1 as a death inhibitor.

Key words: Bax inhibitor-1; Programmed cell death; DNA fragmentation; Plant; Autophagy

1. Introduction

In higher eukaryotes, programmed cell death (PCD) is a normal part of life, playing important roles in many diverse physiological processes, from development to stress responses. While this phenomenon is relatively well understood in animals, our comprehension of plant PCD is only emerging. In mammals, apoptosis is prominently controlled through functionally conserved proteins of the Bcl-2 family, including members that promote cell survival (e.g. Bcl-2, Bcl-X_L) and cell death (e.g. Bax, Bak) [1]. To date, no such genes have been identified in plants, but much evidence argues for the existence of evolutionarily conserved pathways for the control and execution of PCD in both plants and animals [2].

Among evolutionarily conserved PCD actors figures Bax inhibitor-1 (hBI-1), which was first identified in a human cDNA library from its ability to suppress Bax-induced cell death in yeast [3]. hBI-1 also inhibits death induced either by the overexpression of Bax in human HEK293 cells (although not by direct protein–protein interaction) or by other pro-apoptotic stimuli [3]. After the original identification of hBI-1, plant homologues (pBI-1) have been cloned from a number of genera [4–7], and pBI-1 from *Arabidopsis thaliana* and *Oryza sativa* (respectively AtBI-1 and OsBI-1) can suppress Bax-induced lethality in yeast [4,5]. Lethality induced by the ectopic expression of Bax in *A. thaliana* can also be suppressed by AtBI-1 [8]. Moreover, it has been shown that NtBI-1 and BnBI-1 (from *Nicotiana tabacum* and *Brassica napus*) could suppress Bax-induced lethality in human HEK293 cells [7], although it has been reported that AtBI-1 induces death when expressed in human HT1080 cells [9]. Thus, it appears that BI-1 could be a participating actor of an evolutionarily conserved cell death pathway.

Both plant and animal BI-1 are mainly located in membranes of endoplasmic reticulum and the nuclear envelope [3,7,8]. In plants, pBI-1 seems relatively ubiquitous in all organs [5,7], and higher accumulations have been observed in flowers undergoing senescence [7] or in leaves following wounding or pathogen attack [5]. However, its precise physiological function in plants remains to be determined, since no sequence homologues of *Bax* have been identified to date. Considering that antisense (AS) down regulation of hBI-1 induces apoptosis [3], we wondered whether it would be the case in plants. We thus report here the effects of constitutive AS down regulation of NtBI-1 in tobacco BY-2 cells.

2. Materials and methods

2.1. Generation of AS cell lines

Cell suspension cultures of *N. tabacum* cv. BY-2 were cultured as described [7]. A pBI121 vector carrying the *NtBI-1* cDNA in AS orientation was constructed using primers Nt4 (5′-CTTGTTCAAGTTCGAGGAGCTCGATT) containing a *Sst*I site and Nt5 (5′-TTCACTCGAGAACAAGAAAATGCCAG) to amplify the entire coding region of *NtBI-1* from plasmid pBSK-NtBI-1 [7]. The PCR product was then subcloned in the *Xba*I and *Sst*I sites of pBI121. The proper orientation of the AS construct was confirmed by automatic sequencing. This construct, together with the original pBI121 vector, was then used to achieve *Agrobacterium tumefaciens* mediated transformation as described [7] for the generation of AS or β-glucuronidase (GUS) stable transformed cell lines.

2.2. Immunoblot analysis

BY-2 cells were ground in liquid N_2 and boiled in lysis buffer (0.1 M Tris–HCl pH 6.8, 5% sodium dodecyl sulfate (SDS), 20% glycerol, 5 mM EDTA, 70 mM 2-mercaptoethanol) for 10 min before being centrifuged (10 000 × *g*, 5 min). Supernatants were collected and protein contents were determined [10]. Separation of proteins (50 μg per lane) into SDS–12% polyacrylamide gels, transfer to polyvinylidene difluoride membranes and immunodetection were performed according to standard procedures.

Polyclonal anti-pBI-1 antibodies were generated in rabbit using a synthetic peptide corresponding to the C-terminal end of AtBI-1 protein conjugated to keyhole limpet hemocyanin (KLH) by its N-terminal extremity (a cysteine was added for conjugation purposes; KLH-CKNSADKEEKKKKRRN). Serum was used at dilution 1:800. Bound antibodies were detected with a goat anti-rabbit IgG (horse-

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Abbreviations: AS, antisense; Suc, sucrose; Man, mannitol; TUNEL, terminal deoxynucleotidyl transferase-mediated dUTP nick end labeling; PCD, programmed cell death; (p, h)BI-1, (plant, human) Bax inhibitor-1; GUS, β-glucuronidase

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radish peroxidase conjugated) and a chemiluminescent substrate (BM Chemiluminescence Blotting Substrate, Roche Molecular Biochemicals). Labeling specificity was confirmed by a drastic signal reduction following preadsorption of the antibodies to the synthetic peptide.

2.3. Sucrose starvation and cell death assays

Four-day-old, logarithmically growing cells were allowed to settle and washed with phosphate buffered saline (PBS) before being resuspended in either the original volume of standard growing medium [containing 3% sucrose (suc)], medium without suc or medium without suc supplemented with 2% mannitol as an osmoticum (man medium). The percentage of dead cells was determined using Evans blue as described [11].

2.4. DNA fragmentation analysis

For terminal deoxynucleotidyl transferase-mediated dUTP nick end labeling (TUNEL) assays, cells were fixed in 4% paraformaldehyde (in PBS) at room temperature for 45 min, washed and digested in PBS containing 0.1% pectolyase and 8 mM $MgSO_4$ for 2 h at 30°C. Digested cells were spread on microscope slides, air dried overnight and subjected to TUNEL using fluorescein-dUTP according to the manufacturer's instructions (ApoAlert® DNA Fragmentation Assay Kit, Clontech), except that membranes were permeabilized using 0.5% Triton X-100. Nuclei were counterstained with 0.5 μg/ml propidium iodide (PI) and visualized under confocal laser scanning microscopy using a Zeiss LSM310 instrument.

For DNA isolation, cells were ground in liquid N_2 and approximately 200 mg were incubated in 750 μl CTAB buffer [2% CTAB (hexadecyltrimethyl ammonium bromide), 1.4 M NaCl, 0.2% β-mercaptoethanol, 20 mM EDTA, 100 mM Tris–HCl pH 8] at 65°C for 30 min in 1.5 ml tubes. Subsequently, one volume of chloroform:isoamyl alcohol (24:1) was added and the samples were gently agitated at room temperature for 10 min and centrifuged (1600×*g*, 5 min). The aqueous phase was collected, mixed with 0.66 volume of isopropanol and centrifuged (3000×*g*, 5 min). Pellets were resuspended in 400 μl RNase buffer (10 mM Tris–HCl pH 8, 1 mM EDTA, 50 μg/ml RNase A) and incubated at 37°C for 30 min. DNA was ethanol precipitated, air dried, resuspended in 10 mM Tris–HCl pH 8, 1 mM EDTA and separated by electrophoresing 2 μg/lane on 1.5% agarose gel.

3. Results and discussion

3.1. Generation of AS cell lines and NtBI-1 analysis

To investigate the in vivo functions of BI-1 in plants, we transformed tobacco BY-2 cells either with a construction harboring the *NtBI-1* gene in the AS orientation or with the *GUS* gene, both under the control of the constitutive CaMV 35S promoter. Five different AS stable cell lines were used throughout this study and compared to three different GUS cell lines (controls). Levels of NtBI-1 protein were monitored with polyclonal anti-pBI-1 antibodies that specifically recognize a single band of the expected apparent molecular weight (M_r) for pBI-1, i.e. approximately 28 kDa, in leaf extracts of *N. tabacum*, *A. thaliana* or *B. napus* (Fig. 1A). Intriguingly, an M_r of 33 kDa was observed in cultured tobacco cells of all tested cultivars (BY-2 and Xanthi, Fig. 1A; SR-1, data not shown). Since the length of *NtBI-1* mRNA is the same whatever its origin [7], post-translational modifications should occur in cultured cells. In our experiments, the M_r of NtBI-1 remained unchanged upon deglycosylation experiments with BY-2 protein extracts (data not shown), leaving questionable the nature of the post-translational modification.

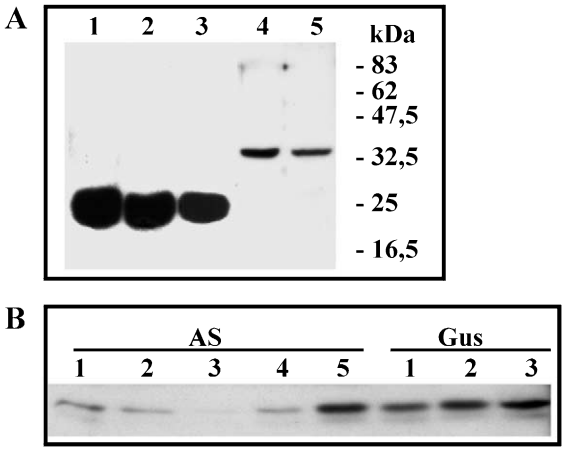

Fig. 1. Immunodetection of pBI-1 in plant leaves and cultured cells. A: Basal expression of pBI-1 in leaves of *A. thaliana* ecotype Columbia (1), *N. tabacum* cv. Xanthi (2) or *B. napus* cv. Westar (3) and in cultured cells of *N. tabacum* cvs. BY-2 (4) and Xanthi (5). B: NtBI-1 levels in different AS and control cell lines.

AS cell lines exhibited low levels of NtBI-1 when compared to controls (Fig. 1B). However, NtBI-1 levels in AS lines were relatively variable from week to week. As an example, line AS5 had the lowest NtBI-1 level when we started our experiments (data not shown), but was found to exhibit the highest thereafter (Fig. 1B). Thus, in order to get representative results, we conducted our experiments using different lines at a time rather than a single one.

3.2. Observation of AS cell lines under standard growing conditions

Observation of AS lines in the exponentially growing phase allowed the visualization of a normal morphology (Fig. 2A). Their growth rates were also comparable to those of control cell lines (data not shown). However, with the aging of cultures (i.e. 9–14 days after the transfer to fresh medium), AS lines became slowly grayish, while control lines maintained their usual light yellow color (not shown). Furthermore, in the majority of AS cells, a number of morphological changes were observable (Fig. 2A), including a drastic reduction (10 days) to a complete disappearance (12 days) in the number of transvacuolar strands and the confinement of the cytoplasm just beneath the plasma membrane and the perinuclear region. Such morphology was only observed in a small proportion of control cells, particularly in older cells (12 days or more), the majority being relatively normal or presenting a reduced number of transvacuolar strands. These morphological features are closely related to those reported for BY-2 cells undergoing autophagy in response to suc starvation [12]. BY-2 cells are heterotrophic and thus depend on their growing medium to supply them with a carbon source. In aged cultures, growing medium was depleted of its sucrose content, and therefore we can speculate from our observations that AS lines are more sensitive to carbon starvation than control cells.

3.3. Observation of AS cell lines under carbon starvation

3.3.1. Evaluation of mortality. To confirm and quantify the relation between autophagy and NtBI-1 level, exponentially growing AS and control cells were transferred to suc-free growing medium or fresh medium and progression of mortality was followed over time (Fig. 3A). As expected, the percentage of dead cells remained low for both AS and control cells transferred to fresh medium, but it reached over 30% for control and 60% for AS cell lines (after 48 h) when transferring cells to suc-free medium. These data indicate an inverse correlation between levels of NtBI-1 and cell death. However, transfer of plant cells to suc-free medium triggers not only a nutrient stress but a hypo-osmotic shock as well.

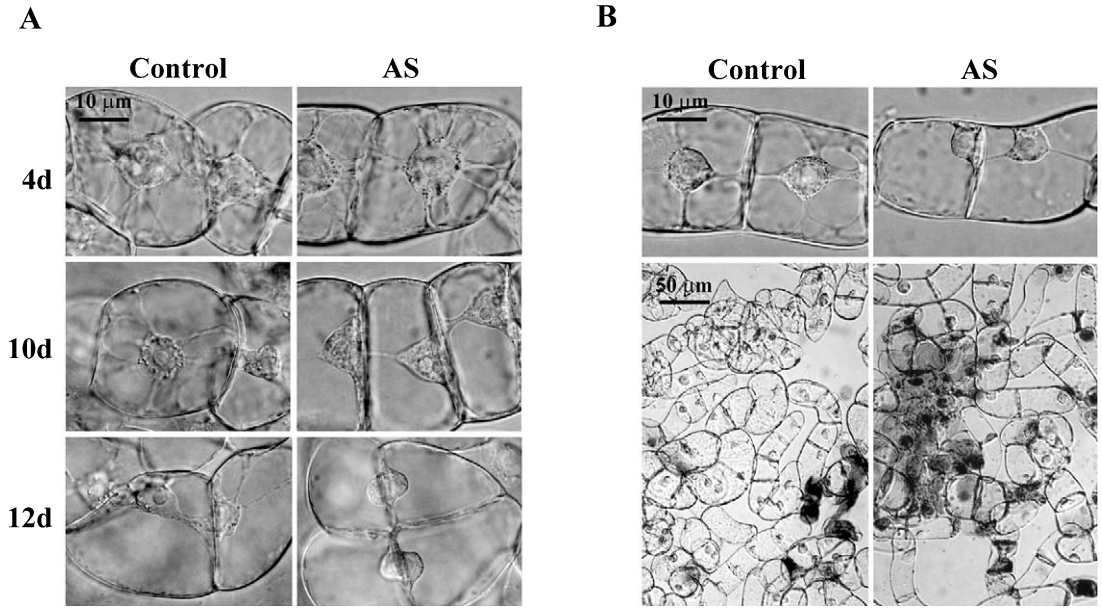

Fig. 2. Representative fields showing the effects of NtBI-1 down regulation on tobacco BY-2 cell morphology. A: Time course observations of AS and control cells growing under standard conditions 4–12 days after the weekly transfer to fresh medium. B: Observations 96 h after transfer to man medium. Cells in the lower panel were photographed in the presence of 0.05% Evan's blue.

Interestingly, we observed that AS lines presented a higher proportion of dehydrated cells than control lines following transfer to suc-free medium (data not shown). This observation raised the hypothesis that increased death of AS lines could be partly or completely attributable to an increased susceptibility to hypo-osmotic shock.

In an attempt to discriminate carbon starvation from hypo-osmotic shock, cells were transferred to man medium, an osmoticum commonly used because it is very slowly taken up by plant cells [13]. In these conditions, no appreciable increase in control cell's mortality was observed until 120 h after the transfer (25%, Fig. 3B), which is similar to data reported for carrot (*Daucus carota* L.) suspension cells subjected to the same carbon starvation conditions [14]. On the other hand, mortality of AS cell lines started earlier than control cells, i.e. 72 h after transfer (over 30%), to reach over 60% after 120 h (Fig. 3B). These data clearly show that carbon starvation can be sufficient to promote cell death in AS lines.

Observations of cells under light microscopy 96 h after transfer to man medium in the presence of Evan's blue to visualize dead cells (Fig. 2B) confirmed that a large proportion of AS cells were already dead by this time, while most control cells remained able to exclude the stain. Moreover, AS cells showed morphological features characteristic of autophagy, with almost complete disappearance of the cytoplasm, while control cells remained relatively normal, exhibiting only a reduced number of transvacuolar strands. Similar morphological differences between AS and control cells were observed when cells were transferred to suc-free medium (data not shown), except that the phenomenon was accelerated and dehydrated cells were frequently observed in AS cells. Taken all together, these data clearly indicate that BY-2 cells expressing a low level of NtBI-1 die prematurely upon carbon starvation or a combination of carbon starvation and hypo-osmotic shock. This is further confirmed by the unstable behavior of line AS5, which, following transformation, exhibited a very low level of NtBI-1, together with a high percentage of cell death (data not shown). However, after 6 months of culture, we observed that these cells displayed a higher NtBI-1 content (Fig. 1B), and presented only 30% of dead cells after 120 h in man medium (data not shown). This clearly illustrates that mortality correlates with the NtBI-1 level and is not attributable to the transformation procedure.

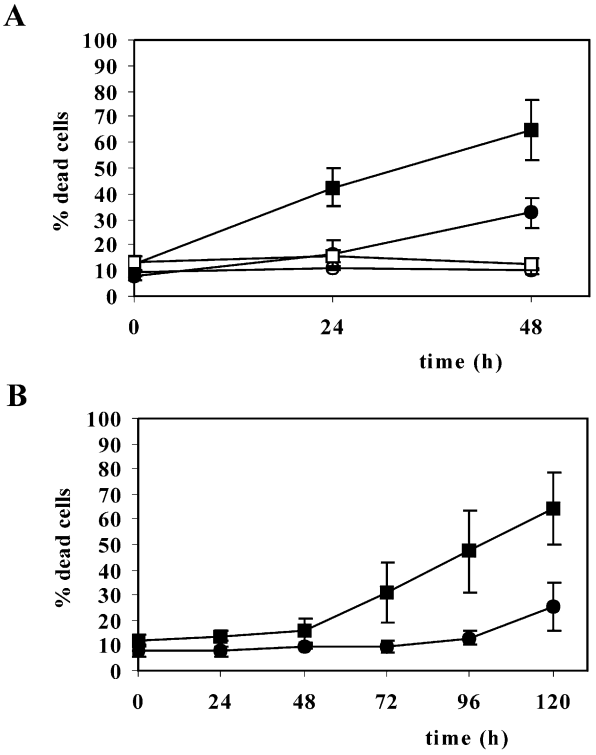

Fig. 3. Death assessment in AS (■, □) and control (●, ○) cell lines upon suc starvation. Cells were transferred to suc-free (■, ●) and fresh (□, ○) media in the absence (A) or presence (B) of man. Data are the mean values (±S.D.) of three independent experiments using all control lines and AS lines 1–4.

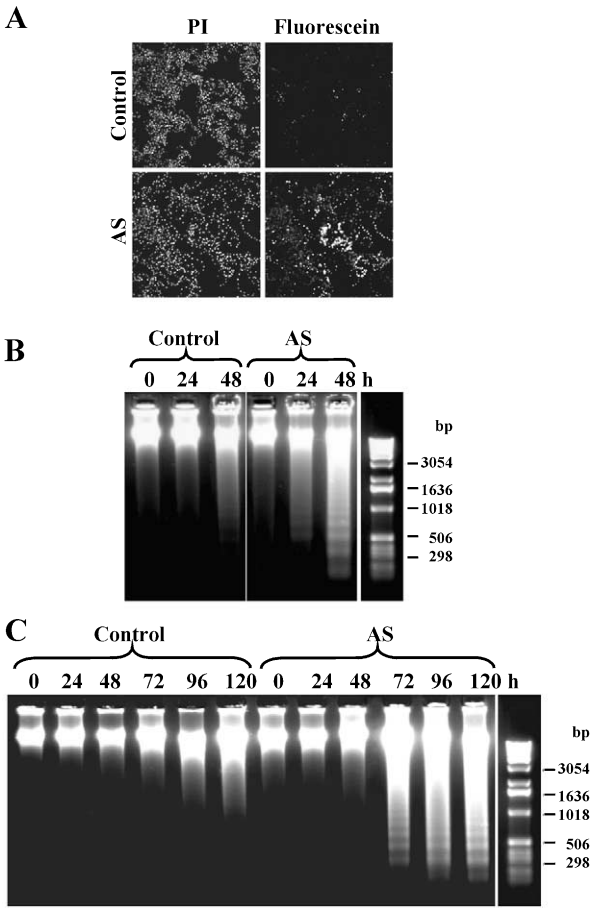

Fig. 4. DNA fragmentation analysis in AS and control cell lines following transfer to suc-free medium (A,B) or man medium (C). A: TUNEL assay on cells harvested 48 h after transfer. B,C: Time course analysis of internucleosomal DNA fragmentation. Typical data from AS lines 1–4 and control lines 1–3 are presented.

3.3.2. DNA fragmentation analysis. To determine whether death in AS lines could be associated with PCD, we investigated the occurrence of internucleosomal degradation of nuclear DNA, a typical biochemical marker of some forms of PCD. Nuclear degradation was first analyzed by a TUNEL assay on cells harvested 48 h after transfer to suc-free medium. As seen in Fig. 4A, DNA was poorly labeled in control cells, while a large proportion of cells from AS lines were TUNEL positive. To further confirm the internucleosomal DNA cleavage characteristic of PCD, DNA isolated from cells cultured under suc starvation was subjected to agarose gel electrophoresis (Fig. 4B,C). For both treatments (±2% man), a DNA laddering could be observed in AS lines but not in control lines within the time frame under study. Moreover, the first appearance of DNA laddering in AS lines correlated with an increased cell death, i.e. 24 h (without man) or 72 h (with man; Fig. 3). In the case of line AS5, which failed to present a significant increase in cell death, no DNA laddering could be observed (data not shown). Taken all together, our data clearly establish a direct correlation between accelerated cell death, internucleosomal DNA degradation and low levels of NtBI-1.

3.4. Concluding remarks

The physiological function of BI-1 in plant cells is still unclear. In this paper, we have demonstrated that AS down regulation of the *NtBI-1* gene in tobacco BY-2 cells affects cell viability and prematurely induces internucleosomal degradation of nuclear DNA when these cells are subjected to stresses such as carbon starvation and hypo-osmotic shock. These observations, which correlate with those reported for AS suppression of the *hBI-1* gene in human cells [3], confirm the anti-apoptotic role of pBI-1 in plants, and suggest its potential implication in the regulation of autophagy. Interestingly, it has been previously reported that hBI-1 suppressed apoptosis induced by a number of pro-apoptotic stimuli, including growth factor deprivation [3], suggesting a similar role in mammals. At the plant level, *AtBI-1* mRNA accumulation was delayed in *A. thaliana coi1* mutants compared to wild type [5], and the authors suggested that reduced AtBI-1 levels may contribute to the enhanced susceptibility shown by *coi1* plants to infections by various fungal pathogens. These data together with all available information on pBI-1 [5–8] strongly indicate that pBI-1 may have a ubiquitous role in responses to biotic and abiotic stress and thus would play a general protective role against significant metabolic perturbations, as previously suggested [5]. However, the elucidation of molecular mechanisms underlying this protective effect will need further investigations.

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