Non-apoptotic programmed cell death with paraptotic-like features in bleomycin-treated plant cells is suppressed by inhibition of ATM/ATR pathways or NtE2F overexpression

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Abstract

In plants, different forms of programmed cell death (PCD) have been identified, but they only partially correspond to those described for animals, which is most probably due to structural differences between animal and plant cells. Here, the results show that in tobacco BY-2 cells, bleomycin (BLM), an inducer of double-strand breaks (DSBs), triggers a novel type of non-apoptotic PCD with paraptotic-like features. Analysis of numerous PCD markers revealed an extensive vacuolization, vacuolar rupture, and chromatin condensation, but no apoptotic DNA fragmentation, fragmentation of the nuclei, or sensitivity to caspase inhibitors. BLM-induced PCD was cell cycle regulated, occurring predominantly upon G2/M cell cycle checkpoint activation. In addition, this paraptotic-like PCD was at least partially inhibited by caffeine, a known inhibitor of DNA damage sensor kinases ATM and ATR. Interestingly, overexpression of one NtE2F transcriptional factor, whose homologues play a dual role in animal apoptosis and DNA repair, reduced PCD induction and modulated G2/M checkpoint activation in BY-2 cells. These observations provide a solid ground for further investigations into the paraptotic-like PCD in plants, which might represent an ancestral non-apoptotic form of PCD conserved among animals, protists, and plants.

Key words: ATM/ATR pathways, BY-2 cells, cell cycle, double-strand break response, E2F, paraptotic-like, programmed cell death.

Introduction

Apoptosis is a recent evolutionary mode of programmed cell death (PCD), which evolved to eliminate damaged or unwanted animal cells through activation of the caspase family proteases and phagocytic clearance (Hengartner, 2000). In addition, at least three other non-apoptotic forms of PCD have also been described in metazoans and were designated as autophagy, paraptosis, or mitotic catastrophe, respectively. However, none of them completely fulfils the typical features of apoptosis (Bröker et al., 2005). In plants, an apoptotic strategy does not exist due to the presence of the cell wall that impedes phagocytosis by other cells. Instead, autophagic PCD takes place when the final proteolytic activity resides in the cell’s own autophagosome or lytic vacuole (van Doorn and Woltering, 2005). Plant metacaspases are distant relatives of caspase which have been recently described to play a role during the hypersensitive response (HR; Coll et al., 2010). Strikingly, they do not exhibit the same proteolytic activity as do animal caspases (Vercammen et al., 2007) since caspase-like activities can participate in execution of plant PCD (Hatsugai et al., 2006). Another, very little known type of PCD is paraptosis, which has so far been identified in animals and protists (Sperandio et al., 2000; Jimenez et al., 2009). This process involves cytoplasmic vacuolization and mitochondrial swelling, in the absence of apoptotic markers such as caspase activation, oligonucleosomal DNA cleavage, or...
nuclear fragmentation (Sperandio et al., 2000, 2004; Jimenez et al., 2009). Despite the elucidation of morphological characteristics, the precise molecular basis of non-apoptotic PCD types remained unknown, especially in plants.

In eukaryotes, the phosphatidylin-3 kinase (PI-3K) family members ATM (ataxia telangiectasia mutated) and ATR (ataxia telangiectasia and Rad3-related) are the main sensors of DNA damage. While ATM senses DNA double-strand breaks (DSBs), ATR is activated in response to single-stranded DNA (Brown and Baltimore, 2003; Lee and Paull, 2007). In mammals, DNA damage signalling also involves checkpoint kinases such as Chk1 and Chk2, which activate downstream effectors such as the E2F1 or p53 transcriptional factors, ultimately leading to cell cycle checkpoint activation, DNA repair, or apoptosis (Chaturvedi et al., 1999; DeGregori, 2002; Lee and Paull, 2007). Ectopic expression of the transcriptional activator E2F1 induces not only S phase entry in quiescent cells, but also p53-dependent or p53-independent apoptosis, which is enhanced upon DNA damage (Qin et al., 1994; Kowalik et al., 1995; Hsieh et al., 1997). The pro-apoptotic E2F1 function was also described to occur in Drosophila melanogaster and Caenorhabditis elegans, thus confirming that the E2F1 function seems to be conserved in animals (Asano et al., 1996; Schertel and Conradt, 2007). The apoptotic role of E2F1 was affirmed by transcriptional data showing that it controls the expression of numerous core pro-apoptotic genes (Muller et al., 2001). Besides its pro-apoptotic role, E2F1 has been recently shown to contribute directly to DNA repair and genome maintenance (Chen et al., 2011). However, all the molecular mechanisms underlying the cell’s decision to proliferate or to die are not yet fully understood (Buchakjian and Kornbluth, 2010).

Plants had to evolve a specific control mechanism for the DNA damage response, among others due to their sessile life. As compared with animals, they developed different strategies to cope with this type of stress. Such an adaptation is the high capacity to regenerate or replace damaged tissues and organs, according to the indeterminate growth of meristems. In comparison with mammals, some players in the DNA damage response are conserved in plants, for example ATM, ATR, and E2Fs (Garcia et al., 2003; Culligan et al., 2004; Roa et al., 2009), but, strikingly, many ATM and ATR targets such as p53, Chk1, or Chk2 are missing (Cools and De Veylder, 2009). In Arabidopsis, the roles of ATM and ATR were demonstrated during DNA repair, cell cycle checkpoint activation (Culligan et al., 2004, 2006; Ricaud et al., 2007), and more recently during DNA damage-induced PCD (Fulcher and Sablowski, 2009; Furukawa et al., 2010). Altogether, in plants the study of cell death induction in response to DNA damage is still emerging and has not yet been investigated during cell cycle progression.

In plants, the E2F factors are transcriptional activators or repressors sharing similar complexity with the animal E2Fs (Lincker et al., 2008). E2Fs have been shown to regulate genes implicated in cell cycle control and DNA repair (Chaboute et al., 2000; Lincker et al., 2004; Vandepoele et al., 2005; Roa et al., 2009; Radziejwoski et al., 2011), but their role in the regulation of PCD has never been investigated. In Arabidopsis, the overexpression of the transcriptional activator E2Fa, with its transactivating partner DPa, induces ectopic cell division, endoreduplication, or endomitosis rather than PCD (De Veylder et al., 2002; Henriques et al., 2010). In addition, available microarray data do not suggest any up-regulation of PCD-related genes in Arabidopsis overexpressing E2Fa/DPa, compared with wild-type plants (Vandepoele et al., 2005; Naour et al., 2009). These results do not exclude a possible role for E2Fa in plant PCD regulation, because a E2Fa/DPa-dependent transcription of PCD-related genes might occur only in response to genotoxic stress, or the PCD response might be mediated by other E2F members. The only tobacco NtE2F factor described is a transcriptional activator (Sekine et al., 1999) similar to AtE2Fa and AtE2Fb (Lincker et al., 2008). NtE2F is up-regulated in response to genotoxins (Lincker et al., 2004), but its role during PCD regulation has not yet been established.

To characterize the genotoxic-induced PCD in plants, dividing tobacco BY-2 cells were used as a model for cellular and cell cycle analyses. This could extend previous results obtained in Arabidopsis root meristem, which is confined to a few developmentally specified cells only (Fulcher and Sablowski, 2009; Furukawa et al., 2010). In the present work, a novel type of non-apoptotic PCD induced by the DSB-inducer bleomycin (BLM) was characterized in plants sharing features with paraptotic PCD. Besides its regulation by ATM/ATR, such PCD was mainly induced upon the activation of the G2/M checkpoint, which was modulated by NtE2F overexpression.

Materials and methods

Cultivation of plant material and BLM treatment
Tobacco BY-2 (Nicotiana tabacum cv. Bright Yellow-2) cells were maintained by weekly subculture in modified Murashige and Skoog medium (Sigma, St Louis, MO, USA) as described (Nagata et al., 1992). Cells were cultivated at 26 °C in darkness on an orbital shaker set at 130 rpm. Before BLM treatment, mid-log phase cells were diluted (1:3). BLM was added to wild-type or BLM-resistant tobacco BY-2 cells at specific concentrations, or endomitosis rather than PCD (De Veylder et al., 2002; Henriques et al., 2010). In addition, available microarray data do not suggest any up-regulation of PCD-related genes in Arabidopsis overexpressing E2Fa/DPa, compared with wild-type plants (Vandepoele et al., 2005; Naour et al., 2009). These results do not exclude a possible role for E2Fa in plant PCD regulation, because a E2Fa/DPa-dependent transcription of PCD-related genes might occur only in response to genotoxic stress, or the PCD response might be mediated by other E2F members. The only tobacco NtE2F factor described is a transcriptional activator (Sekine et al., 1999) similar to AtE2Fa and AtE2Fb (Lincker et al., 2008). NtE2F is up-regulated in response to genotoxins (Lincker et al., 2004), but its role during PCD regulation has not yet been established.

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Generation of the NtE2F line
The open reading frame of NtE2F (BAA86386) was amplified by PCR with specific attb1 and attb2 primers
Viability assay, BCECF staining, and ROS detection

The FDA (fluorescein diacetate) viability assay was used to detect cell death in BY-2 cells. A 50 μl aliquot of cell suspension was mixed with 50 μl of FDA working solution (1/1000 in distilled water; stock solution 0.2% FDA in acetone, Sigma) and incubated for 2 min before observation by epifluorescence microscopy (Nikon E800, Champigny sur Marne, France or Olympus Provis AX 70, Hamburg, Germany) using a GFP filter (excitation, 460–500 nm; emission 510–560 nm). Viable cells cleave FDA into a fluorogenic product detected as a green signal, whereas dead cells do not give a signal. Arabidopsis plantlets were incubated in 1000-fold diluted 10 mg ml⁻¹ water–propidium iodide (Sigma) solution for 3 min, rinsed in water, and immediately observed using a red fluorescent protein (RFP) filter (Zeiss Axioscope 100M with Zeiss LSM 510 Software, Le Pecq, France). The integrity of the vacuoles was determined after 5 min of double staining with 6 mM BCECF (2',7'-bis-carboxyethyl)-5(6)-carboxyfluorescein; Sigma) and 0.05% Evans blue as described (Matsuoka et al., 1997). For reactive oxygen species (ROS) detection, cells were incubated with DCFH-DA (2',7'-dichlorfluorescein-diacetate; Sigma) at a final concentration of 2 μM. The images were captured after 120 s at a single 600 nm exposure (Basler A101c Camera, Buckinghamshire, UK). The relative ROS accumulation is presented as mean pixel intensity per cell measured with ImageJ software. Five hundred cells were counted in at least three independent experiments.

TUNEL assay and DNA laddering

TUNEL (terminal deoxynucleotidyl transferase dUTP nick end labelling) assay was performed according to the manufacturer’s protocol (TMR red in situ cell death detection kit, Roche Diagnostics GmbH, Heidelberg, Germany) with slight modifications. Just before the TUNEL reaction, two steps were added. First, the samples were incubated in 10 mM CaCl₂ and subsequently treated with 40 μM H₂O₂. Second, the samples were incubated with 0.1% Triton and the nuclei were stained with 20 μg ml⁻¹ 4',6-diamidino-2-phenylindole (DAPI; Sigma). The MI was determined as the percentage of mitotic cells among 500 cells analysed by fluorescence microscopy (Nikon E800 or Olympus Provis AX 70). In parallel, the nuclear morphology of at least 300 nuclei was analysed in at least three independent experiments.

Results

Induction of paraptotic-like PCD in tobacco BY-2 cells after DNA damage

To ascertain whether and how BLM can induce PCD in cultured BY-2 cells, mid-log phase cells were submitted to different doses of BLM, and cell viability was analysed in the following 72 h using the FDA test. Upon BLM treatment,
the cell mortality progressively increased in a dose- and time-
dependent manner, while the mortality of control cells was
essentially negligible (Fig. 1). To distinguish whether cell death
occurred by PCD or in a non-programmed manner, the
presence of several PCD hallmarks was investigated: cyto-
plasm shrinkage, chromatin condensation, DNA laddering,
and ROS accumulation. Finally, vacuolization and vacuolar
disintegration were analysed, leading to leakage of hydrolytic
enzymes into the cytoplasm during autophagic PCD execution
in plants (van Doorn and Woltering, 2005).

First, BLM-treated cells were stained with Evans blue to
analyse cytoplasm shrinkage in dead cells. This cytoplasm
shrinkage was found in 87±4% and 92±3% of dead cells
treated for 72 h with 10⁻⁵ M and 10⁻⁴ M BLM (P < 0.01),
respectively (Fig. 2A), whereas control cells exhibited normal
morphology. Formation of apoptotic bodies containing the
fragments of cytoplasm and organelles could not be observed.

However, a high proportion of cells exhibited an exten-
vacuolization, mainly after 10⁻⁴ M BLM treatment. BLM also reduced the number of cytoplasmic strands due to
apparent vacuolar fusion to form one central vacuole
occupying the majority of the cellular inner space (Fig. 2B).
To monitor the integrity of the tonoplast and its relation-
ship to cell viability, BCECF-AM and Evans blue double
staining was used. BCECF-AM is cleaved in the fluorogenic
product BCECF which was localized to the vacuolar lumen
and excluded from the cytoplasm and nuclei of viable cells.
Conversely, Evans blue was accumulated in dead cells,
whereas it was actively exported out of viable cells. Thus,
three different cell categories can be discerned according
to their staining patterns by both dyes (Higaki et al., 2007):
type I, intact viable cells with intact plasma membrane and
tonoplast had Evans blue-negative and BCECF-positive
signals in the vacuole; type II, viable cells with intact
plasma membrane and collapsed vacuole exhibited Evans
blue-negative and BCECF-positive signals in the cytosol
and the nucleus; and type III, dead cells with disintegrated
vacuolar and plasma membranes showed Evans blue-
positive and BCECF-negative signals (Fig. 2A, C). During
72 h of BLM treatment, the number of type I cells
progressively increased while the number of type III cells
increased. The number of type II cells that were alive but
contained a collapsed vacuole was relatively high after BLM
treatment (Fig. 2D). In the non-treated control, 99% of cells
belonged to type I (data not shown). Importantly, the
relatively high number of type II cells means that cells can
survive for a certain period even with a disintegrated vacuole.
Therefore, it was suggested that the vacuolar collapse is not
a crucial step during execution of BLM-induced PCD as was,
for example, reported to happen during elicitor-induced or
developmental PCD (Obara et al., 2001; Higaki et al., 2007).

Vacuolar collapse may be linked to internucleosomal
DNA fragmentation in BY-2 cells (Hatsugai et al., 2004;
Kuthanova et al., 2008b). However, when BLM was
supplied during 3 d or 7 d to BY-2 cells, no apoptotic
DNA fragmentation was observed using TUNEL or DNA
laddering assays, respectively (Supplementary Fig. S1 avail-
able at JXB online), even though most of the cells were
dead (data not shown). Both vacuole rupture-mediated and
apoptotic-like PCD could depend on caspase-like activities.
However, PCD induction by BLM was insensitive to the
most commonly used caspase inhibitors, z-YVAD-FMK
and z-DEVD-FMK, when compared with the positive
control treated with 50 μM 6-benzylaminopurine (6-BAP;
Supplementary Fig. S2), which is a potent inducer of
apoptotic-like PCD in BY-2 cells (Mlejnek and Prochazka,
2002; Danon et al., 2004; Hatsugai et al., 2004).

In parallel, by DAPI staining, chromatin morphology was
analysed after 24 h of BLM treatment in the early phases
of cell death induction (Fig. 3A). At 10⁻⁴ M BLM, the
chromatin was highly condensed into one or several bodies in
81±1% of the cells. In contrast, the control exhibited normal
nuclear morphology, with large nucleoli being surrounded by
uniformly stained chromatin. After 10⁻³ M BLM treatment,
no significant difference in nuclear morphology was observed,
albeit that a fraction of cells (5±5%) exhibited more granular
chromatin (data not shown).

Finally, in light of the crucial role played by ROS in PCD
and DNA damage (Gadjiev et al., 2008; Roldan-Arjona and
Ariza, 2009), ROS production was monitored using DCFH-
DA, which is a fluorescent detector of a broad range of
oxidizing reactions that may be increased during intracellu-
lar oxidative stress (Hempel et al., 1999). After 24 h of BLM
treatment, DCFH fluorescence apparently increased as
compared with control cells, which exhibited only a very
weak signal (Fig. 3B). DCFH fluorescence was more
pronounced at 10⁻³ M than at 10⁻⁴ M, most probably due
to different kinetics of ROS accumulation after both BLM
treatments (Fig. 3C).

Taken together, these results demonstrate that BLM-
induced PCD is a unique type of PCD, which does not fit to
the criteria for apoptotic-like PCD or for autophagic PCD,
but rather shares characteristics with paraptotic-like PCD.
Caffeine inhibits BLM-induced PCD

Previous work has reported that DSB-induced PCD is ATR and/or ATM dependent in *Arabidopsis* (Fulcher and Sablowski, 2009; Furukawa *et al.*, 2010). To define the role of DNA damage signalling during the BLM-induced PCD in tobacco cells, the ATM/ATR inhibitor caffeine was used. Although caffeine affects other cellular processes such as cytokinesis (Yasuhara, 2005), it is also successfully used for ATM/ATR inhibition in mammals (Sarkaria *et al.*, 1999). In plants, caffeine is also able to override the DNA damage G2/M checkpoint arrest (Hartley-Asp *et al.*, 1980; Gonzalez-Fernandez *et al.*, 1985), but seems not to affect the replication checkpoint control in S phase (Amino and Nagata, 1996; Pelayo *et al.*, 2001; Weingartner *et al.*, 2003). The possibility that caffeine is active also towards ATR in tobacco BY-2 cells cannot be ruled out, and thus caffeine may inhibit both the ATM and ATR pathways.

Mid-log phase BY-2 cells were inoculated into MS medium supplemented with 5 mM caffeine 1 h prior BLM addition. As a positive downstream target of ATM/ATR signalling, the well-characterized transcriptional induction of the *RNR1* gene (Roa *et al.*, 2009) was investigated. In tobacco, the two *NtRNR1a* and *b* genes were shown to be induced by DSBs (Lincker *et al.*, 2004, 2008) and, as expected, the BLM-induced *RNR1a* expression was significantly reduced by caffeine (P < 0.01) (Supplementary Fig. S3 at *JXB* online). However, this *RNR1a* down-regulation was only partial, suggesting that under the experimental conditions used either caffeine did not completely inhibit ATM/ATR activities, or there might exist another ATM/ATR-independent regulation of *NtRNR1a*.

To analyse the effect of ATM/ATR inhibition on paraptotic-like PCD, the MI (the percentage of cells in mitosis), chromatin condensation, vacuolar dynamics, and, finally, induction of cell death were evaluated. As expected, BLM caused a rapid arrest of cell division already after 24 h of treatment (Supplementary Fig. S4 at *JXB* online). Fluorescence-activated cell sorting (FACS) analysis performed on nuclei from BLM-arrested cells showed an increase of 4C nuclei versus control nuclei, and a concomitant reduction of
order to study the role of the tobacco NtE2F activator factor on BLM-induced PCD (called NtE2F cells or the NtE2F line). This fusion protein was localized in the nucleoplasm (Fig. 5A), and it was proved that GFP does not alter proper NtE2F transcriptional function using the NtRNR1b promoter as a target (J. Lang et al., unpublished results). In addition, this was confirmed by increased expression of the E2F target gene NtRNR1b in non-treated and BLM-treated NtE2F cells compared with the wild type (Fig. 5B). GFP::NtE2F overexpression did not modify the mortality of untreated and 10⁻⁵ M-treated cells, but significantly decreased PCD in 10⁻⁴ M-treated cells (P > 0.05) (Fig. 5C). At high BLM concentration, GFP::NtE2F not only reduced PCD, but almost totally inhibited chromatin condensation (Fig. 5D).

Because deregulation of the pRB/E2F pathway in animal cells leads to increased genome instability (Pickering and Kowalik, 2006; Srinivasan et al., 2007), the effect of NtE2F overexpression on chromatin integrity and mitosis progression was examined. A total of 27±3% (P < 0.01) of 10⁻⁵ M BLM-treated NtE2F cells displayed DAPI-stained micronuclei, which were never observed to such an extent in 10⁻⁴ M BLM-treated cells or control cells, where their number did not exceed 2±0.5%. This suggested that micronuclei appeared as a result of abnormal mitotic division with lagging chromosomal fragments during anaphase and telophase. This was confirmed by FISH (Fig. 6), using a probe specific to telomeric sequences. Another hallmark of compromised genome integrity in NtE2F cells, even without genotoxins, was the formation of anaphase bridges, which were observed neither in untreated cells nor in cells treated with BLM or caffeine alone. The induction of PCD, together with chromatid condensation (Fig. 4D) and vacuolar disintegration (Fig. 4E, F), was significantly reduced, but only in response to 10⁻⁴ M BLM (P < 0.05) (Fig. 4G). Thus, the inhibition of the ATM/ATR pathways may suppress G₂/M checkpoint activation, leading to entry into mitosis even with damaged DNA. The detection of numerous mitotic catastrophes linked to DNA damage may be a result of the caffeine-mediated inhibition of cytokinesis (Yasuhrara, 2005). These results strongly suggest that paraptotic-like PCD induced by high BLM doses is at least partly ATM/ATR dependent.

PCD and genome integrity in BY-2 cells is altered by pRB/E2F pathway deregulation

A BY-2 cell line overexpressing the GFP::NtE2F fusion under the control of the constitutive 35S promoter was generated in order to study the role of the tobacco NtE2F activator factor on BLM-induced PCD (called NtE2F cells or the NtE2F line). This fusion protein was localized in the nucleoplasm (Fig. 5A), and it was proved that GFP does not alter proper NtE2F transcriptional function using the NtRNR1b promoter as a target (J. Lang et al., unpublished results). In addition, this was confirmed by increased expression of the E2F target gene NtRNR1b in non-treated and BLM-treated NtE2F cells compared with the wild type (Fig. 5B). GFP::NtE2F overexpression did not modify the mortality of untreated and 10⁻⁵ M-treated cells, but significantly decreased PCD in 10⁻⁴ M-treated cells (P > 0.05) (Fig. 5C). At high BLM concentration, GFP::NtE2F not only reduced PCD, but almost totally inhibited chromatin condensation (Fig. 5D).

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GFP::NtE2F overexpression modulates the G₂/M DNA damage checkpoint for PCD induction

To investigate further how GFP::NtE2F overexpression affects the DNA damage checkpoint, mitotic entry and FACS analysis were investigated in synchronized cells where BLM (10⁻⁵M) was applied at different cell cycle stages (Fig. 7A). APC, a specific inhibitor of DNA polymerase α and δ, reversibly blocks the cells in early S phase (Litvak and Castroviejo, 1985). Wild-type cells reached the maximum of mitosis at 9 h after APC release (0 h) and then progressed into G₁, whereas NtE2F cells exhibited the peak of mitosis at 10 h, indicating a slightly slower cell cycle progression. Therefore, BLM treatments of synchronized NtE2F cells at G₂ and M phases were modified accordingly (see the Materials and methods).

As expected, wild-type cells treated with 10⁻⁵ M BLM in S or G₂ phases did not enter mitosis (Fig. 7B). In contrast, NtE2F cells treated under the same conditions partially entered mitosis, with a more pronounced effect when BLM was added in G₂. In addition, as compared with the wild...
type, the MI dropped more slowly in NtE2F cells when BLM was added in M phase.

In order to correlate the lack of entry into mitosis with G2/M checkpoint activation, FACS analyses were performed 24 h after APC release. Wild-type cells treated with BLM in S or G2 mainly exhibited a 4C DNA content, whereas cells treated in the M or G1 phase essentially presented a 2C DNA content as for control cells (Fig. 7C). This confirmed that BLM applied in S or G2 wild-type cells led to G2/M checkpoint activation. In contrast, treatment of NtE2F cells in any cell cycle phase led to a major accumulation of 2C DNA-containing cells. These data indicate that ectopic GFP:NtE2F expression impairs G2/M checkpoint activation in response to 10⁻⁵ M BLM, thereby leading to cell accumulation in G1 (Fig. 7C).

Previous studies have demonstrated that PCD induction in BY-2 can be regulated throughout the cell cycle (Herbert et al., 2001; Kuthanova et al., 2008a). Therefore, dead cells were counted in synchronized culture after 3 d of 10⁻⁵ M BLM treatment applied during cell cycle progression. Wild-type cells exhibited the highest mortality when BLM was applied in G2 (Fig. 7D), whereas the mortality of NtE2F cells was significantly reduced (P < 0.01). Partial inhibition of cell death in NtE2F cells was also found during S or M phase treatments (P < 0.05), but to a lesser extent (Fig. 7D). These data indicate that when applied in G2, 10⁻⁵ M BLM induces maximal cell death which is significantly impeded by GFP:NtE2F overexpression.

After a 10⁻⁴ M BLM treatment applied in G2, cell death induction was down-regulated by NtE2F overexpression (Fig. 8C), but entry into mitosis was not modified (Fig. 8A). Similarly, FACS analysis showed that most of the cells had 4C DNA content in both the NtE2F and wild-type lines (Fig. 8B), suggesting that G2/M checkpoint activation was not altered by NtE2F overexpression in response to 10⁻⁴ M BLM. Thus, at high BLM concentration, NtE2F overexpression does not modify G2/M checkpoint activation but only modulates the subsequent PCD induction.

Discussion

This study provides the first evidence of a non-apoptotic PCD in higher plant cells that most closely matches to paraptotic-like PCD. The implication of the NtE2F activator factor during this process was determined using a novel model system based on synchronizable tobacco BY-2 cells treated with BLM. In contrast to mammals, Drosophila, or...
*Caenorhabditis*, overexpression of NtE2F fused to GFP prevents BLM-induced PCD, which is at least partly dependent on ATM/ATR pathways. It should be underlined that BLM-triggered PCD appeared to be strongly dependent on G2/M checkpoint activation, which could be modulated by GFP:NtE2F overexpression.

**BLM induces non-apoptotic PCD**

The current studies have shown that genotoxic compounds can induce PCD in various plant model systems. Apoptotic-like PCD after UV-C or camptothecin treatments was characterized by typical apoptotic hallmarks such as oligonucleosomal DNA fragmentation, involvement of caspase-like activities, fragmentation of the nucleus, or oxidative burst (De Jong *et al.*, 2000; Danon *et al.*, 2004; Gao *et al.*, 2008). Other authors, using zeocin or γ-irradiation as PCD inducers in *Arabidopsis* root tips, were not so focused on PCD morphology, thus its classification is more than difficult (Fulcher and Sablowski, 2009; Furukawa *et al.*, 2010).

In tobacco BY-2 cells, BLM-induced PCD was characterized by some typical apoptotic features such as cell shrinkage, chromatin condensation, and ROS accumulation; however, apoptotic oligonucleosomal DNA fragmentation was not detected either by TUNEL assay or by DNA electrophoresis. Moreover, specific caspase inhibitors did fail to block BLM-induced cell death as described, for example, for UV-C- or 6-BAP-induced PCD in *Arabidopsis* mesophyll or BY-2 cells, respectively (Mlejnek and Prochazka, 2002; Danon *et al.*, 2004). These data suggest that caspase activities are not implicated in the BLM-induced PCD. Therefore, this type of PCD does not present a classical apoptotic character, in spite of the apoptotic-like features mentioned above.

Vacuolar collapse was described as a key step of autophagic PCD, resulting in a leakage of vacuolar hydrolases such FIG. 5. NtE2F overexpression affects BLM-induced cell death and vacuolar collapse. BY-2 cells were stably transformed with the 35S::GFP:NtE2F construct, and GFP-positive clones, named NtE2F, were selected (A). Mid-log wild-type and NtE2F cells were transferred into MS medium with 10⁻⁵ M and 10⁻⁴ M BLM. After 24 h, the expression of NtRNR1b was analysed using real-time qPCR (B). After 72 h, the number of dead cells was quantified using the FDA viability test (C); chromatin condensation was analysed and quantified after nuclear DAPI staining (D). The number of vacuole-collapsed cells (type II, see text) in the culture was observed after BCECF-AM and Evans blue double staining during 72 h (E and F). Data represent the mean values (±SD). Asterisks indicate the values that are significantly different from the control (*P < 0.05; **P < 0.01). Bar=18 μm.
as vacuolar processing enzymes (VPEs) into the cytoplasm. This mechanism ensures a rapid cell death to prevent the pathogen spreading, as was shown for vacuole-mediated PCD during the HR induced by plant elicitors such as cryptogein or by virus infection (Hatsugai et al., 2004; Kuroyanagi et al., 2005; van Doorn and Woltering, 2005). Similarly, vacuole-mediated cell death during xylem development in Zinnia also occurs within a few minutes after vacuolar disintegration (Obara et al., 2001). Thus, if vacuolar collapse mediates PCD, the number of cells with disintegrated vacuoles but still alive (type II) should be very limited. Taking into account that PCD processing was relatively slow (days compared with minutes or hours during HR or developmental PCD) and that PCD did not appear immediately after vacuolar rupture, it is very likely that BLM-induced cell death is distinct from the previously reported vacuole-mediated PCD (Obara et al., 2001; Hatsugai et al., 2004). Moreover, as the VPE-specific inhibitor z-YVAD-FMK did not modify the amount of dead cells, this group of vacuolar hydrolases, being crucial for vacuole-mediated

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**Fig. 6.** Formation of micronuclei in NtE2F cells after BLM treatment. Wild-type and NtE2F cells were transferred to MS medium with BLM. The FISH experiment was performed on interphasic nuclei (left) and during telophase (right) 72 h after $10^{-5}$ M BLM treatment. Aliquots of BY-2 cells were hybridized with a telomere-specific probe labelled with Cy3 (red) and stained with DAPI (blue). Arrows indicate micronuclei; arrowheads indicate chromosomal bridges. Representative pictures of each sample are shown. Bar=10 μm.

**Fig. 7.** Mitotic index progression, DNA content, and cell death induction in wild-type (WT) and NtE2F cells after $10^{-5}$ M BLM treatment. WT and NtE2F cells were synchronized with APC blocking the cells at the entry of S phase. After the release of APC (0 h), BLM was added at different time points (S, G2, M, and G1; arrowheads) within the cell cycle (A). Mitotic index progression was analysed during 12 h after APC release (B). Flow cytometry analysis revealed the DNA content of the cells in the culture 24 h after APC release (C). At 72 h after the APC washing, the number of dead cells (±SD) was analysed with the aid of the FDA viability test (D). Representative values of three independent experiments are presented, due to some variable synchronization rates between each experiment. Asterisks indicate the values that are significantly different from the control (*$P < 0.05$; **$P < 0.01$).
PCD, is most probably not involved in the BLM-induced PCD.

Until now, paraptotic PCD was considered as an alternative non-apoptotic form of PCD in animals, for example in some tumours or during neurodegenerative diseases (Bröker et al., 2005; Fombonne et al., 2006; Zhang et al., 2011). This type of cell death is accompanied by vacuolization, phosphatidylserine exposure to the extracellular side of the plasma membrane, absence of oligonucleosomal DNA cleavage, pyknosis, and insensitivity to caspase inhibitors or a requirement for transcription (Sperandio et al., 2000, 2004; Wang et al., 2004). However, not all these hallmarks are always present during paraptotic PCD, showing that a wider range of paraptotic PCD mechanisms may exist (Sun et al., 2010). Taking into account all morphological and biochemical analyses, BLM-induced cell death most resembled paraptosis, mainly due to the apparent inefficiency of caspase inhibitors, lack of DNA fragmentation, and extensive vacuolization. However, the signalling involved in this alternative PCD remains to be elucidated in plants in order to confirm whether BLM-induced PCD is strictly homologous to animal paraptosis.

Paraptotic-like PCD is inhibited by caffeine and GFP:NtE2F overexpression

When caffeine, an inhibitor of the PI-3Ks ATM and ATR, was applied, a significant decrease of PCD, chromatin condensation, and vacuolar disintegration was observed, but only at a high BLM concentration. These data suggest that BLM-induced paraptotic-like PCD is at least partially dependent on ATM/ATR kinases. This phenomenon has already been reported for Arabidopsis meristematic cells that were subjected to DSB inducers (zeocin and γ-irradiation) (Fulcher and Sablowski, 2009; Furukawa et al., 2010), but the novelty of these results is that ATM/ATR could regulate a paraptotic-like PCD in plant cells. This hypothesis finds further support in results obtained with Arabidopsis, where caffeine inhibits the ATM/ATR-dependent PCD in root meristematic cells as observed in atm (Supplementary Fig. S5 at JXB online). These observations may indicate some conservation of the ATM/ATR control of DSB-induced PCD in higher plants. A low concentration of BLM induced only a minor decrease in cell viability, which was not affected by caffeine. There are two possible explanations: this PCD is mediated either by residual ATM/ATR activity in the case of incomplete caffeine inhibition, or by some other pathways, independent of ATM and ATR. Indeed, the existence of such a pathway may be supported by Furukawa’s findings that DNA damage-induced PCD is not totally inhibited in the atm atr double mutant (Furukawa et al., 2010).

In contrast to animals, where E2F1 is a positive regulator of apoptosis through transcriptional regulation of pro-apoptotic genes (p53, Apaf1, and caspases) (Müller et al., 2001), NtE2F may have no pro-cell death function in plants since its overexpression prevents cell death induction. However, it cannot be ruled out that some other uncharacterized tobacco E2F factors may have a pro-cell death role. Such a discrepancy in E2F-mediated PCD between plants and animals may be due mainly to evolutionary constraints. Indeed, sedentary plants, in view of their inherent developmental plasticity, can generally tolerate much higher doses of DNA damage and therefore they may not need such strict regulation of PCD as for animal apoptosis (Yokota et al., 2005). This could be illustrated by decreased genomic integrity induced by NtE2F overexpression at low BLM concentrations, but without a subsequent increase of PCD. One has to keep in mind that these experiments based on GFP:NtE2F overexpression could have a limited biological relevance. Nevertheless, this approach could be considered as a tool to study DNA damage checkpoint regulation in relation to PCD.
BLM-induced PCD requires G₂/M checkpoint activation

Even though cell cycle-dependent PCD has already been studied using various models and conditions of PCD induction (Herbert et al., 2001; Kadota et al., 2004), the results are hardly comparable. Cadmium was shown to promote cell death when applied in all cell cycle phases of synchronized BY-2 cells, but to induce DNA fragmentation and apoptotic-like PCD only when applied prior to mitotic entry (Kuthanova et al., 2008b). In contrast, BLM-induced DNA fragmentation was never observed, even when BLM was applied during G₂ phase (data not shown). Complementary experiments using a broad range of genotoxic compounds to induce PCD will now be required to define the character and the underlying mechanism of G₂/M-related PCD in plant cells.

BLM treatment of synchronized cells activates both G₁/S and G₂/M checkpoints, suggesting the induction of DNA repair pathways; however, a certain cell fraction undergoes PCD. This cell death induction mainly resulted from the BLM concentration-dependent activation of the G₂/M checkpoint in order to remove heavily DNA-damaged cells. Simultaneously, BLM treatment triggers an increased expression of NtRNR1b, which is in agreement with previous data showing the key role of RNR during DNA repair (Wang and Liu, 2006; Roa et al., 2009) (Fig. 9A, B).

The requirement for G₂/M checkpoint activation for PCD induction upon high BLM doses is further supported by the fact that stationary cells that are mainly in G₁ phase exhibit low PCD induction under the same conditions of genotoxic treatment (Fig. 5C; Supplementary Fig. S6 at JXB online).

When NtE2F is overexpressed, besides its capacity to induce DNA repair due to NtRNR1b up-regulation as reported previously (Lincker et al., 2008), it also modulates DNA damage G₂/M checkpoint activation in a BLM dose-dependent manner. With a low BLM concentration, G₂/M checkpoint activation is partially overridden, thus leading to mitotic entry with unrepaired DNA detected as micronuclei and decreased PCD as well (Fig. 9C). These data do not preclude that DNA repair might exist in G₁/S. Using a high BLM concentration (10⁻⁴ M), NtE2F overexpression does not impair G₂/M checkpoint activation, as clearly documented by the accumulation of 4C nuclei and the absence of formation of micronuclei. However, even at this high BLM concentration, NtE2F overexpression significantly reduced the mortality, most probably by increased repair efficiency due to E2F-mediated up-regulation of DNA repair genes, as shown for NtRNR1b (Fig. 9D).

In tobacco BY-2 cells, G₂/M transition is linked to increased CDKB1 activity and protein levels, suggesting a role for CDKB1 in G₂/M checkpoint regulation (Poredudu et al., 2001; Sorrell et al., 2001). The expression and activity of the mitotic CDKB1 have been shown to be induced by both AtE2Fa and AtE2Fb (Boudolf et al., 2004; Magyar et al., 2005; Sozzani et al., 2006). It is therefore tempting to suggest that bypass of the BLM-induced G₂/M checkpoint induced by NtE2F overexpression might be related to increased CDKB1 activity controlled by NtE2F, which is a close homologue of AtE2Fa and b (Lincker et al., 2008).

Fig. 9. Model of PCD regulation in wild-type (A, B) and NtE2F (C, D) cells upon 10⁻⁵ M (A, C) and 10⁻⁴ M (B, D) BLM treatment. Thick arrows indicate preferential pathways under the given conditions.

Is paraptotic-like PCD an evolutionarily conserved ancestral mode of cell death in eukaryotes?

In conclusion, based on morphological criteria, BY-2 cells have the capacity to undergo non-apoptotic cell death with paraptotic-like features, which is impaired by E2F overexpression and ATM/ATR inhibition. It seems that paraptotic-like PCD could be conserved among animals (Sperandio et al., 2000), protists (Jimenez et al., 2009), and plants (this study), and therefore might represent an ancestral form of PCD, notably as compared with the evolutionarily more recent apoptosis. In contrast to apoptotic PCD, the molecular basis of paraptotic cell death is very limited. For example, mitogen-activated protein kinases (MAPKs) regulate paraptosis in animals and algae (Sperandio et al., 2004; Jimenez et al., 2009) and, interestingly, their plant homologues participate in a general stress-activated pathway including DNA damage response (Bulavin et al., 2001; Ulm et al., 2001; Reinhardt et al., 2007). These data raise an interesting question, namely whether the MAPK pathway might be involved in the BLM-induced PCD. An identification of such general paraptotic regulators is needed to demonstrate the conservation of paraptosis among eukaryotes.

Supplementary data

Supplementary data are available at JXB online.

Figure S1. Detection of specific DNA fragmentation in BY-2 cells after BLM treatment.

Figure S2. Effect of caspase inhibitors DEVD and YVAD on BLM-induced PCD.
Figure S3. Inhibition of BLM-induced NtRN1a expression by caffeine.

Figure S4. Time and concentration-dependent effect of BLM on the mitotic index.

Figure S5. Effect of caffeine on PCD induction in Arabidopsis root tips.

Figure S6. Cell death induction in stationary wild-type and NtE2F cells.

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