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Molecular and cellular aspects of programmed cell death in response to genotoxics in plants

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by

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Declaration

I submit this dissertation for review and defense in partial fulfillment of the requirements for the degree of Doctor of Philosophy at the Charles University in Prague, Czech Republic and at the University of Strasbourg, France.

I hereby declare that this doctoral thesis is completely my own work and that I used only the cited sources.

Prague, August 8, 2010 Ondřej SMETANA

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Preface

Plants are continuously exposed to different endogenous or exogenous stresses. Compared to animals, plants defense against adverse environment effects must be highly developed due to their sessile life. In the context of genome integrity maintenance, numerous spontaneous DNA mutations are introduced into the genome but in addition a high DNA damage is induced by UV light, an invisible component of a sunlight. Plants had to evolve specific mechanisms to control DNA damage and adapt their development to environmental stresses and to solve the dilemma coming from the need of the sunlight with DNA damage ability for photosynthesis. In addition they lack a reserved germline and often produce the meiotic cells at the end of their development, so mutations accumulated in somatic cells during the lifetime may be represented in gametes. At the molecular level, basic DNA damage pathways from a common Eukaryotic ancestor have been conserved between animals and plants: two kinases ATM and ATR were identified across the kingdoms and are activated specifically in response to different genotoxics stresses and initiate DNA damage response. After DNA damage the signal is transduced to effectors involved in the regulation of the cell cycle arrest, transcription of DNA damage responsive genes or programmed cell death (PCD) according to the severity of damage (Figure 1.1). Regulation of DNA damage-induced PCD was intensively studied in mammals (Bernstein et al. 2002). In plants the DNA damage response started to be investigated since last 6 years in Arabidopsis with the main focus on the DNA repair mechanisms and cell cycle checkpoints (Ricaud et al. 2007; Ramirez-Para et al. 2007; reviewed in Cools and de Veylder 2009). However, since the main DNA damage sensors and several effector proteins homologous to their animal counterparts were identified in plant, plant DNA damage response harbors number of specificities which remain to be discovered (Cools and de Veylder 2009). Especially, PCD induction as a one outcome of DNA damage response was till now out of focus of plant biologists and thus this process is still poorly understood.

Introduction

In the introduction of this manuscript, I will focus on cell cycle regulation and I will summarize our knowledge about DNA damage response linked to cell cycle checkpoints as well as PCD. Finally, I will present recent data about genotoxics-induced PCD in plants.

1.1. Cell cycle

Most of the cells of living organism have an origin in one maternal cell which divided into two daughter cells. This process of growing and division is called the cell cycle which regulation and organization is highly conserved among all Eukaryotes. Basic features of the cell cycle are the replication of genetic information during S phase which is followed by mechanical redistribution of duplicated chromosomes into daughter cells followed by cell division (cytokinesis) during M phase. This basic cell cycle also called "embryonic cell cycle" could be observed during the first several division of animal zygote with rapid cell division and virtually no growth. To avoid reducing of the cell size, two "gap" phases are introduced when the cell growth and increases in volume, first G1 phase precedes S phase whereas G2 phase is before M phase. During these two G phases, the cell also controls if the precedent phase was successfully terminated and prepares for the progression into the following phase. There are at least two major cell cycle checkpoints G1/S and G2/M whose core regulatory machinery is highly conserved across the Eukaryotes. Two other checkpoints could be activated in response to DNA damage. After stalled replication fork during S phase, cell cycle arrests at the intra-S checkpoint whereas abnormal progression of mitosis can induce M-phase checkpoint (Cools and de Veylder 2009).

1.1.1. Regulation of the cell cycle

Cell cycle is highly complex system regulated by number of genes which expression and regulation of encoded proteins is cell-phase-specific (Combettes et al. 1999). Eukaryotes, the central regulators of the cell cycle are cyclin-dependent kinases (CDKs) which are serine/threonine kinases (Figure 1.2.) (Morgan et al. 1997; Mironov et al. 1999). The four major regulatory inputs affecting the CDKs activity are: the binding of regulatory proteins (i.e. cyclins, CDK inhibitors) (Pines et al. 1999), positive and negative phosphorylation of certain conserved amino acid residues (Dunphy et al. 1994) and also by cell cycle-dependent proteolytic degradation of regulatory proteins (King et al. 1996; Peters et al. 1998). For the first time, the CDK kinase activity was shown to be the active component of mitosis-promoting factor (MPF), an autocatalytic protein that could drive an interphasic cell into the mitosis (Gautier et al., 1988). The catalytic subunit of CDK recognizes a specific target motif (a serine or threonine followed by proline) whereas CDK interaction with regulatory cyclin determines target proteins of cyclin/CDK complex. Different cyclin/CDK complexes phosphorylate a wide range of

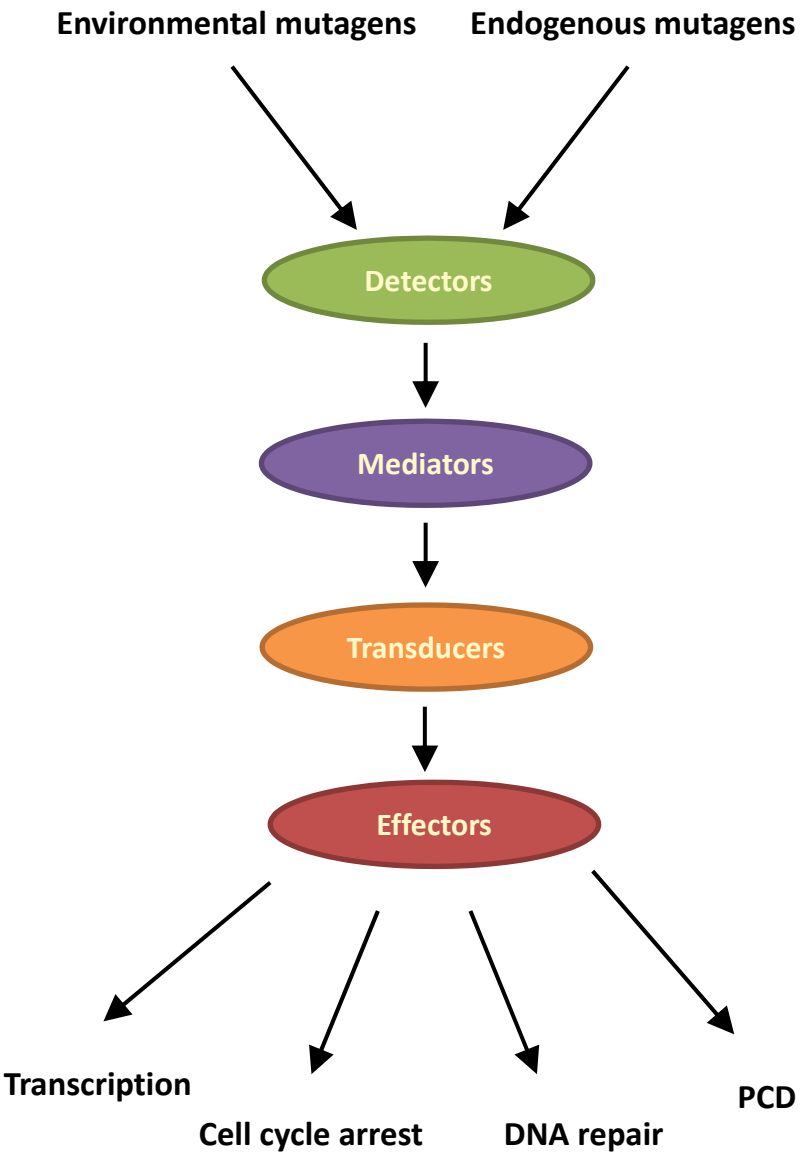


Figure 1.1: Scheme of signal transduction after DNA damage. DNA lesion is first recognized by detector proteins. Then the signal is sensed via a cascade of proteins (notably kinases) which activate target proteins through protein/protein interactions involving BRCT domain proteins recognizing phosphorylated residues. These proteins are also CDKs and their phosphorylation at specific residues induces cell cycle arrest at different control points of the cell cycle allowing DNA repair or other cellular responses.

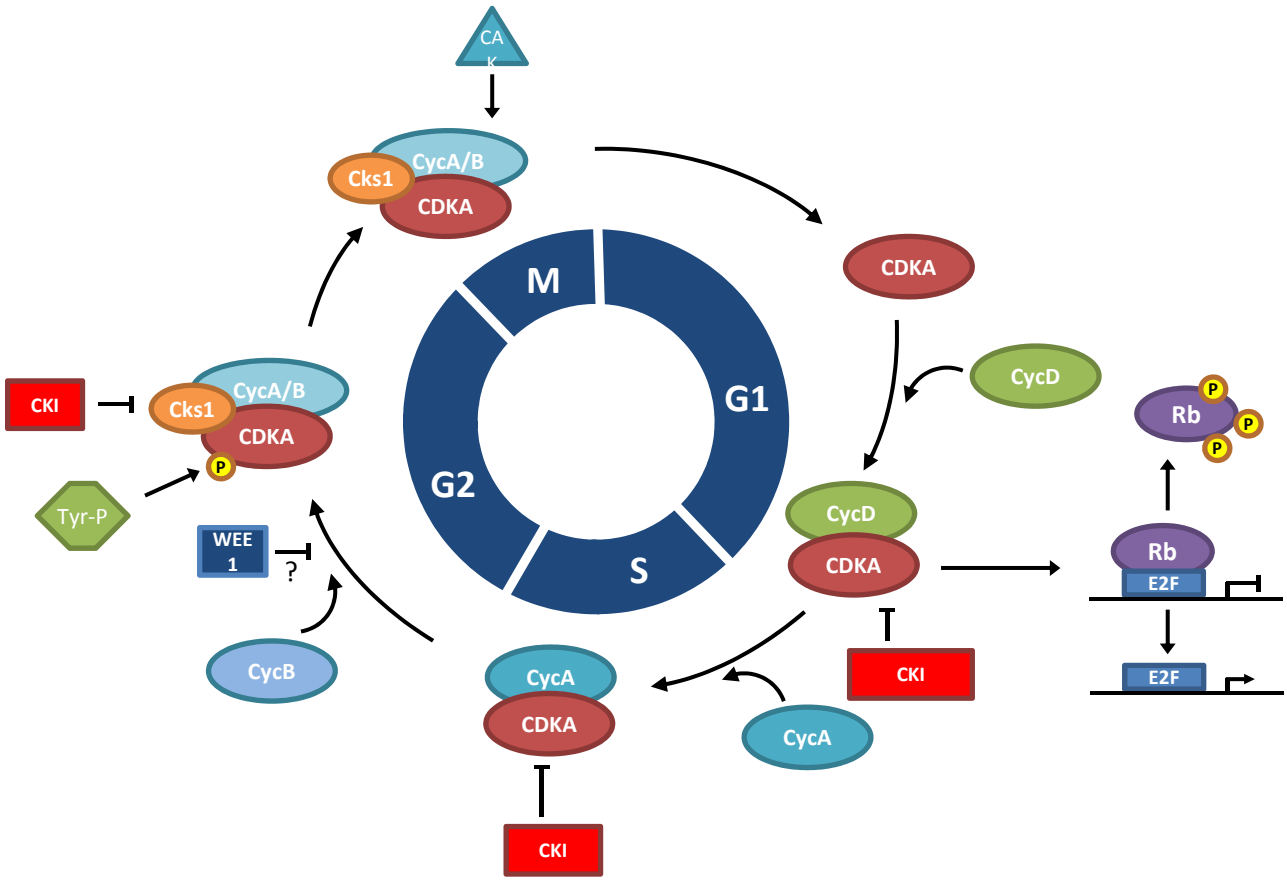


Figure 1.2: Simplified model of cell cycle regulation in plants. Progression of the cell cycle is controlled by different Cyclin/CDK complexes. At the G1/S transition, CycD/CDK complex phosphorylates RBR factor releasing E2F transcriptional factor and activation of transcription of S phase-specific genes. At the G2/M transition the M-phase-specific CDK activity is regulated by phosphorylation and dephosphorylation of specific amino acid residues. The activity of Cyclin/CDK complexes is also regulated by different CKIs.

substrates at the G1/S and G2/M transition checkpoints triggers DNA replication or entry into the mitosis (Francis 2007).

1.1.1.1. Cyclin-dependent kinases (CDKs)

Cdc2 was a first CDK identified in fission yeast *Schizosacharomyces pombe* and later its orthologue Cdc28 was identified also in budding yeast *Saccharomyces cerevisiae* (Hartwell et al. 1974, Nurse et al. 1980, Hindley et al. 1984). Cdc2 is the only one CDK in *S.pombe,* but since its identification multiple cdc2/CDC28-related genes were identified in other species. They are characterized by distinct sequences within the cyclin binding motif (mainly PSTAIRE) which is common for principle mitotic CDKs in animals and yeasts. In plants, there is only one CDK containing PSTAIRE which is designated as CDKA1 but also other CDKs – CDK B, C, D, E and F (Fobert et al. 1996; Joube et al. 2000, Vandepoele et al. 2002). CDKA1 appears to be a main plant CDK involved in the regulation of both G1/S and G2/M checkpoints and similar to other PSTAIRE CDKAs identified animals and yeasts, mRNA and protein levels of CDKA1 are constant throughout the cell cycle (Reichheld et al. 1999; Menges and Murray, 2002). It is necessary for cell cycle progression, because Arabidopsis T-DNA insertion mutant for CDKA1 is embryonic lethal, however for proper plant development only low CDKA activity is needed (Nowack et al. 2006, Dissmeyer et al. 2009). Four CDKBs are plant specific and instead of PSTAIRE motif contain other unique motifs, either PPTALRE (B1-type – CDKB1;1, CDKB1;2) or PPTTLRE (B2-type – CDKB2;1, CDKB2;2). Unlike typical CDKs, the expression of CDKBs is strictly cell cycle dependent – CDKB1s are present from S phase to mitosis whereas CDKB2s are produced in more restricted period from G2 to mitosis with the peak at the G2/M boundary (Porceddu et al. 2001). Arabidopsis CDKB1 activity is necessary for correct stomatal formation (Boudolf et al. 2004) and its role is also required for meristem organization and cell cycle progression in shoot apical meristem (Andersen et al. 2008). Other CDKs described in plants seem not to have direct role in cell cycle regulation but are rather considered as CDK-activating kinases (CAKs) or they are implicated in regulation of transcription, cell expansion or cell fate determination (Barrôco et al. 2003, Wang et Chen, 2004).

1.1.2. Regulation of Cyclin dependent kinases

1.1.2.1. Cyclins

CDK activity and substrate specificity is given by interaction with regulatory cyclin which is the first level of CDK regulation (Figure 1.2.). The name cyclin was given to these proteins in mammals due to the different waves of cyclin expression occurring during cell cycle progression. High number of cyclins has been identified in various plant species. For example Arabidopsis genome encodes 50 members of cyclin family which are classified into three major groups – cyclins D, A and B. They share the homology with their animal and yeast counterparts, except for yeasts D-type cyclins.

Cyclins C, H, L, P and T are other members of plant cyclin family (Vandoepole et al. 2002, Wang et al. 2004).

D-type cyclins regulate the G1/S checkpoint and work in a mitogen-dependent manner in association with CDKA1. It means that they are proposed to be integrators of external and internal signals such as sucrose or phytohormones leading eventually to cell division. For example, the expression of *CycD3* is reduced under sucrose starvation whereas under higher concentration of cytokinines its expression is upregulated (Helay et al 2001; Riou-Khamlichi et al. 1999). Overexpression of *CycD3* also drives cell cycle progression in cell culture (Menges et al. 2006).

Plant A-type cyclins are divided into 3 classes. They associate with CDKA1 and CDKBs and they play a role during both S phase and M phase control (Roudier et al. 2000). Their expression in synchronized tobacco BY-2 cells is induced during G1/S transition but then their mRNA levels progressively decrease during mitosis (Reichheld et al. 1996). Mutation of *CycA3* results in increased ploidy levels in Arabidopsis (Imai et al. 2006). On the contrary, *CycA* overexpression results in ectopic cell division, delayed differentiation and increased expression of S-phase specific genes (Yu et al. 2003; Takahashi et al. 2010). These results indicate that A-type cyclins play an important regulatory role during G1/S transition and are also important for the control of endoreduplication.

B-type cyclins are divided into two groups - Cyclins B1 and B2. They are tightly linked to the G2/M checkpoint and the control of M phase progression and therefore they are suitable markers of G2/M checkpoint and cell division. They interact both with CDKA1 and CDKBs (Weingartner et al., 2004, de Almeida Engler et al. 2009) and in Arabidopsis their role in positive regulation of cell division have been shown by ectopic expression of cyclin B1;1 promoting root growth (Doerner et al. 1996).

1.1.2.2. Protein degradation

Another level of cell cycle control is the modulation of cell cycle regulators activity by protein degradation in the proteasome. Specific protein degradation ensures that the cell cycle progress unidirectionally and thus provides an irreversible mechanism that drives the cell cycle forward. Proteins addressed for degradation via ubiquitin-26S proteasome pathway are marked by ubiquitinylation which consist of three steps. E3 ubiquitin ligase is able to bind target protein and E2 ubiquitin-carrying enzyme (Pickart 2001). There are two E3 ubiquitin ligase complexes - APC and Skp1/Cullin/F-Box (SCF)-related complex which are involved in basic regulation of the cell cycle (Vodermaier 2004). CDH1/FZR is an activator of APC and in plants two types are present, CCS52A and CCS52B (Tarayre et al. 2004). Their deregulation inhibits entry into mitosis and also induces endoreduplication (Vinardell et al. 2003). A, B and D-type cyclins are good example of cell cycle regulators whose activity is controlled by protein degradation in 26S proteasome. The degradation is

facilitated by the presence of conserved destruction box (D-box) in their sequence. For example B1-type cyclin are target for ubiquitin ligase complex strongly resembling APC but the degradation of B2-type cyclin at prometaphase seems to be proteasome independent (Criqui et al. 2000). Moreover constitutive expression of a non degradable cyclin B2 with mutated its D-box results in G2/M checkpoint misregulation and increased ploidy levels (Weingartner et al. 2003).

1.1.2.3. Phosphorylation and dephosphorylation

Next level of CDK regulation is phosphorylation and dephosphorylation of specific amino acid residues. For CDK activation, Thr14 and/or Tyr15 are needed to be dephosphorylated by CDC25 phosphatase whereas Thr160 is needed to be phosphorylated by some CAKs or CAKs-activating kinase (CAKAK) (Lew et Kornbluth, 1996). This model of CDK regulation is suggested to be common for all organisms investigated so far, but it seems that in plants this regulation is particular.

Phosphorylation and dephosphorylation of Thr14 and Tyr15 is made by two antagonists who function as molecular switchers – WEE1 kinase and already mentioned CDC25 phosphatase. WEE1 kinase was identified also in plant genomes of corn *Zea mays* (Sun et al. 1999) or Arabidopsis (Sorrell et al. 2002). Simultaneously, one Arabidopsis gene has been identified as a homologue of animal and yeast Cdc25; however encoding protein lacks N-terminal regulatory domain but can still dephosphorylate plant CDKs (Landrieu et al. 2004). More recently, it was demonstrated that Arabidopsis plants lacking or overexpressing this shortened Cdc25 homologue do not exhibit any cell cycle regulation defects supposing that AtCdc25 do not play an important role in plant cell cycle regulation (Dissmeyer et al. 2009). These results show plant cell cycle specificities and a divergence in cell cycle regulation which is generally well conserved between animals, yeasts and plants.

Plant CAKs or CAKAKs represent another mode of CDK regulation by phosphorylation and they could be divided into two groups according to their substrate specificity and cyclin dependence. First group consists of heterodimeric CDKD which are homologous to animal CDK7 and for their activity they need to heterodimerize with CycH (Joubes et al. 2000). Monomeric CDKF belongs to the second group which is plant-specific (de Jager et al. 2005). In Arabidopsis, this CAKAK (CDKF) phosphorylates CDKD3 and CDKD4 which in turn positively regulates the cell cycle (Umeda et al. 2005).

1.1.2.4. CKIs – CDK inhibitors

CDK inhibitors (CKIs) regulate the cell cycle by binding and inhibiting Cyclin/CDK complexes or CDK alone (Figure 1.2.) (de Clercq et Inzé, 2006). Their homologs are present in various Eukaryotes including animals, yeasts and plants. In Arabidopsis 7 different CKIs have been characterized and named as Inhibitor of Cyclin-dependent Kinases (ICKs) also known as Kip-Related Proteins (KRPs) – ICK1/KRP1, ICK2/KRP2, KRP3-7. Although the function between animal and

plants is conserved, these proteins share only partial sequence similarity with their animal counterparts Cip/Kip inhibitors (de Veylder et al. 2001). In ICK1/KRP1 several conserved regions and functional motifs are needed for CDK inhibition, nuclear localization and protein stability (Wang et al. 2007). In Arabidopsis, KRP/ICKs interact with CDKA1;1, D-type cyclins or both (Schnittger et al. 2003). The interaction of KRPs with plants specific CDKB2/CycD2 complex in insect cells was previously demonstrated but this interaction needs to be confirmed in plants (Nakai et al. 2006). The members of ICK/KRP family proteins are important for cell cycle regulation and endoreduplication (Jasinski et al. 2001). Homologs of INK4, another family of CKIs characterized in mammals, have not been yet identified in plants.

Recently, another small group of CDK inhibitor represented by the SIAMESE (SIM) has been described in plants. This protein is a key factor during the control of endoreduplication in trichomes. Walker et al. (2000) showed that *sim* mutants show inhibition of endoreduplication and formation of multicellular trichomes because of mitosis induction. On the contrary, SIM overexpression induces endoreduplication and leaf cell enlargement as a result of SIM-mediated inhibition of CDKA1;1/cyclin D complex in cooperation with a component of anaphase promoting complex (APC) CCS52A1, Kasili et al. 2010). In Arabidopsis this family consists of four members and seems to be unique to plants (Peres et al. 2007). Compared to ICK/KRPs the knowledge about SIM regulation and function is still poorly understood.

1.1.3. G1/S transition

Entry into S phase and thus beginning of the new cell cycle onset is regulated in Eukaryotes by the highly conserved pRb/E2F pathway (Figure 1.3.). Retinoblastoma (pRb) protein is able to recruit and thus block E2F transcriptional factors which are necessary for activation of S-phase specific genes (de Gregori et al. 1995; Luo et al. 1998). Second barrier for S-phase entry is the existence of different CKIs which inhibits CDK activity required for G1/S transition. In plants, the external signals as sucrose or phytohormones induce the expression of cyclin D3 that allow the formation of active CycD3/CDKA1 complex. E2F transcription factor is released from inhibitory interaction with Retinoblastoma-related protein (RBR, a plant homologue pRb) through pRB hyperphosphorylation by CycD3/CDKA1 complex. Then E2F factor activates the expression of S phase-specific genes or it can also positively regulate other pathways implicated in cell cycle progression and growth (Connell-Crowley et al. 1997; Chabouté et al. 2002; De Veylder et al. 2002). Downregulation and loss of Arabidopsis RBR function uncouples division and differentiation of meristemoid cells in leaves and disrupts the appropriate division and maintenance of meristem stem cells. Thus RBR has a critical function in the homeostasis of stem cells and organ production (Borghi et al. 2010). Deeper insight into E2F transcriptional factors as key players of E2F/RBR pathway, their description and function, will be summarized in special paragraph (see below).

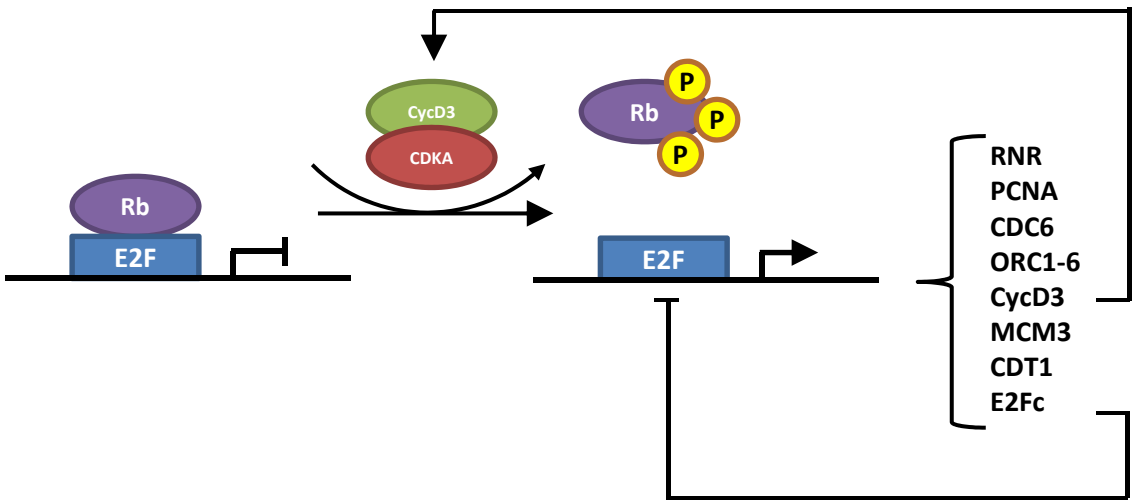
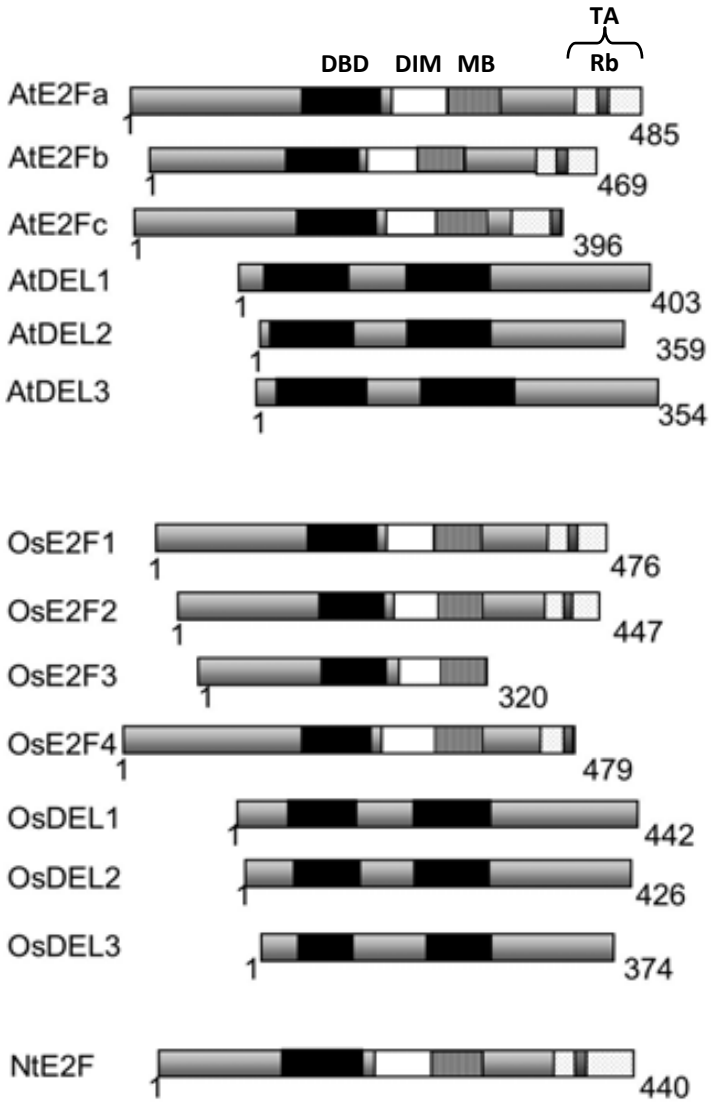


Figure 1.3: Model of G1/S transition control by E2F. After perception of mitogen signal, CycD3/CDKA complex is activated and phosphorylates Rb pocket protein. Rb phosphorylation leads to release of E2F and activation of S phase-specific genes. The control of G1/S transition encompasses positive and negative control of E2F-mediated transcription via CycD3 and E2Fc, respectively (according to Gutierrez et al. 2002).



DBD – DNA binding domain DIM – dimerization domain MB – marked box
Rb – Rb binding domain TA – transactivation domain

Figure 1.4: E2F transcriptional factors in *Arabidopsis thaliana*, O*ryza sativa*, *Nicotiana tabacum*. Typical E2Fs harbor similar domain organization - DBD, DIM, MB, Rb and TA domain whereas atypical E2Fs or DELs harbor only two DBD. Number of aminoacid residues of each E2F TF is indicated (Lincker et al. 2008).

1.1.4. G2/M transition

In plants, G2/M transition is controlled by different CDKs interacting with B-type cyclins. During G2 phase, B-type cyclins expression increases and therefore active CycB/CDK complexes are formed. Transcriptional regulation of M-phase specific genes is controlled by Myb transcription factors which recognize MBA sequence within the promoter of M phase-specific genes. For example, tobacco MybA1 and MybA2 bind to MBA of B-type cyclin and activate CycB expression whereas MybB acts competitive repressor of MybAs (Menges et al. 2005; Ito et al. 2001). It seems that not all species present these repressors in their genome as it was reported for rice or Arabidopsis (Haga et al. 2007). The initial signal activating Myb factors remains unknown but it was already demonstrated that the Myb activity depends on their phosphorylation by cyclin/CDK complexes. The presence of MBA elements in cyclin promoters make the amplification loop that super activates the Myb activity (Araki et al. 2004).

The regulation of CDK activity necessary for G2/M transition is made of course by specific phosphorylation and dephosphorylation governed by WEE1 kinase and CDC25 phosphatase. In plants, WEE1 can phosphorylate CDKA, CDKB and CDKD (Shimothono et al 2006) however the importance of CDKA1 phosphorylation during the control of G2/M checkpoint is under debate and seems to be not important (Dissmeyer et al. 2009). It is interesting to note, that in spite of lack of functional Cdc25 homologue in plant genome, the ectopic overexpression of Cdc25 from *Schizosacharomyces pombe* bypassed G2/M checkpoint induced by inhibition of cytokinin signaling (Orchard et al. 2005). Experiments with dominant negative mutants of both CDKA1 and CDKB1 have shown that CDKA1 is not required for entry into mitosis whereas inhibition of CDKB1 activity induces accumulation of cells in G2 phase (Dissmeyer et al. 2007; Porceddu et al. 2001). CDKB activity is also controlled by E2F pathway since E2F overexpression up regulates CDKB1 suggesting a possible crosstalk between regulation of G1/S and G2/M checkpoints (Boudolf et al. 2006). Linked to the G2/M checkpoint, CDKB phosphorylates also ICK2/KRP2 which then releases CDKA from its inhibition by KRP2 (Inzé et de Veylder 2006).

1.1.5. E2F transcriptional factors

As major regulators of cell cycle, apoptosis and differentiation, E2F transcription factors have been studied extensively in a broad range of organisms including mammals, worm, frog, fly and plants as well. E2F transcription factors are divided into two classes – typical E2Fs and atypical E2Fs also called DELs. They are one of the key regulators of G1/S transition and S phase progression in all Eukaryotes.

1.1.5.1. E2Fs and DELs characterization

In plants, E2Fs were identified based on their sequence homology to mammalian E2Fs and the first E2F transcription factors were identified in tobacco and wheat (Sekine et al. 1999; Ramirez-Parra

et al. 1999). A nice review summarizing the progress in the knowledge of E2F factor in plans was recently published by Lincker et al. (2008). E2Fs can be divided into two categories according to their specific domain organization (Figure 1.4.). In Arabidopsis, the first class includes typical E2Fs (E2Fa-c) containing a DNA binding domain (DBD), dimerization domain (DIM) with a leucin zipper, required for its heterodimerization with DP protein, and marked box (MB) involved in specific protein/protein interactions. Typical E2Fs also contain transactivation domain (TA) containing a RBR binding domain (RBD) for repression of E2F activity. For the transactivation of E2F target genes, E2Fs require to associate with a dimerization partner (DP) protein which harbors second DBD domain necessary for the binding to E2F promoters (van den Heuvel et Dyson 2008). In Arabidopsis, two DPs (DPa and DPb) have been identified (Magyar et al. 2000). E2Fa and E2Fb are transcriptional activators (Rossignol et al. 2002) whereas E2Fc with a truncated TA domain is a transcriptional repressor (del Pozo et al. 2002).

Second class of "atypical" E2Fs (E2Fd-f) have been first identified in plants and later also in animals by the presence of two E2F DBD motifs in their sequence (Lincker et al. 2008). Low overall sequence similarity between typical and atypical E2Fs implies important structural differences between these two classes. Because of their phylogenic position between E2Fs and DPs, atypical E2Fs were designated also as E2F-DP-like (DEL1-3) (Lammens et al. 2009). DEL factors posses two DBD domains that bind E2F elements in a DP-independent manner and act as transcriptional repressors probably by competition with activating E2Fs on the same E2F-binding site (Lammens et al. 2009).

In other plants species, E2F factors have been identified as well – moss *Physcomitrella patens* contains only one first class E2F whereas other fully sequenced genome of unicellular algae *Ostreococcus tauri* harbors one typical OtE2F factor with simple domain organization and one OtDEL. Rice have a similar E2F organization as in Arabidopsis – four members of typical OsE2Fs and three members of OsDEL factors have been described (Guo et al. 2007).

In other organisms which are not fully sequenced, we can only estimate the total number of E2Fs. NtE2F is the only E2F factor characterized in *Nicotiana tabaccum* and is similar to E2Fa and E2Fb thus it is considered as a transcriptional activator. However, tobacco EST database analysis suggests the existence at least of two other NtE2Fs (Chabouté et al. 2000).

1.1.5.2. Physiological role of E2Fs

Target E2F genes are characterized by specific canonical DNA binding site in their promoter – TTTC/GC/GCGC which is conserved between plants and animals (Lincker et al. 2008). In Arabidopsis genome about 23% genes contain this E2F binding site however not all these genes are E2F-regulated and only a small fraction has been demonstrated to be an E2F-target (Ramirez-Para et al. 2003; Chabouté et al. 2000, Lincker et al. 2004; Naouar et al. 2009). In mammals and plants,

another specific sequence GGCGG, called CDE element (cell cycle-dependent expression), was shown to be also an E2F target sequence important for transcriptional repression, notably outside of S phase (Lincker et al. 2006). Several *in silico* and ChIP analyses have identified about 334 candidates for E2F target genes involved in cell cycle regulation, DNA replication and chromatin dynamics (Vandepoele et al. 2002; Naouar et al. 2009).

The genes upregulated by E2F are similar between plants and animals. In plants, the first E2F target gene identified in plants was genes encoding Ribonucleotide Reductase (RNR), the enzyme providing dNTP pool needed for DNA synthesis (Chabouté et al. 2000, 2002). Among other genes controlled by E2F are *PCNA*, *CDC6*, *MCM3*, *ORC*, *CDT1* or DNA polymerase ε – coding for proteins implicated in DNA replication or DNA repair (Lincker et al. 2008; Naouar et al. 2009). For example, overexpression of *E2Fa* or *E2Fb* in both Arabidopsis and tobacco plants induces the expression of E2F target genes such as CDC6, ORC1 or MCM5 (Koshugi and Ohashi 2005).

Interestingly, not only E2F-target genes are controlled in a cell-cycle specific manner, but also some E2Fs exhibit cell cycle-dependent transcriptional regulation. While *E2Fa* is specifically expressed at G1/S transition, *E2Fc* is mainly expressed during S and S/G2 transition whereas *E2Fb* is constitutively expressed throughout the cell cycle. It has been also proposed that E2Fa controls the expression of *E2Fb* and negatively regulates *E2Fc* creating a negative feedback control of *E2Fa* (Magyar et al. 2005; Sozzani et al. 2006, Vandepoele et al. 2005). Auxin is a hormone which coordinates cell division and cell growth and differentiation. It was shown that *E2Fb* expression and protein stability are positively regulated by auxin and *E2Fb* ectopic expression can overcome cell cycle block caused by auxin deprivation (Magyar et al. 2005).

The role of E2F factors during plant growth, development or stress response was investigated using T-DNA insertion mutants, RNAi approach or ectopic overexpression. In quiescent Arabidopsis protoplasts, it was shown that *E2Fa* overexpression can induce re-entry into S phase (Rossignol et al. 2002). Arabidopsis plants overexpressing *E2Fa* induces ectopic cell division and even endoreduplication when coexpressed with *DPa*. Increased *E2Fb* transcripts were detected in dividing tissue such as meristem but also in cotyledons with high level of endoreduplication (de Veylder et al. 2002; Sozzani et al. 2006). In contrast to *E2Fa* and *E2Fb,* overexpression of *E2Fc* reduces cell division and induces endoreduplication and thus E2Fc controls with other E2Fs the balance between cell division and endoreduplication (del Pozo et al. 2006).

1.1.5.3. Physiological role of DELs

Transcriptional regulation of DELs differs between animals and plants. In animals, the highest expression of DELs is at the G1/S transition which is consistent with their function during DNA replication. DELs are unique repressive arm of E2F transcriptional network that is important for

regulating cellular proliferation (Logan et al., 2004-2005). In plants, the expression of DEL factors is also cell cycle regulated, but they reach the highest level at the G2/M boundary with another additional peak at G1/S for *DEL1* and *DEL3* (Lammens et al. 2009). These two genes are also believed to be controlled by E2Fa (Vandepoele et al. 2005; Naouar et al. 2009). The expression of *DEL1* and *3* is also tissue specific; the highest levels were detected in young growing tissue, such as young leaves and immature flower buds whereas their expression remains low in mature tissue (Kosugi and Ohashi 2002). *DEL2* transcripts are present in both developing and mature tissues but the protein preferentially accumulates in mature tissues (Sozzani et al. 2010).

DEL1 and 3 (E2Fe, E2Fd) are considered as competitive repressors of E2Fa-b factors because they are able to inhibit the transactivation of an E2F-responsive reporter gene activated by E2Fa or E2Fb (Mariconti et al. 2002, Koshugi et Ohashi 2002). Loss of DEL1 function results in increased ploidy levels, whereas ectopic expression of *DEL1* reduces endoreduplication, probably via negative regulation of a component of APC CCS52A2 (Lammens et al. 2008). Ploidy changes were correlated with altered expression of the genes implicated in DNA replication. Therefore DEL1 is an important inhibitor of the endocycle (Vlieghe et al. 2005). DEL3 is implicated in cell size control and cell wall biosynthesis (Ramirez-Para et al. 2004). Recently, it was shown that DEL2 (E2Ff) has an unexpected ability to promote plant cell proliferation, possibly by up-regulating *E2Fa* and downregulating the repressors of the cell cycle (Sozzani et al. 2010). These results indicate that DEL2 has a distinct role among other plant DEL factors.

1.1.6. Endoreduplication

Endoreduplication or endocycle is an alternative mode of the cell cycle when the endocycling cells undergo subsequent rounds of DNA replication without mitosis resulting in increased DNA ploidy. Endoreduplication is a common process in both animal and plants and is connected to terminal differentiation and often correlates with increased cell size. In *Arabidopsis thaliana*, mature parts of the plant can reach the ploidy up to 32C but in other species as *Arum maculatum* the endosperm can have up to 24576C.

1.1.6.1. Role of endoreduplication in plants

The physiological role of endoreduplication is still under debate but D. Inzé and L. de Veylder (2006) reviewed several hypotheses. Endoreduplication probably plays a role during differentiation process because the onset of the endocycle often characterizes the switch between cell proliferation and cell differentiation. Plant species that endoreduplicate have often a rapid cell cycle thus endoreduplication might support fast development. Endoreduplication was also suggested to maintain the balance between cell volume and nuclear DNA. Another hypothesis expects that endoreduplication might buffer mutations accumulated in the genome during plant sessile life and thus help to keep the integrity of the genetic information (Inzé et de Veylder 2006).

1.1.6.2. Regulation of endoreduplication onset

Normal mitotic cycle and endocycle have DNA replication in common. Interestingly, constitutive overexpression of genes promoting G1/S transition drives the cells in both, cell division and endoreduplication. This was reported for activating E2F transcriptional factors (de Veylder et al. 2002; Koshugi et Ohashi 2003). Because both processes were activated it was hypothesized that the switch to endocycle is controlled at G2/M boundary. This was confirmed by an observation that during the switch to the endocycle coincides with a decrease of M-phase specific CDK activity. For example, coexpression of dominant-negative *CDKB1* mutant with *E2Fa/DPa* increases endoreduplication (Boudolf et al. 2004b). Contrary, overproduction of several cyclin genes (*CycA3*, *CycA2*, *CycD3*, *CycB*) enhance M-phases specific CDK activity and thus inhibits endoreduplication onset (Dewitte et al. 2003; Imai et al. 2006; Yu et al. 2003, Schnittger et al. 2002).

As mentioned previously, CDK activity could be negatively regulated also by CKIs. Inhibition of mitotic CDKs by CKIs might therefore modulate the entry to the endocycle what was demonstrated for CDK inhibitor ICK2/KRP2 specifically binding CDKA1 (Verkest et al. 2005). Similar results were observed after overexpression of *ICK1/KRP1* resulting in block of mitosis but not of S-phase entry leading to increased levels of endoreduplication (Weinl et al. 2005). These data are in agreement with previous observations that the expression level of *ICK1/KRP1* is increased in endoreduplicating tissue (Ormenese et al. 2004).

Another regulator of endocycle onset is a part of APC CCS52A which marks mitotic cyclins for degradation via 26S proteasome pathway. CCS52A upregulation leads to inhibition of M phase-specific Cyclin/CDK complexes and thereby to induction of endoreduplication (Cebolla et al. 1999; Vinardell et al. 2003). Possible target of CCS52A could be a cyclin partner of CDKB1 (Inzé et de Veylder 2006). Indeed, CCS52A1 participates in endocycle onset by down-regulating CDKB1 activity through the destruction of CYCA2;3 (Boudolf et al. 2009).

Recently, another 26S proteasome subunit RPT2a was shown to be important regulator of endoreduplication. The Arabidopsis knockout mutant *rpt2a* shows an increased ploidy in the leaves probably by regulating degradation of positive endocycle regulators (Sako et al. 2010).

Another documented regulator of endoreduplication onset might be the WEE1 kinase, inhibiting mitotic CDK by phosphorylation. Elevated WEE1 levels were associated with endoreduplicating tissue during tomato fruit development suggesting that endoreduplication may require elevated *Wee1* transcript levels. However little is known if WEE1 kinase activity correlates with the endoreduplication process (Gonzales et al. 2007).

Last group of proteins tightly linked to the regulation of endoreduplication are atypical E2F proteins DELs. As already mentioned, elevated levels of DEL1 reduces endoreduplication whereas

loss of DEL1 function results in increased ploidy. The mechanism of this response is probably the repression of genes important for endoreduplication induction and thereby maintenance of mitotic state of proliferating tissue (Vlieghe et al. 2005).

Auxins have a physiological role in coordinating the developmental transition from mitotic cycle to the endocycle in Arabidopsis root which is mediated by the TIR1-AUX/IAA-ARF-dependent auxin signaling pathway. High auxin levels at the proximal root meristem repress the endocycle and promote M phase (Ishida et al. 2010). Depletion of auxin signal does not simply block the mitotic cycle but also allow the alternative cycle and permits endoreduplication linked with organ growth and development. Importance of auxin signaling during regulation of endoreduplication is suggested because depletion of auxin 2,4-D from culture media induces endoreduplication in tobacco BY-2 cells (Quélo et al. 2002; Magyar et al. 2005).

1.2. DNA damage response

1.2.1. Different DNA damage inducers

DNA damaging agents come from internal or external sources. One of them is UV irradiation which is probably one of the most obvious DNA damaging agents for sessile plants, therefore plants had to develop much higher tolerance to UV than animals. Among plant adaptations to UV irradiation are waxy substances on leaf surface, cell wall or intracellular plant pigments (Schmitz-Hoerner et Weissenbock 2003). These compounds often contain conjugated double bonds which are able to efficiently absorb high-energy photons damaging DNA while other substances and leaf structures efficiently reflex UV radiations. Among the most prevalent effect of UV-B and UV-C is production of dimers between pyrimidine bases. UV-A with the lowest energy is less harmful for DNA because it produces DNA damage indirectly by generation of oxygen and hydroxyl radicals (reactive oxygen species - ROS) which in turn react with DNA resulting in photoproducts formation, DNA strand breaks and DNA-protein cross links. Among other exogenous genotoxics are ionizing radiations, chemical compounds such as hydroxyurea (HU), bleomycin (BLM), methyl methane sulphonate (MMS), aphidicolin (APC), mitomycin C (MMC), camptothecin (CPT) or *cis*-platin. Similarly to UV irradiation, some of these chemical genotoxics (BLM, cis-platin) induce DNA damage via secondary production of reactive oxygen species (ROS) (Chen et Stubbe 2005; Brozovic et al. 2010).

Endogenous stress induced DNA damage is represented mainly by ROS which are produced not only during photosynthesis and respiration but also during the response to various environmental challenges. Oxidative damage of DNA can be divided into two types. First is the production of altered bases and second is the damage of sugar residue of the sugar phosphate DNA backbone. Damage of DNA bases generally involves •OH addition to double bonds whereas sugar residues are mainly damaged by hydrogen abstraction from the deoxyribose (Dizdaroglu 1993). This deoxyribose alteration leads to DNA single strand breaks (SSBs) production however ROS can induce also DNA double strand breaks (DSBs) when the radicals induced by ionizing radiation are produced in clusters that damage both DNA strands close to each other (Ward 1988).

Second important endogenous source of DNA damage is spontaneous integration of errors into the DNA sequence during DNA replication by DNA polymerase. Meiosis is another cellular process when DNA damage occurs due to the important rearrangements of chromosomes. The overall scheme of different sources of DNA damage and DNA repair pathways are summarized in Figure 1.5A.

The character of DNA damage response during DNA replication depends on the type of DNA damage. For example inhibition of replication fork progression (called replicative stress) could be induced by direct inhibition of DNA polymerase but also when DNA polymerase cannot override the lesions present in DNA molecule such as DNA base dimers. The most common drugs inhibiting DNA

synthesis are hydroxyurea and aphidicolin. HU is able to block the enzyme Ribonucleotide Reductase (RNR) providing the dNTPs for DNA synthesis (Eklund et al. 2001) whereas APC preferentially blocks DNA polymerase α (Litvak et Castroviejo 1985). This polymerase is implicated in DNA synthesis initiation and its inhibition results in the arrest of replication fork (Chen et al. 1990). It is important to note that both drugs act probably through different checkpoints (Culligan et al. 2004) and that their effects are reversible what is used in different cell cycle studies.

Genotoxics that was used as a main DSB-inducing in this study is the bleomycin (BLM), a member of glycopeptide antibiotics which was for the first time isolated from *Streptomyces verticillus* (Umezawa et al. 1966) and which is widely used in anticancer therapy. The cytotoxic and mutagenic effect of this compound is a complex mechanism comprising SSBs production which could be than converted to DBSs depending on DNA sequence around the lesion. Therefore the ratios of SSBs to DBSs cleavage events within DNA range from 6:1 to 20:1. BLM requires for its proper function to be activated in presence of a reduced metal (Cu^{2+} or Fe^{+}), oxygen and one-electron reductant (Chen et Stubbe 2005). In plant biology, BLM is widely used as a DSB inducer to study DNA DBS response (Menke et al. 2001; Roa et al. 2009) but the cellular response to BLM is better described in animals (discussed bellow, Figure 1.5B.).

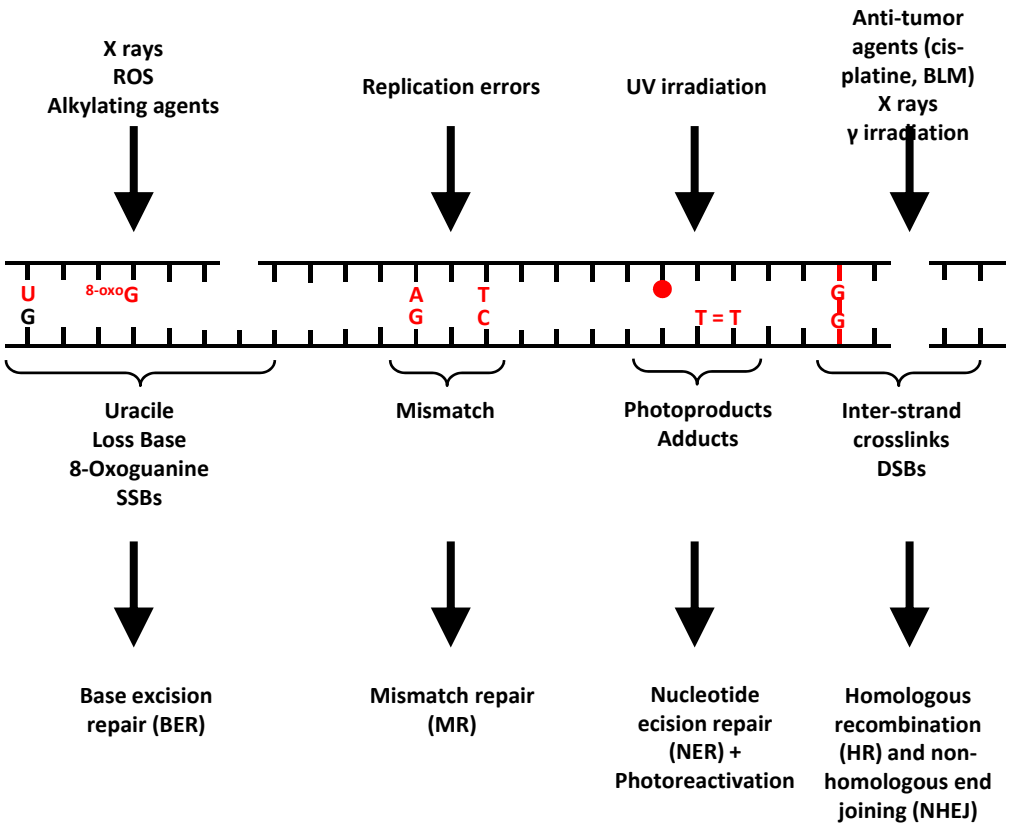
1.2.2. Different DNA repair pathways

DNA damage must be repaired in order to maintain the genome integrity. Modified nucleotide bases or thymine dimers induce replication arrest and therefore the DNA repair is also essential for normal functioning of the plant cell. High conservation of DNA repair pathways among Eukaryotes shows that DNA repair is essential for growth and faithful transmission of genetic information from one generation to the next. We know different DNA repair pathways which are activated according to the type of DNA damage. Photoreactivation, Nucleotide excision repair, Base excision repair and Mismatch repair are mechanisms which are used when only single DNA strand is modified whereas Homologue recombination or Non-homologous-End-joining are implicated during DNA DBS repair (Figure 1.5A).

1.2.2.1. Photoreactivation

UV irradiation results in the formation of photoproducts which are two adjacent DNA bases covalently bounded together. Two major DNA damage products are Cyclobutane pyrimidine dimers (CPDs) and (6-4) photoproducts. DNA damage caused by UV irradiation is repaired by photoreactivation that is carried out by photolyases, a DNA repair enzymes using the energy of blue light. Cyclobutane pyrimidine dimers (CPDs) are directly cleaved by CPD lyase and (6-4) photoproducts are cleaved by (6-4) photolyase. During this enzymatic reaction the DNA lesion is first recognized then the photoproducts are reduced by photolyase cofactor $FADH^{+}$ to monomer pyrimidines and finally the enzyme is released (Tuteja et al. 2009). Photoreactivation is the major

A



B

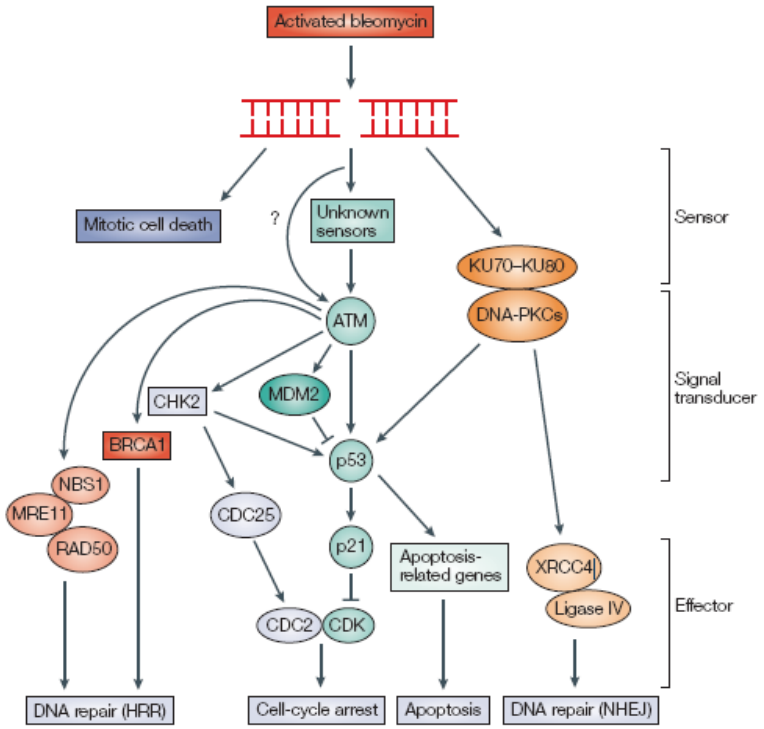


Figure 1.5: A: Different DNA damage and mode of DNA repair. **B:** Intracellular response network to BLM damage in animals (Chen et Stubbe 2005).

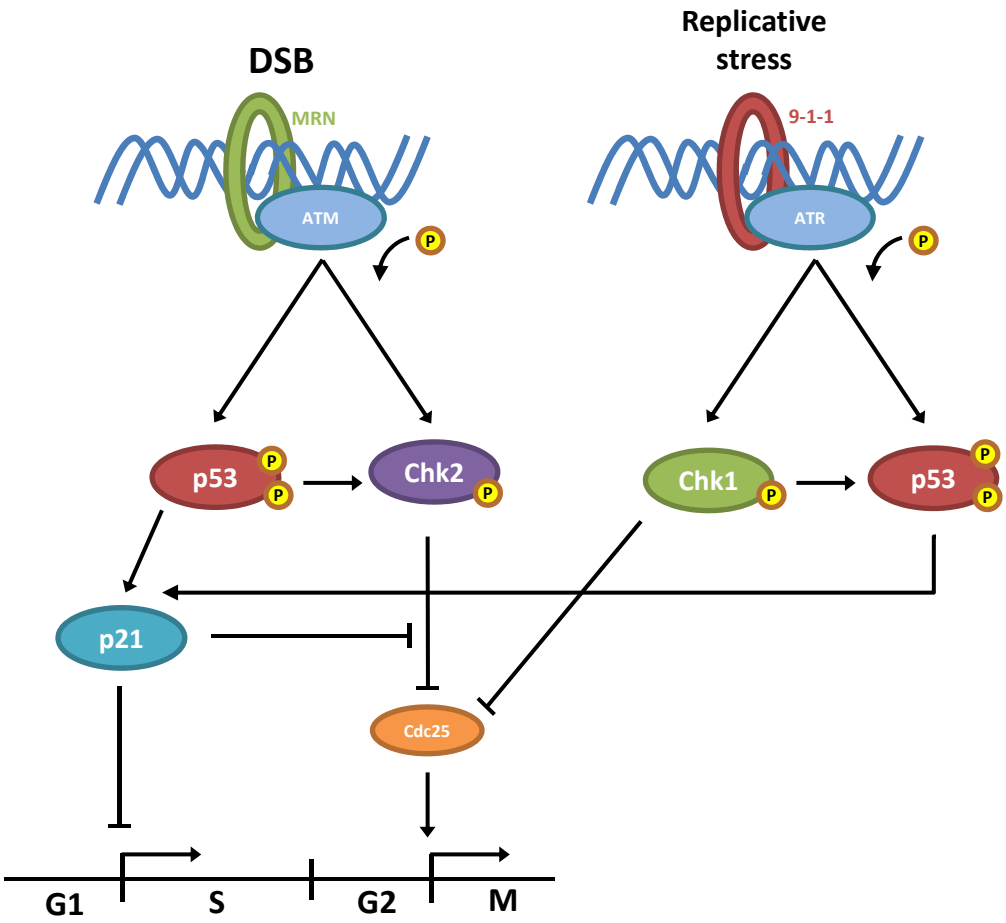


Figure 1.6: DNA damage response to DNA double strand breaks (DSBs) and replicative stress in animals. During DNA DSB response the lesion is recognized by MRN complex, a sliding clump on the DNA molecule facilitating the phosphorylation and activation of sensor kinase ATM. This kinase targets p53 and Chk2 arresting the cell cycle progression at the G1/S or G2/M boundary via p21 or Cdc25.
After SSB or stalled replication forks ATR kinase is activated in cooperation with 9-1-1 complex. ATR phosphorylates and activates its downstream targets Chk1 and p53 which leads to the arrest of cell cycle progression at the G1/S and G2/M boundary similarly as after ATM activation.

DNA repair pathway in non-proliferating cells (Takahashi et al. 2002; Kimura et al. 2004). In cucumber the photoreactivation activity is highest during the afternoon when the plants receive full sunshine.

1.2.2.2. Base excision repair

Base excision repair (BER) is a mechanism which permits to repair DNA damage induced by ROS, ionizing radiation, alkylating agents (*cis*-platin) or by spontaneous mutations. The examples of DNA damages are spontaneous deamination of cytosine into uracil, xanthine or hypoxanthine, oxidation of DNA bases, alkylation or apurinic/apyrimidinic (AP) sites formation where the DNA base is completely lost (Evans et al. 2004). Base modification is widely used in plant mutagenesis when ethylmethane sulphonate (EMS) induces alkylation products which could be recognized during DNA replication as adenine causing G-A mutation (Greene et al. 2003).

1.2.2.3. Nucleotide excision repair

In plants and animals, Nucleotide excision repair (NER) is another conserved mechanism to repair DNA lesions induced by UV or *cis*-platine. First, NER consists of DNA damage recognition and removal of neighboring region of 24-32 nucleotides. The gap is filled by DNA polymerase and finally new DNA molecules are ligated together. We recognize two different types of NER, one designated Global Genome Repair (GGR) which repairs DNA lesions over the entire genome while Transcription-coupled repair (TCR) specifically restores DNA damage on transcribed DNA molecule. Most of the genes implicated in NER were also characterized in plants and by mutational analysis it has been shown that NER is an important mechanism during plant challenge with genotoxic stress as in animals (Jiang et al. 1997).

1.2.2.4. Mismatch repair

In DNA molecule, the mispaired bases arise from replication errors, homologous recombination and as a result of DNA damage (Schofield et Hsieh 2003). The cells defective in mismatch repair (MMR) have a rate of spontaneous mutations highly elevated therefore this DNA repair pathway is an important mechanism for maintenance of genome integrity. The template DNA with correct sequence is recognized by methylation of parental DNA strand whereas the DNA strand with replication error is unmethylated (Kimura et Sakaguchi 2006). In Eukaryotes some of the steps during MMR are not yet fully understood.

1.2.2.5. Repair of double strand breaks

Compared to the repair of single strand breaks, the importance of double strand break (DBS) repair is even higher because the DNA molecule is separated in two fragments and after there is not any complementary DNA to serve as a template. If DNA lesions are not repaired in time, DBSs can lead to the loss of chromosomal parts which could be lethal for the entire cell. DNA DBS could be

induced by exogenous agents as ionizing irradiation γ or X, chemical compounds such as BLM or CPT. DBSs can be also induced by endogenous causes for example during DNA replication or repair of other kinds of DNA lesions and meiosis (Bleuyard et al. 2006).

As already mentioned, DBSs can be repaired by two different strategies based on DNA recombination. Homologous recombination (HR) uses for repair as a template the second homologous DNA molecule therefore this mechanism represents more accurate pathway without any loss of genetic information. During Non-homologous recombination (NHR) repair, two ends of DNA molecule are simply joined together what is by contrast connected with a risk of inaccurate repair because of frequent insertion or deletion of DNA sequences (Lees-Miller et al. 2003). HR can leads to loss of genetic information as well what could happen especially to DNA located between two homologous sequences.

HR between sister chromatids or homologous chromosomes starts by formation of single stranded DNA at DBS site in participation of MRN complex (Mre11/Rad50/Nbs1). Recombination is mediated by RecA homologs including Rad51 through Holliday structure as an intermediate. The Holliday structure is then incised by endonucleases and finally the repair proteins dissociate by an unknown mechanism (Kimura et Sakaguchi 2006). Homologous recombination preferentially occurs during S and G2 phase of the cell cycle when sister chromatids are easily available.

NHR has two different forms according to the implication (or not) of Ku70/Ku80 protein complex. Nonhomologous-end joining (NHEJ) is the major mechanism to repair DNA DBSs based on juxtaposition of the blunt ends or the ends with short overhangs which are joined together in assistance of Ku70/Ku80 heterodimer which is important for recognition and stabilization of DBS ends (Walker et al. 2001).. DNA-PKs is found mainly in vertebrates (and curiously in the mosquito, honey bee, and sea urchin) whereas other NHEJ components (Ku70, Ku80, XRCC4, DNA Lig4) are present also in plants and lower eukaryotes including *C. elegans, Drosophila,* yeasts, and distantly related homologs are present even in bacteria. Micro-homology mediated end joining (MHEJ) is Ku-independent pathway which has been identified in animals, yeasts and plants. The ends at the DBS site are processed into single-stranded DNA and these short overlaps with low sequence “micro” homology complement together. Any resulting flaps are excised and finally the ends are ligated together. In contrast to HR, the DBSs repair by NHR is mainly used during G1 phase and at the beginning of S phase because at these times the chromosomes are formed only by one chromatid. In plants, some members of NHEJ signaling such as DNA-PK were not identified (Bleuyard et al. 2009).

1.2.3. DNA stress checkpoint activation

Once the DNA damage is sensed by specific sensor proteins, the signal is then transduced on effector proteins implicated in the activation of DNA repair pathways and in delaying or arresting cell cycle progression. Activation of cell cycle checkpoint then provides sufficient time for DNA repair.

The activation of programmed cell death occurs if DNA damage is excessive and DNA repair would be too difficult or even more not possible.

ATM (ataxia telangiectasia mutated) and ATR (ataxia telangiectasia and Rad3-related) kinases are the main cellular sensor proteins implicated in DNA damage response. Transduction of the signal from ATM and ATR to other transducers also harboring a kinase activity is highly important to ensure corresponding cellular response (Bakkenist et Kastan 2003; Cortez et al. 2001). ATM and ATR are related kinases having a central role in sensing DNA damage in a specific manner. DNA DSB involves mainly ATM whereas ATR is preferentially activated in response to SSB or stalled replication fork during replicative stress. The mechanism of DNA stress checkpoint control is common for Eukaryotes and in animals its deregulation induces developmental defects such as cancer suggesting the major importance of DNA damage response for growth and cellular viability of living organisms. I will summarize which mechanisms are known to monitor the status and structure of DNA during cell cycle progression in animals and plants.

1.2.3.1. ATM/ATR pathway in animals

The first step in DNA damage response is the recognition of DNA damage (Figure 1.6.). Single strand DNA damage is recognized by the Rad9, Rad1 and Hus1 (9-1-1) heterotrimeric complex resembling a sliding clamp on DNA molecule. The chromatin-bound 9-1-1 complex then facilitates a phosphorylation mediated by ATR kinases (Parrilla-Castellar et al. 2004). Another complex MRN (MRE11, RAD50 and NBS1) is a sensor during ATM specific DSB response. This complex also functions as a molecular bridge between histone H2AX which is then phosphorylated by kinase ATM also known as γH2AX (Rogakou et el. 1998; Lee and Paul 2005). This H2AX phosphorylation permits chromatin remodeling and formation of specific multiproteic structure called foci which regroup the proteins implicated in DNA repair (Lisby et al. 2005). The dynamics of these foci which is related to H2AX posttranslational modifications is a matter for investigation. Besides H2AX phosphorylation, this histone is also ubiquitinated what is important for the DNA damage signal transduction to downstream effectors (Vissers et al., 2008). The loss of H2AX in mammals compromises genomic stability, probably due to a decreased DNA repair efficiency.

Activated ATM kinase also phosphorylates many other effector proteins including BRCA1, NBS1, Chk2 or tumor suppressor transcriptional factor p53 which contributes to G1/S and G2/M checkpoints, senescence and apoptosis and thereby suppresses tumorigenesis. Phosphorylation of p53 increases its stability leading to G1/S checkpoint activation via p21, an inhibitor of CDKs necessary for entry into the S phase. In addition, p21 inhibits Chk2 kinase which mediates CDC25 phosphatase inhibition leading to G2/M checkpoint activation (Giaccia et Kastan 1998). p53 is important for the transcription of DNA repair genes including p53R2 which encodes a subunit of Ribonucleotide

Reductase (Tanaka et al. 2000) and it is also a crucial regulator of apoptotic genes. Thus, p53 is an important regulator during both DNA repair and apoptosis.

ATR kinase plays a role during replicative stress therefore its primary function appears to be during S phase when DNA replication occurs. Once replication fork stalled the replicative helicase MCM continues DNA unwinding ahead of replication fork producing ssDNA stabilized by RPA protein complex. RPA allows the recruitment and activation of ATR and other cofactors which facilitates to load 9-1-1 complex on the DNA molecule (Yang et Zhou 2006). Activated ATR phosphorylates downstream effectors such as TopBP1 protein which stimulates ATR kinase activity by a positive amplification loop (Myers and Cortez 2006). Under genotoxics stress, ATR promotes intra-S checkpoint activation via phosphorylation of Chk1 (Cortez et al. 2001) and CDC25 controlling the initiation of new replicons together with WEE1 (similarly to Chk2 kinase during ATM response) (Jin et al. 2003). Inhibition of ATR and Chk1 evokes CDC25 accumulation and increased initiation of DNA replication resulting in ssDNA and DSBs production (Syljuasen et al. 2005). Similarly to ATM, ATR stabilizes p53 protein leading to the activation of G1/S or G2/M DNA damage checkpoint via p21 (Lakin et al. 1999). Finally, p53 can induce apoptosis if ATR-specific DNA damage is excessive or impossible to repair (Yonish-Rouach et al. 1991). Thus p53 is an integrator of both ATM and ATR pathways.

1.2.3.2. ATM/ATR pathway in plants

Homologues of mammalian DNA stress checkpoint proteins were also characterized in plants especially in the completely sequenced genome of *Arabidopsis thaliana*. ATM and ATR are highly conserved and in plants exhibit similar roles as their animal counterparts. ATM is activated mainly during DBS response and ATR primarily responds to replicative stress (Figure 1.7.) (Garcia et al. 2000; Culligan et al. 2004).

Compared to mammals, *atm* and *atr* mutant plants do not show any developmental defect under normal growth conditions except a partial sterility of *atm* mutant because of abundant chromosomal fragmentation during meiosis. Under stress conditions *atm* plants are hypersensitive to γ irradiation, MMS but not to UV-B and fail to induce the transcription of DSB repair-related genes (Garcia et al. 2003; Ricaud et al. 2007). The importance of ATM during plant development and stress response was demonstrated by large scale transcriptional analysis of ATM-targets upon γ irradiation. Radiosensitive genes encode proteins involved in cell cycle regulation, cell death and repair, DNA replication and recombination, translation and transcription (Ricaud et al. 2007). Plants lacking ATR are hypersensitive to replicative stress induced by HU, APC and UV-B (Culligan et al. 2004). Under stress conditions *atr* plants harbor a G2/M checkpoint activation defect and exhibit changes in regulation of RNR genes *RNR1* and *RNR2A* (Culligan et al. 2004; Roa et al. 2009). Taken these data

together it is evident that ATM and ATR kinases play a pivotal role during DNA damage response in plants.

1.2.3.3. WEE1 kinase and Cdc25 phosphatase

Wee1 kinase and Cdc25 phosphatase play an important role during cell cycle checkpoint activation notably after DNA damage. Under genotoxic stress *wee1* Arabidopsis mutant exhibits growth defects caused by alteration in DNA damage checkpoint activation and DNA repair. WEE1 controls G2/M checkpoint and it is transcriptionaly activated in ATR- or ATM-dependent manner (de Schutter et al. 2007). Until now, CDKA1 was considered as the main Wee1 target upon DNA stress but recent data showed that Arabidopsis CDKA1 phosphorylation on Thr14 and Tyr15 do not have relevance during DNA damage response (Dissmeyer et al. 2009). Based on these results and because a functional CDC25 was not identified in plants it is suggested that WEE1/CDC25 switcher of PSTAIRE-type CDK activity does not operate during cell cycle regulation. Possible role of Wee1 activity in the regulation of plant specific B-type CDKs and its role during DNA damage response remains to be elucidated.

1.2.3.4. Other regulators of DNA damage checkpoint control in plants

SIAMESE (SIM) operates as a negative inhibitor of the cell cycle progression in trichomes by its association to CDKs (Churchman et al. 2006). Interestingly, some SIM-related (SMR) genes are strongly induced in ATM-dependent manner during DNA damage response in Arabidopsis (Culligan et al. 2004) and it was hypothesized that they can potentially operate as a checkpoint regulators (Cools et de Veylder 2009). Another gene which was strongly induced in an ATM- and ATR-dependent manner is CycB1;1 while other G2-phase specific genes were rather downregulated. It is proposed that CycB1;1 do not reflect the accumulation of the cells in G2 but it reflects some other unknown role of CycB1;1 during DNA damage response (Culligan et al. 2006).

Another regulator of DNA damage checkpoint control is Suppressor of Gamma-1 (SOG1) which was identified as a second-site suppressor of radiosensitive phenotype of seeds defective in the repair endonuclease XPF (Preuss et Britt 2003). SOG1 is a putative transcriptional factor and similarly to ATM it is required for transcriptional regulation of DNA repair genes. Simultaneously, checkpoint response in *xpf* mutant is both ATR- and Sog1-dependent. Thus it seems that Sog1 is a downstream target and likely an integrator of both pathways ATM and ATR (Yoshiama et al. 2009).

Putative function in DNA stress checkpoint signaling is attributed to Mitogen-activated protein kinase (MAPK) phosphatase (MPK1). Plants lacking MPK1 are hypersensitive to UV-C and MMS. The regulation of MPK1 is rather posttranslational because there is no change in *MPK1* expression in response to genotoxics (Ulm et al. 2001). However a direct evidence of MPKs role in DNA damage stress checkpoint is still missing.

1.2.3.5. Checkpoint differences between animals and plants

Although number of DNA stress checkpoint regulators is well conserved between animals and plants, the important transducers in the ATM/ATR pathway as Chk1, Chk2 or p53 are not present in plants. Also the mutation of ATM of ATR genes does not affect plant growth and development under normal conditions whereas in animals, these mutations leads to meiotic/developmental defects and lethality, respectively, even under non-stress conditions (Lydall et al. 1996; Cliby et al. 1998).

ATM- and ATR- downstream effector p53 activates G1/S checkpoint which is therefore the main control point of DNA damage response in animals (Bernstein et al. 2002). In plants it seems that the mechanism of cell cycle arrest operates more likely at G2/M transition which is controlled probably by WEE1 or by other unknown mechanism (Carballo et al. 2006; de Shutter et al. 2007; Cools and de Veylder 2009; Dissmeyer et al. 2009). These data show that the control machinery of DNA damage checkpoint has similar regulatory elements but the regulation of the cellular response differed between animals and plants.

Role of E2F transcription factor during DNA damage response

In Eukaryotes, large number of genes implicated in DNA repair, recombination and DNA damage checkpoint regulation were identified by promoter analyses as E2F targets (Dimova et al. 2003, Vandepoele et al 2002). Moreover, overexpression of Arabidopsis *E2Fa* with its transactivation partner *DPa* leads to upregulation of DNA repair-related genes such as *Rad51*, *BRCA1*, *Rad17*, *PARP1* (Vandepoele et al. 2002). A gene encoding a small subunit of RNR *TSO2* is also induced during DNA damage response and its transcription is lost in *atm* and *e2fa* Arabidopsis mutants (Roa et al. 2009). Genotoxics-induced expression of *RNR* was shown to be under the control of NtE2F also in tobacco (Linker et al. 2004). Some of DNA repair-related genes, upregulated after γ irradiation and having an E2F binding element in their promoters, are under the control of ATM (Culligan et al. 2006).

Regulation of E2F members could be altered in response to DNA damage. Transcription of *E2Fa* could is activated after BLM and MMC treatment (Chen et al. 2003) in an ATM-dependent manner (Roa et al. 2009). Cadmium induce DNA damage indirectly by ROS generation and it was reported that cadmium response involves upregulation *E2Fb* (Herbette et al. 2006; Chamseddine Mediouni, personal communication). Induction of *E2Fb* was also observed in response to $CuSO_4$ (Chamseddine Mediouni, personal communication). It is evident that variety of DNA repair genes such as *RNR*s are under the control of E2F suggesting that E2Fs are implicated in the DNA damage response in plants, similarly to mammals. Moreover expression of some E2F-regulated genes is ATM-dependent concluding that these two proteins may act in the same signaling pathway (Roa et al., 2009).

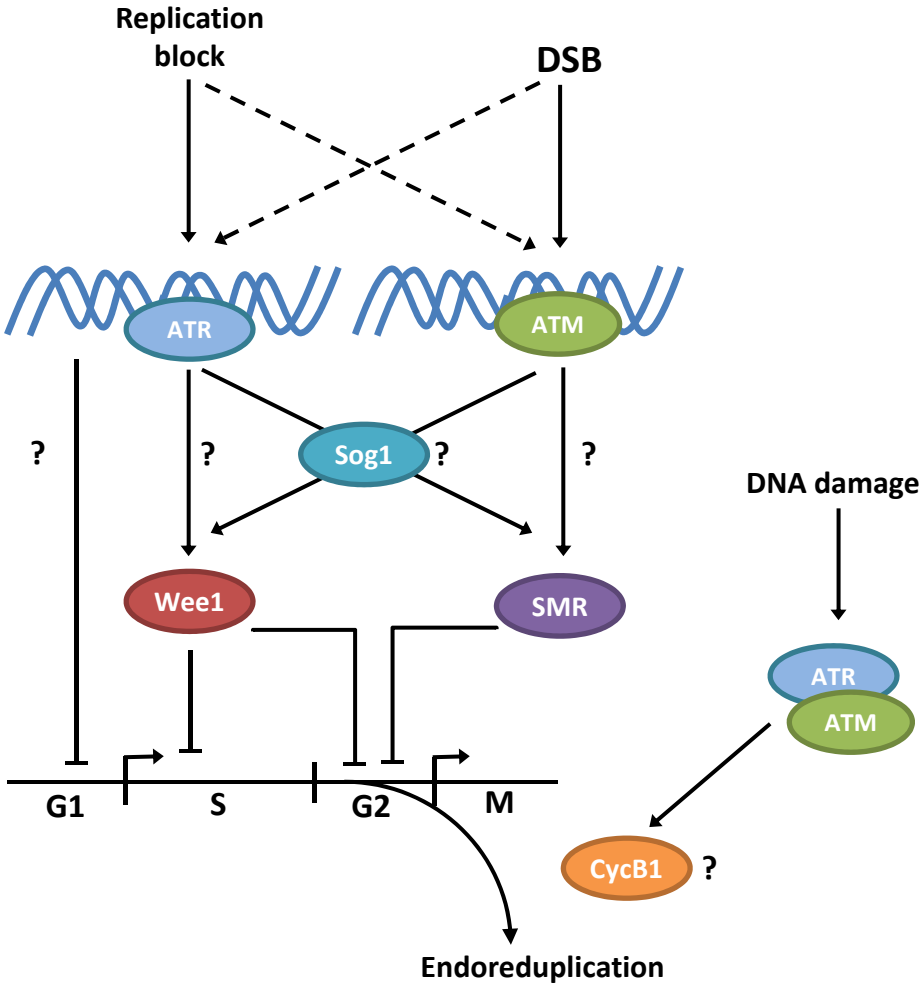


Figure 1.7: Hypothetical model of DNA damage response to DNA double strand breaks (DSB) and replicative stress in plants (Cools and de Veylder 2009). Upon DNA stress, kinases ATM and ATR are activated and inhibit the cell cycle progression at different checkpoints via Wee1 and SMR. Sog1 is an integrator of ATM and ATM pathways but it is not known if it contributes to Wee1 and SMR signaling. G2 checkpoint is controlled by Wee1 and SMR, intra-S by Wee1 and G1 by still unknown mechanism. ATM/ATR dependent stabilization of CycB1 probably prevents premature differentiation of DNA damaged cells.

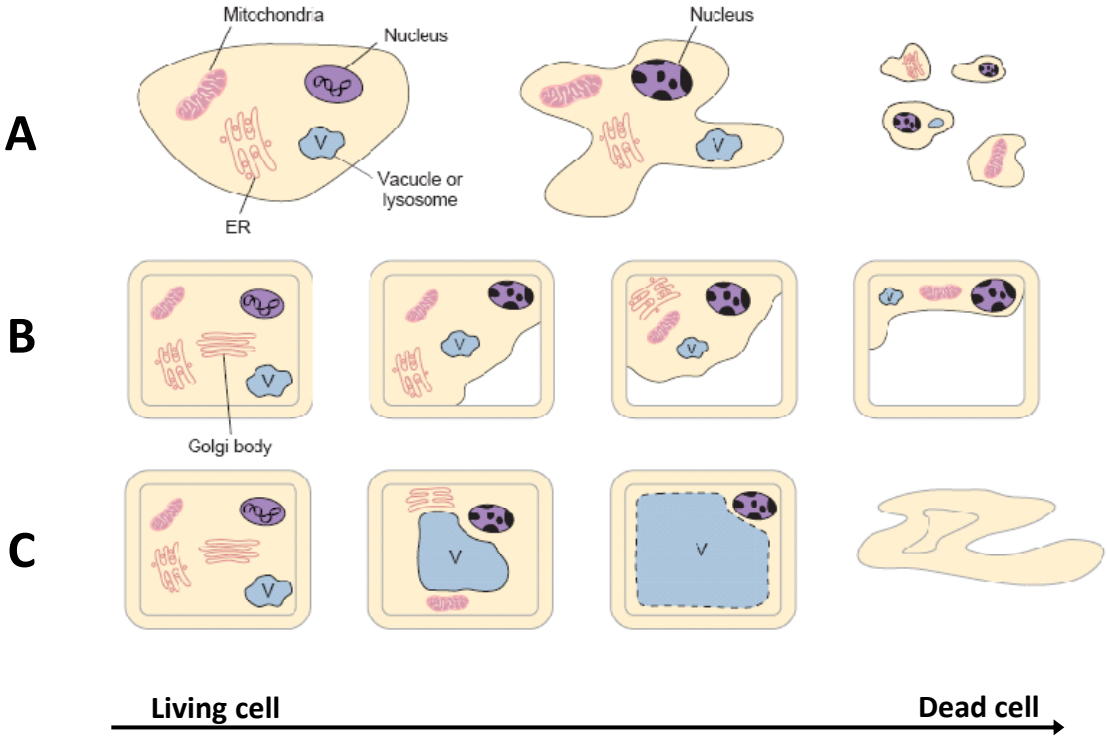


Figure 1.8: Different PCD morphotypes according to van Doorn and Woltering (2005). Cell types are divided according to implication of autophagic compartment. Apoptosis is most common PCD type in animals. **A:** During apoptosis, condensation of nucleus occurs, the cell shrinks and formation of apoptotic bodies occurs. These bodies are engulfed by other cells; **B:** Non-lysozomal PCD; **C:** Autophagic PCD is characterized by vacuolization and rupture of the vacuole leading to leakage of vacuolar hydrolytic enzymes into the cytosol and digestion of cellular components. Autophagic PCD is the mechanism described mainly during plant PCD.

1.3. Programmed cell death

Programmed cell death (PCD) plays an essential role during ontogenesis and interaction of multicellular organisms with the environment. Activation of PCD pathways results in highly controlled and coordinated set of reaction leading to degradation of cellular structures which is accompanied by characteristic morphological and biochemical changes. This altruistic suicide is a normal part of the physiology and homeostasis of multicellular organism. In the literature, we can often find a term apoptosis (Greek: *apoptosis, apo-* from and *ptosis-* falling), which is very often used as a synonym of PCD. Originally, this word means a leaf cast.

A model system to study the origin and evolution of PCD is a sporulation of *Bacillus subtilis* during unfavorable conditions. Bacterial sporulation is initiated by activation of specific genes resulting in asymmetric cell division on one mother cell and endospore. At the end of maturation, in mother cell specific enzymes are activated that results in mother cell autolysis and release of maturated endospore (Amiens 2002). This example shows that PCD cannot be positively selected in unicellular organism but this process is important for the control and survival of cell populations which form colonies during the reproduction. Therefore certain fraction of the cells undergoes altruistic suicide to maintain the stem cell line.

Programmed cell death has been extensively studied in animals because of its role during animal development. It was demonstrated that similar programmed suicide can occur also in plants sharing not only morphological and cytological similarities with animal PCD but also the conservation of this process at the molecular level suggesting a high conservation among Eukaryotes. Considerable progress of research was made in the field of animal PCD also because deregulation of animal PCD signaling pathways often leads to cancer demonstrating that the ability to undergo PCD has a protective function. In this section, I will first focus on several morphological PCD types which are distinguished in animals and plants and then on PCD signaling pathway mainly in the context of DNA damage. Finally, recent progress in the research of plant PCD induced by DNA damage will be summarized.

1.3.1. Different PCD morphotypes

Based on animal studies, three different PCD morphotypes are distinguished according to the implication of autophagic pool – apoptosis, autophagic cell death and non-lysozomal cell death (Figure 1.8.) (Clarke 1990; Jones 2000).

1.3.1.1. Apoptosis

In animals, the apoptosis is the most common PCD type. It is characterized by three steps: nuclear fragmentation, formation of apoptotic bodies – simple membrane vesicles containing

fragments of nucleus and cytoplasm, and finally phagocytosis and degradation of apoptotic bodies by surrounding cells. Other apoptotic features are chromatin condensation, implication of caspases and nucleosomal DNA fragmentation (Kerr et al. 1972). DNA fragmentation was observed also during other PCD types, however in recent years the suitability of this marker is under debate, especially in plant cells because specific DNA fragmentation can occur also in cells which died in a non-programmed manner (Kuthanová et al. 2008). In plants, typical apoptosis cannot be present at least due to the structural specifities of plant such as rigid cell wall which does not permit phagocytosis of dead protoplast by adjacent cells.

1.3.1.2. Autophagic PCD

Autophagy is a mechanism to degrade and recycle cellular components in all Eukaryotes. During typical autophagy, small vesicles containing parts of the cytoplasm are targeted for degradation into the lysosome (Klionsky et al. 2000). According to the volume of cytoplasm degradated at once we can distinguish micro-, macro- and mega-autophagy. Microautophagy involves pinocytosis of small amounts of cytoplasm in the lysosome. Formation of autophagosome which is an intracellular double-membrane structure containing elements of cell´s own cytoplasm and its fusion with a lysosome characterize macroautophagy. Last, megautophagy supposes rupture of lysosome or lytic vacuole and leakage of hydrolytic enzymes into the cytoplasm leading to degradation of cellular content (Bassham et al. 2006). In plants, autophagy has an established role during development, environmental stress, starvation and senescence (Hayward et al. 2009).

Autophagy is a mechanism how the cell can restore the nutrients necessary during particular developmental steps such as germination of monocotyledon plants. Aleurone is a cell layer enclosing endosperm rich storage proteins which provide an important pool of energy for embryo development. PCD of aleurone cells is mediated through vacuole rupture and leakage of hydrolases required for the breakdown of reserves in the endosperm. Compared to apoptosis, autophagic PCD does not necessary present typical DNA fragmentation (Fath et al. 2000). A good plant model for vacuole-mediated (autophagic) PCD has been established and well described during tracheary element (TE) formation (Obara et al. 2001). TE-specific PCD is accompanied by specific DNA fragmentation typical for apoptosis (Mittler et Lam 1995). Therefore, DNA fragmentation typical for apoptosis is not a hallmark of plant autophagic PCD and it cannot be used as a specific marker for PCD.

1.3.1.3. Non-lysosomal-type PCD

Third type of Eukaryotic PCD is called necrosis-like PCD or non-lysosomal PCD and involves neither lysosome of dying cell nor lysosome of other cell. The cells slowly die by inhibition of major biosynthetic pathways, membrane destabilization or by other unknown ways (van Doorn and Woltering 2005). It seems that this type of PCD is not so common compared to the frequency of

apoptosis or autophagy in Metazoans so the knowledge about non-lysosomal PCD is weak (Baehrecke 2003).

1.3.1.4. Oncosis and necrosis

Oncosis is viewed as accidental cell death where metabolic homeostasis fails. It is characterized by membrane and organellar swelling resulting in leakage of cytoplasm in the extracellular space (Jones 2000). Old or damaged cells undergo necrosis which is a non-physiological process requiring no energy, transcription or *de novo* protein synthesis. Similarly to oncosis, necrosis result in cell membrane rupture and leakage of cellular contain into the tissue (Cohen 1993; Havel et Durzan 1996).

1.3.2. PCD during development and interaction of the plant with environment

In plants, PCD process is employed in vegetative and generative phase of development: formation of different leaf shape, death of aleurone layer in endosperm of monocotyledons, autolysis of parenchymatic tissue and aerenchyma formation, differentiation of tracheary elements, abscission of leaves, flowers, during sex determination, anther rupture, death of incompatible pollen, degradation of haploid megaspores, synergids or suspensor cells (Greenberg 1996; Jones 2001; Rogers 2005).

PCD is also induced by number of biotic or abiotic stresses such as pathogen attack or hypoxia. Such pathogen attack is often accompanied by rapid cell death in and around the infection site to block the spreading of the pathogen to adjacent cells. This reaction called hypersensitive response is one of the mechanisms of the plant innate immune response sharing lot of characteristics to autophagic PCD (Hayward et al. 2009).

1.3.3. Effectors of cell death

1.3.3.1. Caspases in animals

Caspases has a crucial role during initiation and executionary phase of animal PCD (Cohen 1997). These enzymes are cysteine proteases demonstrating specificity toward aspartic acid. Caspases recognize specific tetrapeptide motifs and cleaves on the carboxyl site of aspartic residue (Talanian et al. 1997). These proteases are divided into initiatory caspases having a role during early PCD signaling and executive caspases digesting the cell components during executionary phase of PCD. Caspases are synthesized as zymogens activated by other caspase molecules during autolysis or by initiator caspase processing upstream in the cell death pathway. Caspase regulation is mainly under the control of Bcl-2 family proteins (Hengartner 2000) and after the activation they target several proteins important for cell integrity e.g. poly (ADP-ribose) polymerase (PARP), lamins or gelsolins (Duriez et al. 1997). Destruction of these key substrates leads to morphological changes and cell death. In particular, caspase-3 is implicated in the activation of caspase activated DNAse (CAD) which is synthesized in the inactive complex with the Inhibitor of CAD (ICAD). After caspase-3-mediated

cleavage of ICAD, CAD forms a scissor-like dimer cleaving DNA with minimal sequence specificity (Nagata et al. 2003). It is important to note that during PCD signaling, a specific point of no return exists. Once the cell enters the executionary phase of cell death and intracellular proteases and nuclease starts to degrade cellular components the PCD is not reversible as during the initiatory phase of cell death.

1.3.3.2. Caspase activities in plants

In plants, the activation of specific proteases is also required during PCD execution. As caspases have a unique role during animal apoptosis, a lot of effort has been made to find structural and functional homologs in plants. In spite of conservation of PCD signaling pathways between animals and plants no functional homologs of caspases were identified in plants, notably in the completely sequenced genome of Arabidopsis. Surprisingly, caspase activity has a crucial role during various types of plant PCD as it was demonstrated by analysis with specific fluorogenic substrates and inhibitors of animal caspases. The implication of caspase-like activities in plant PCD was observed e.g. in tomato suspension cells treated with CPT, staurosporine, or Fumonisin B1 (de Jong et al. 2000), in UV-irradiated Arabidopsis (Danon et al. 2004), in BY-2 cells treated with high doses of cytokinines (Mlejnek et Procházka 2002), or during hypersensitive response in tobacco (del Pozo et Lam 1998).

Cleavage of PARP, implicated in DNA repair, by caspase 3 is one of the hallmark and useful marker of animal apoptosis. PARP seems to be structurally and functionally conserved between animal and plants and it was reported that plant PARP is cleaved by plant proteases in caspase specific sites (Lam et del Pozo 2000). In addition, ectopic expression of the general caspase inhibitor p35 from Baculovirus can effectively block plant cell death in response to pathogen toxin or UV exposure (Lincoln et al. 2002; Danon et al . 2004).

1.3.3.3. Caspase-like proteins

Although plants lack classical caspases, some other enzymes which exhibit caspase activity or are similar to caspases by the tertiary structure were described. Among these “caspase-like” proteins (CLPs), we regroup metacaspases, legumains and subtilisin-like proteases (Figure 1.9.) (Piszczek et al. 2007).

Metacaspases are cysteine proteases that show structural similarity to caspases but instead of cleavage after aspartic residue, they show the specifity against arginine and lysine. Plant metacaspases are divided into two groups according to their domain organization as metacaspase type I and metacaspase type II. This domain is probably important during protein-protein interactions necessary for the autoactivation. In Arabidopsis genome there are 3 genes encoding metacaspases I (*MCA1-3*) and 6 genes encoding metacaspases II (*MCA4-9*) (Uren et al. 2000). Metacaspase structural characteristics are similar to that of animal caspases, in addition to N-terminal prodomain of

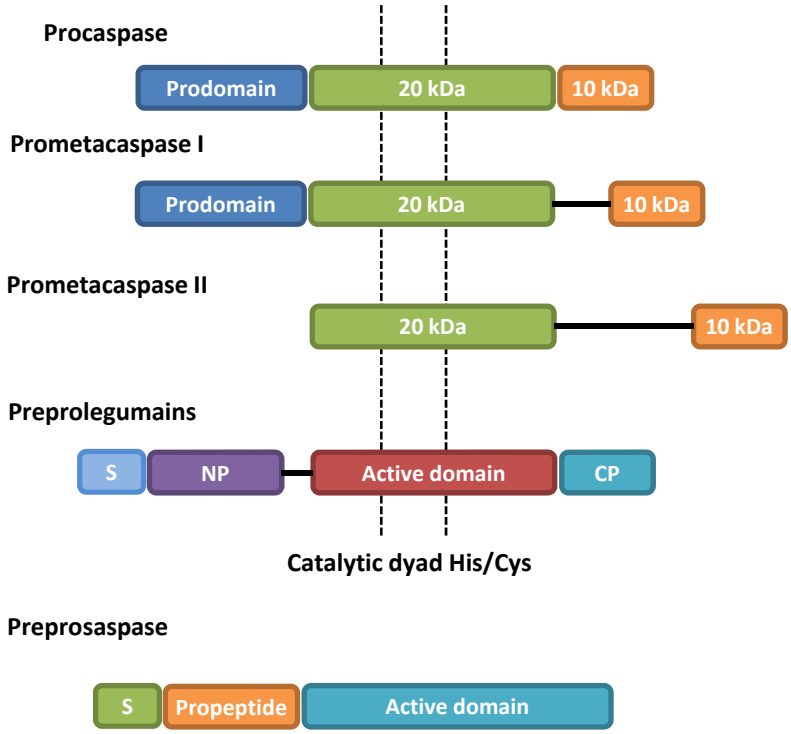


Figure 1.9: Domain structure of caspase-like proteins. S – signal peptide, NP – N-terminal propeptide, CP – C-terminal propeptide. Animal caspase share a structural and sequence homology with plant metacaspase I and II but they do not exhibit the same proteolytic activity. These enzymes are synthesized as inactive proenzymes (procaspase or prometacaspases). Legumains encompass Vacuolar processing enzymes and saspases are synthesized as preprolegumains and preprosaspase do not share sequence homology but exhibit caspase-like enzymatic activities (Piszczek et al. 2007).

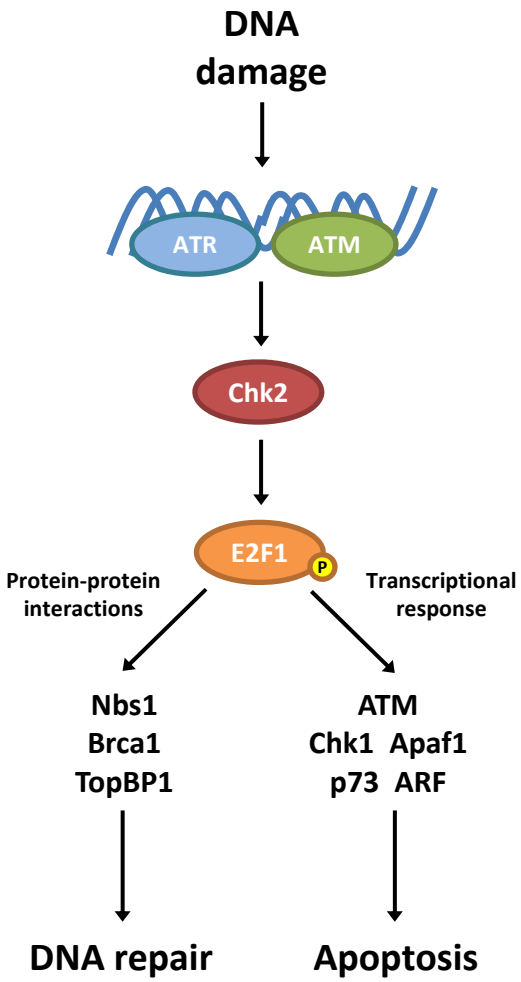


Figure 1.10: Role of mammalian E2F1 during the control of DNA repair and apoptosis. When DNA damage occurs, E2F transcriptional factor is phosphorylated and it is implicated in the regulation of DNA repair pathways via protein-protein interactions with Nbs1, Brca1 or TopBP1. Apoptosis could be triggered by transcriptional activation of PCD genes including Apaf1, ARF or p73.

metacaspases I, both metacaspases I and II harbors two conserved domains resembling to the large and small subunits of animal caspases. N-terminal prodomain of metacaspases I is likely important for autoactivation sharing some homology with animal caspases (Vercamenn et al. 2006). In spite of structural similarities, it seems that metacaspases does not cleave animal caspase substrates but their activity could be inhibited by specific caspase inhibitors (Vercamenn et al. 2004; Watanabe et Lam 2005). The involvement of metacaspases during development or hypersensitive response has been demonstrated (Suarez et al. 2004; Lam 2004) and recently also direct implication of metacaspase8 has been shown during PCD induced by oxidative stress caused by UV-C, H_2O_2 or methyl viologen (He et al. 2008). In Arabidopsis the overexpression of *MCAs* did not result in obvious phenotypes during normal or under several biotic or abiotic stresses (Vercamenn et al. 2007) but, contrary, KO mutants of *MCA* genes slightly did. It has been demonstrated that a type II metacaspase from Norway spruce, mcII-Pa, is essential for developmental PCD during somatic embryogenesis (Bozhkov et al. 2005). The role of metacaspases during plant PCD or other biological processes is still not so well understood in plants. Identification of pathways involved in metacaspase signaling would give us a better insight into the metacaspase role during both plant PCD and development.

Vacuolar Processing Enzymes (VPEs) are cysteine endopeptidases belonging to legumains and showing significant structural homology to animal caspases. They were identified in various animal and plant species including Arabidopsis with 4 *VPE* genes (VPEα-δ) (Kuroyanagi et al. 2005) and tobacco with similar complexity (VPE1a, VPE1b, VPE2, VPE3) (Hatsugai et al. 2006). VPEs cleave a peptidic bond preferentially after asparagine residue (Hatsugai et al. 2006) but under *in vitro* conditions these enzymes have been shown to be able to cleave also after aspartic residue e.g. in a caspase-1 specific sequence YVAD, but not caspase 3 sequence DEVD (Kuroyanagi et al. 2005). VPEs and caspase-1 have a common catalytic dyad (His, Cys) and three amino acid residue forming a pocket for a substrate (Arg, Arg, Ser). Both enzymes also harbor a prodomain needed for autoactivation, and similar large and small subunits. Thus, VPEs exhibit functional similarities to animal caspases although their do not share similar sequence homology. VPEs are targeted to the vacuole where they participate to the maturation of the vacuole-targeted proproteins (Yamada et al. 2009). For cell death execution, they act as key molecules during mega-autophagy through regulation of proteins involved in tonoplast rupture (Hatsugai et al. 2006; Hara-Nishimura et al. 2005). Role of VPEs has also been demonstrated during maturation of seed proteins, senescence or during various stress responses (Shimada et al. 2003; Kinoshita et al. 1999).

Last group of CLPs are saspases which are serine proteases belonging to the subtilisin-like proteases family. Cofeen and Wolpert (2004) identified two genes – *SAS1* and *SAS2* from *Avena sativa* which are implicated during victorin-induced PCD. These cytosolic proteases are sensitive to caspase inhibitors and also can cleave caspase substrates. However their natural substrates are not known (Cofeen et Wolpert 2004). Phytaspase (plant aspartate-specific protease) is another new member of

subtilisin-like proteases identified in tobacco and rice. This enzyme exhibit caspase activity but which is distinct from other known caspase-like activities identified in plants till now. The role of phytaspase was demonstrated during tobacco mosaic virus- or abiotic stress- induced PCD (Chichkova et al. 2010).

26S proteasome is involved in the degradation and recycling of the majority of cell proteins and its role during PCD regulation was demonstrated in animals, yeasts and plants. Interestingly, RNAi plants with a defective proteasome subunit PBA1 have reduced DEVDase activity, typical for animal caspase 3 suggesting that PBA1 may act as a caspase-like enzyme in plants (Hatsugai et al. 2009).

1.3.4. PCD signaling

A huge progress in understanding animal PCD regulation and signaling pathways has been made during the last two decades however the knowledge of plant cell death signaling remain sill poorly understood. First, we will focus on the well defined animal signaling pathways and more particularly on those induced by DNA damage leading to apoptosis and then what is known in plants. Partial conservation of PCD permitted the identification of similar regulators in plants to unravel some mechanisms of plant PCD regulation. However, plants exhibit a lot of particularities making a plant PCD regulation distinct from the animals.

1.3.4.1. Animal apoptosis

It has been demonstrated that mitochondria, an energetic center of the cell, are coordinating multiple external and internal signals which could result in the initiation of cell death through the release of cytochrome c from mitochondrial inter membrane space and caspase activation. This was first suggested in the experiments when mitochondria were required for morphological changes typical for apoptosis in the nuclei isolated from frog eggs (Newmeyer et al. 1994). Caspases must be highly regulated and be activated only when appropriate signals are received. This signal could be DNA damage, growth factor deprivation, ER stress, detachment from neighboring tissues or engagement of cell surface death receptor on a plasma membrane (Kuwana et Newmeyer 2003).

The signal perception leads to the formation of channels in outer mitochondrial membrane under the control of Bcl-2 family proteins resulting in the leakage of cytochrome c from intermembrane space to the cytosol. Cytochrome c is along with Apaf1 (Apoptosis inducing factor-1) and dATP necessary for the activation of inactive procaspase 9. This complex called apoptosome releases active initiator caspase 9 which cleaves and activates other downstream caspases (Zhou et al. 1999). Such example of integration of cellular stresses by mitochondria could be DNA damage dependent leading to the activation of p53 which can activate proapoptotic members of Bcl-2 family. During normal condition, p53 is maintained at low levels by targeted degradation by ubiquitin 26S-

proteasome pathway. Upon DNA damage, p53 is stabilized via p19 and also *p53* expression is enhanced resulting in increased p53 protein levels. Second example is related, as already mentioned, to the membrane death receptor. Ligand binding activates caspase 8 which is also an upstream regulator of Bcl-2 proteins. Caspase 8 can directly activate caspase 3 that is a cytochrome c-independent (or mitochondria-independent) pathway (Kwan et Newmeyer 2003). This illustrates that mitochondrion are integrators of endogenous and exogenous cell death signals.

1.3.4.2. Role of E2F in regulation of apoptosis

The role of E2F/Rb pathway during cell cycle regulation, DNA repair, recombination and differentiation in animals and plants was already discussed earlier (summarized in Figure 1.10.). However, in animals E2F is also an important regulator of apoptosis which is mainly known for animal the best studied member E2F1 exhibiting both oncogenic and tumor-suppressive activities (Iaquinta et Lees 2007). The regulation of the E2F bivalent role is complex and the final cellular response whether it promotes cell division or apoptosis depends on cellular context.

The proapoptotic role of E2F1 has been shown by ectopic expression of E2F1 that leads to p53-dependent apoptosis in quiescent fibroblasts (Qin et al. 1994). However E2F1-induced apoptosis could be also processed in a p53-independent manner (Hsieh et al. 1997). On the contrary, E2F1 deficient mice show thymus hypertrophy due to the reduced ability of these cells to undergo apoptosis (Field et al. 1997).

Since the discovery, that E2F can promote apoptosis, many pro-apoptotic E2F target genes have been identified. Among these targets the most remarkable are several members of Bcl-2 family or both initiator (caspase 9 and 8) and some effector caspases (caspase 3 and 7) (Nahle et al. 2002).

Along the E2F1-mediated increase of p53 via p19, E2F1 is also able to act in a p19-independent manner. E2F can regulate p53 activity with other cofactors which enhances the ability of p53 to transactivate pro-apoptotic genes such as the Bcl-2 protein Bax. Similarly to Bax, p53 can also directly target mitochondrial membrane resulting in the leakage of cytochrome c to the cytosol (Bernstein et al. 2002). E2F may also act in a p53-independent manner via p53 homologue p73 which also controls some pro-apoptotic genes (Iaquinta et Lees 2007). This multiple modes of regulation mediated by E2F1 probably serve to ensure the activation of PCD in the presence of apoptotic stimuli.

E2F does not regulate pro-apoptotic genes only directly but also indirectly through other interacting co-factors. These genes could not be discovered by simple *in silico* promoter analysis on the presence of E2F consensus. For example Jab1 whose expression enhances the ability of E2F1 to induce PCD (Hallstrom et Newins 2006).

1.3.4.3. Regulation of plant PCD

In plants, lot of crucial regulators of animal apoptosis are not present that is most likely linked to the specific morphology and physiology of plants. Among these genes are *Bcl-2 family*, *p53*, *caspases* or *Apaf1*. However the conservation of animal and plant PCD signaling was demonstrated by production of transgenic plants expressing animal apoptotic proteins. This approach demonstrated the functionality of Bcl-2 members in plants. For example, hypersensitive response PCD was induced in plants after ectopic expression of the animal Bcl-2 family member, the pro-apoptotic Bax. Bax-induced cell death is correlated with accumulation of a defense-related protein PR1 (Pathogenesis-related protein 1), suggesting the activation of an endogenous cell death program (Lacomme et Santa-Cruz 1999). On the contrary, plants harboring anti-apoptotic Bcl-2 proteins Bcl-xL or CED-9 are resistant to biotic and abiotic stresses as paraquat, menadione, UV or fungal pathogens (Dickman et al. 2001). These results show that basic mechanisms of signaling pathways are conserved among animals and plants.

Compared to Bcl-2 family proteins, which were not identified in plants, several groups of Bcl-2 cofactors are conserved in plants, animal and yeasts. The BAG proteins (Bcl-2-associated athanogene) were discovered in a screen for Bcl-2 protein binding partners and their role was indicated as molecular co-chaperones which regulate cytoprotective processes from pathogen attack to abiotic stress and development (Doukhanina et al. 2006). BAG1 enhances cell survival synergistically with Bcl-2 suggesting their involvement in PCD pathways (Takayama et al. 1995). Another evolutionary conserved protein that potentially regulates PCD in all Eukaryotes is ER-resident protein Bax-Inhibitor 1 (BI-1). BI-1 modulates cell death induction in response to multiple types of cell death signals. Bax inhibitor-1 was first identified due to its ability to suppress Bax-induced cell death (Xu and Reed 1999; Kawai et al. 1999). In plants, its homolog was characterized in a wide range of species and as ER-reside protein its function may be associated with ER-related cellular activity such as homeostasis control or ER stress response where the ROS or Ca^{2+} signals could be generated (Chae et al. 2004). In plants, BI-1 has been shown to reduce PCD induced by fungal pathogens, fungal elicitors, H_2O_2, salicylic acid, heat or cold (Kawai-Yamada 2004). On the other hand, plants lacking functional BI-1 are more sensitive to PCD induction after heat and cold stress (Watanabe et Lam 2006).

These reports indicate that the signaling pathways in plants have conserved basic regulation but our knowledge of the genetic mechanisms that regulate and execute plant cell death is still limited. Although the number of plant cell death modulators is increasing, they need to be placed in a specific position within the complex regulatory network.

1.3.4.4. ROS function during plant PCD signaling

PCD regulation is a result of complex signaling pathway. In addition to phytohormones or Ca^{2+} during PCD signaling, reactive oxygen species (ROS) have similar important role. ROS are

considered to be key modulators of PCD as well as many other biological processes. They are produced in plants and other aerobic organisms as a result of O_2 reduction which is localized to mitochondrion, chloroplasts or peroxysomes that was well documented during plant pathogen interaction. Indispensable ROS production is also as a result of DNA damage (see section DNA damage response). In recent years, increasing number of reports suggest that ROS function also as a signal molecule (Gechev et al. 2006). Numerous ROS signal transduction components have been shown to regulate plant PCD including protein kinases, phosphatases or transcriptional factors. These regulators are often plants specific, suggesting a distinct role of ROS signaling during plant PCD compared to mammals. Because of high levels of ROS production during photosynthesis, plants developed an antioxidant system composed of antioxidant enzymes and molecules as a means of protection against excessive ROS production (Gadjev et al. 2008). The cellular response to altered ROS levels depends on the type of ROS, the intensity of the signal and on the localization of ROS production.

One of the most important signal ROS molecules is H_2O_2 due to its relative stability and ability to diffuse through the tissue. H_2O_2 is a mediator of a number of cellular stress responses suggested from its elevated levels during development or after plant exposure to different biotic and abiotic stresses. Exposure to H_2O_2 induces different cellular responses including activation of MAPK, differential gene expression, increase of intracellular Ca^{2+}, NO synthesis and also accumulation of ethylene and acid salicylic. H_2O_2-mediated PCD was reported during aleurone cell death, tracheary element maturation, trichomes formation, organ senescence (Gechev et al. 2006), during hypersensitive response or plant challenge to cold, salt or xenobiotic stress (Swidzinski et al. 2002). PCD can be also triggered by other forms of ROS including singlet oxygen or superoxide radicals (Op den Camp et al. 2003). Arabidopsis lacking catalytic subunits of NADPH oxidase, an enzyme of ROS metabolism, are more tolerant to cell death induction after pathogen attack (Torres et al. 2002). Thus, it is clear that ROS is an important signal molecule during PCD however the underlying mechanisms are not completely understood.

1.3.5. Plant PCD induction in DNA damage context

In animals, PCD induced by DNA damage is extensively studied but the knowledge about plant PCD pathways induced by DNA damage are not well understood. Several reports demonstrated the presence of cell death after exposure of plant cell to different classes of genotoxics. Among the first reports showing that DNA damage induces PCD is the induction of apoptotic cell death in Arabidopsis. UV-C irradiation causes lesions similar to those after pathogen attack in Arabidopsis leaves and also it induces light-dependent cell death of protoplasts suggesting the implication of ROS signaling. The characteristic of cell death was described as apoptotic-like due to the presence of apoptotic DNA fragmentation and also caspase 1 and 3 activities suggested form experiments with caspase specific substrates and inhibitors (Danon et al. 2004). Similar characteristics of PCD were

shown in tomato cell suspension after the exposure to CPT (de Jong et al. 2000). Cell death was also detected after γ irradiation of Arabidopsis roots or in *rnr* and *atm* Arabidopsis mutants with increased genomic instability and upregulation of several PCD-related genes and DNA fragmentation as well but without any further characterization of cell death or genes possibly implicated in this pathway (Wang et al. 2006; Ricaud et al. 2007). Cadmium ions as a stressor with pleiotropic effects including ROS generation and subsequent DNA damage was shown to be also potent inducer of PCD characterized by specific DNA fragmentation (Fojtová et al. 2000; Kuthanová et al. 2008) or induction of DNA repair genes (Meddiouni et al. 2008). These data shows that similarly to animals, DNA damage induces cell death in plants but the understanding of mechanism of signal transduction from DNA damage sensing to PCD execution remains to be elucidated.

Only a few studies have investigated the role of genes implicated in DNA damage-induced PCD in Arabidopsis. Plants lacking ATR kinase were hypersensitive to APC, but not HU, and exhibited cell death in root tips suggesting that ATR is implicated in the replicative stress response leading to cell death (Culligan et al. 2004). The authors characterized cell death just as a disappearance of nuclei which is not really convincing. Another recent report show that cells in the root apical meristem are hypersensitive to a DSB inducer zeocin and this PCD response is mediated through ATM and ATR kinases but not WEE1. Compared to PCD induction after UV irradiation, this cell death seems to have rather autophagic characteristics (Fulcher et Sablowski 2009). Thus, it is clear, that DNA damage signaling leading to cell death are conserved through evolution and it will be interesting to unravel the mechanisms underlying these pathways.

1.4. Aims of the study

Our knowledge of the PCD signaling induced by genomic instability or DNA damage is still weak. This work tries to integrate previous investigations obtained in our laboratory on DNA damage signaling and more particularly the role of the E2F transcriptional factor in the DNA damage response. Thus the objective of my thesis was to identify which factors are important for the decision in the induction of PCD after DNA damage in plants.

For this purpose we needed first to establish and characterize a simplified PCD system at the unicellular level – using the tobacco BY-2 cell line and the double strand breaks inducer bleomycin (BLM). These results are presented in Chapter I.

E2F is involved in both PCD and DNA repair signaling in animals, and that in plants the implication of E2F in the DNA damage response was demonstrated (Lincker et al. 2004; Roa et al. 2009). The purpose of Chapter II is to determine the role of E2F factor during BLM-induced PCD in plants.

Finally, to test whether our findings obtained at the unicellular level in BY-2 cells are valid also *in planta*, we investigated PCD induction mainly in the root system of Arabidopsis which has a well defined structure. We have tested several candidates which could be involved in the regulation of PCD upon genotoxics. These regulators were implicated in DNA damage signaling (ATM, ATR, SOG1, BRCA1, BARD1), cell cycle regulation, endoreduplication (WEE1, CDC25, CDKs) or cell death execution (MCAs, VPEs). All these results are summarized in Chapter III of the results.

Materials

2.1 Laboratory material

All laboratory glass and metallic materials was surface sterilized at 180 °C during 2 h in the kiln whereas plastic material was sterilized at 120 °C during 30 minutes in humid autoclave.

Medias for cultivation of living material, buffers and other stock solutions were sterilized at 120 °C during 30 minutes in humid autoclave.

2.2. Living material

2.2.1. Plant material

2.2.1.1. Tobacco BY-2 cell line

Tobacco cell line BY-2 was initially isolated by Kato et al. (1972) from *Nicotiana tabacum* L. cv. Bright yellow-2 (BY-2). This cell suspension was kindly provided to our laboratory by Professor Toshiyuki Nagata (Tokyo University, Japan).

2.2.1.2. Arabidopsis PSB-V cell line

Arabidopsis cell culture PSB-V was kindly provided by Geert de Jaeger (Ghent University, Belgium) (van Leene et al. 2007). This cell line is derived from *Arabidopsis thaliana* ecotype Landsberg erecta.

2.2.1.3. Plants *Arabidopsis thaliana*

Different WT or *Arabidopsis thaliana* plants with different genetic background were used during this work. *Arabidopsis thaliana* is widely used as a model plant in plant biology because of several reasons: its small genome consisting from 125 Mbp is completely sequenced, short generation period only of 4 weeks, self-pollination, production of high amount of seeds, low space requirements during the cultivation, no economic interest of this plant permitting the exchange of information between the laboratories and availability of several mutant collections.

In our work, we used several T-DNA insertion Arabidopsis mutants leading to loss of function of the target gene or Arabidopsis plants transformed with specific constructs and characterized in our or other laboratories.

2.2.2. List of different Arabidopsis genotypes used during the present work:

atm2 (At3g48190, ecotype Columbia (Col-0)) and *atm3* (At5g40820, ecotype Wassilewskija (Ws)) lines were previously characterized by K. Culligan and V. Garcia (Culligan et al. 2004; Garcia et al.

2003). *sog1-1* (At1g25580, ecotype Col-0) line was a gift from A.B. Britt previously identified and characterized by S.B. Preuss (Preuss et Britt 2003).

brca1-1 (At4g21070, ecotype Col-0, SALK_014731) and *bard1-2* (At1g04020, ecotype Col-0, SALK_031862) lines were previously characterized by W. Reidt (Reidt et al. 2006). *to12* and *to24* (At4g02110, ecotype Col-0, SAIL_610E01) lines were characterized in the laboratory by L. Sanchez-Calderon.

e2fa (At2g36010, ecotype Col-0, GABI N348E09) line and *NtE2F* line overexpressing *E2F* (from *Nicotiana tabaccum* was characterized in the laboratory by J. Lang. *e2fb* (At5g22220, ecotype Col-0, SALK_131062) line was characterized by me during this thesis.

wee1 (At1g02970, ecotype Col-0, GABI 270E05) was obtained from Dr. Nico Dissmeyer (IBMP, Strasbourg) and was previously characterized by K. de Schutter (de Schutter et al. 2006). SpCdc25 line overexpressing *Ccd25* (CU329670) from *Schyzosacharomyces pombe* was a kind gift from Dr. D. Francis (Cardiff University, United Kingdom).

CDKA1;1 phospho-mimicry lines named d7 and de48 were characterized and provided by Dr. Nico Dissmeyer (IBMP, Strasbourg) (Dissmeyer et al. 2007 and 2009).

vpeα (At2g25940) mutant was kindly provided by Dr.Hara-Nishimura (Kuroyanagi et al. 2003). Mutants of *mca2* (At4g25110, SALK_084393) and *mca8* (At1g16420, RATM13-5036) were characterized by me during my thesis.

2.2.3. Bacterias

2.2.3.1. *Escherichia coli* DH5α

This bacterial strain has the genotype *fhuA2* Δ*(argF-lacZ)U169 phoA glnV44 Φ80* Δ*(lacZ)M15 gyrA96 recA1 relA1 endA1 thi-1 hsdR17*. The *endA1* mutation inactivates an intracellular endonuclease that degradated plasmid DNA in many minipreps methods. Δ*(lacZ)M15* is the alpha acceptor allele which is necessary for blue-white screening with many *LacZ* based vectors. *recA1* is important for reduced frequency of homologous recombination of this bacterial strain.

2.2.3.2. *Agrobacterium tumefaciens* LBA4404

Bacteria *Agrobacterium tumefaciens* is widely used to introduce DNA into the plant genome with the highest efficiency because only one or a few copies of the transfer DNA (tDNA) are integrated into the host genome (Smith et Hood, 1995). These bacteria naturally causes crown gall disease when it transfers one part of Ti (tumor inducing) plasmid or T-DNA placed between right

(RB) and left border (LB) which are repetitive into the plant genome by the mechanism of non-homologous recombination.

Strain LBA4404 (the Netherlands culture collection of bacteria, NCCB) was used for transformation of both tobacco BY-2 cells and Arabidopsis plants. LBA4404 contains pAL4404 Ti plasmid with inactivated oncogenic genes and also active *vir* gene of avirulence responsible for integration of T-DNA into the plant genome (Ooms et al. 1998). This strain also contains a gene for the resistance to rifampicine which was used for the selection.

2.2.4. Vectors

2.2.4.1. Vector pENTR201

This vector is derived from pDONR201 and served as a Gateway™ entry vector (Figure 2.1A). The presence of recombination sites attL1 and attL2 permits the introduction of desired PCR product into the vector during the first step of Gateway® cloning via BP reaction. This plasmid harbors a kanamycin resistance for the selection of bacterial clones.

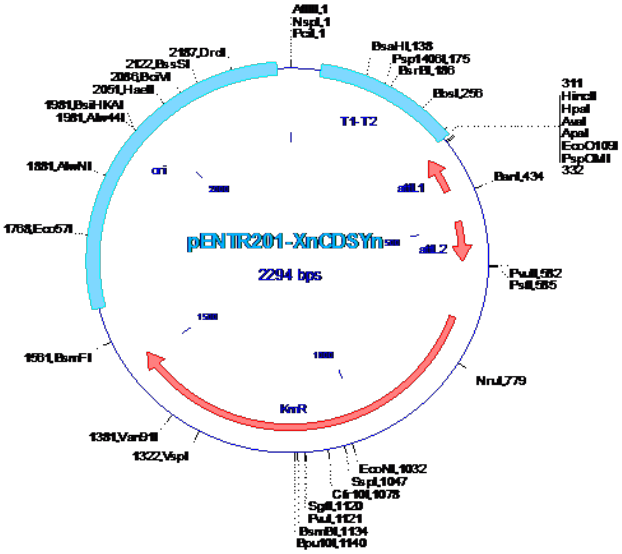
2.2.4.2. Vectors pK7FWG2 and pK7GWF2

Plasmids pK7FWG2 and pK7GWF2 are binary destination vectors Gateway™ (Figure 2.1B) (Karimi et al. 2002). This vectors permit to produce the fusion protein were the eGFP (Enhanced green fluorescent protein) is placed on N- or C- terminus, respectively, of the target protein. CcdB cassette is surrounded with attR1 and attR2 recombination sites which permit to replace this cassette by the coding region of our gene of interest using Gateway® cloning technique. In these vectors, two genes encoding resistance to antibiotics spectinomycin and kanamycin are placed which allow the selection of transformed bacterial or plant clones, respectively.

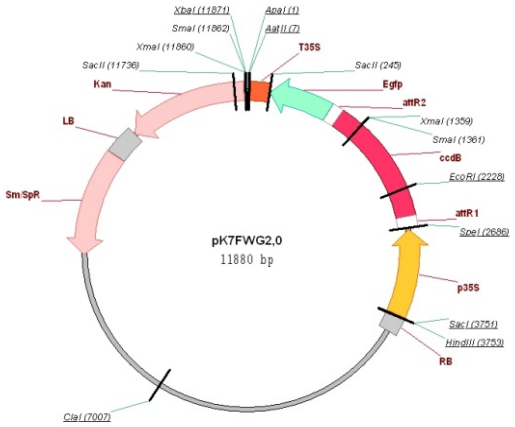
Green fluorescent protein (GFP) is a protein of 27 kDa previously isolated from the jellyfish *Aequoria victoria*. When excited with UV lights it emits light in the green part of the spectra. The peak of the maximal excitation is at 395 nm but another minor peak is at 475 nm what is close to the excitation ability of confocal microscopes which are generally equipped with 488 nm excitation laser. The peak of emission for GFP is at 509 nm. GFP chromophore is constituted from 3 amino acid residues – serine 65, tyrosine 66 and glycine 67 (Yang et al. 1996a). This triad is autocatalyticaly formed in the middle of barrel-like structure which is formed by 11 β sheets and 4 α helixes as revealed from crystallography analysis (Ormo et al. 1996). The fluorescence could be also observed after simple excitation with UV or blue light.

Native GFP protein does not have good characteristics to be used as a protein marker in different heterologous systems. That is why several mutations in the coding sequence were introduced to improve native GFP resulting in different modifications of emission spectra and the intensity of the

A



B



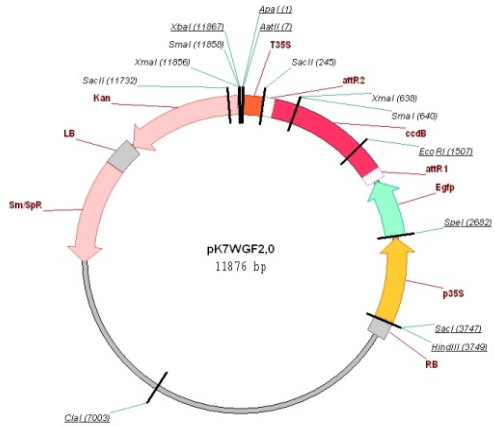


Figure 2.1: Maps of vectors. **A:** Entry vector pENTR201; **B:** Binary destination vectors pK7FWG2 and pK7WGF2.

fluorescence. The coding sequence of Enhanced Green Fluorescent protein (eGFP) used in vectors pK7FWG2 and pK7GWF2 differ from native GFP form in 2 types of mutations: mutation of isoleucine 167 by threonine modify the absorption spectrum by strong increase of excitation at 476 nm or mutations which affect excitation and emission spectra and characteristics of the protein (amino acids of the chromophore or close to the chromophore (Yang et al. 1996b).

The barrel-like structure gives to the GFP high stability permitting to resist to different denaturizing environment without obvious inhibition of the fluorescence (Bokman et Ward, 1981) including wide range of pH (5,5 – 12). Moreover, one of the biggest advantages is that GFP fluorescence could be observed directly in the transformed cells *in vivo*.

2.2.5. Oligonucleotides and bioinformatics

Oligonucletotides were ordered from Sigma (www.sigma-aldrich.com). Tm temperatures were calculated using online internet tools for primer design called Primer3 (http://frodo.wi.mit.edu/primer3/). The list of all primers used during this work is presented in the annex. Comparisons, alignments, analysis of all sequences and searches in gene databases were performed using BLAST on NCBI web page (http://www.ncbi.nlm.nih.gov/ blast/Blast.cgi) or ClustalW2 alignment tool (http://www.ebi.ac.uk/Tools/clustalw2/index.html).

Methods

2.3. Plant material culture

2.3.1. Sterilization of Arabidopsis seeds, *in vitro* culture and treatment

One ml of sterilization solution was added up to 100 µl of Arabidopsis seeds in 2 ml tube and mixed for 10 min. The seeds were rinsed twice with 96 % ethanol. After the second wash, maximal amount of ethanol was eliminated with a pipette tip for fast drying of the seeds under laminar hood. This technique permits to sow high quantities of the seeds and also to conserve stock of sterile seeds up to 1 month without reduction of germination efficiency.

Seeds were sown on the agar MS medium (MS 0255, Duchefa) supplemented with 0,5 g/l MES and 10 g/l sucrose (pH 5,7) and agar (12 g/l for vertical, 9 g/l for horizontal cultivation, Duchefa). The plates were placed in obscurity at 4 °C for 2 days for vernalization. *In vitro* plants were cultivated in the cultivation room at 21°C with 14 h photoperiod (minimum 2500 lux).

Ex vitro Arabidopsis plants were cultivated in pots placed in the greenhouse at following conditions: temperature 23°C/18°C (day/night), 12 h photoperiod. Plants were regularly watered and maintained by gardeners of the Greenhouse platform of IBMP.

Different genotoxics treatment of 5 DAG (for CDKA1;1 phospho-mimicry lines of 10 DAG) Arabidopsis plants was made in liquid MS Arabidopsis culture medium (mainly for transcription analysis) or on MS agar plates (mainly for cell death induction in roots, flow cytometry analysis) supplemented with genotoxics. Bleomycin (Bellon), Hydroxyurea (Sigma), Camptothecin (Sigma) or Aphidicolin (Sigma) were used in doses mentioned in the chapter Results.

Modified MS medium for Arabidopsis (for 1 liter, pH = 5,7): MS medium including vitamins and MES buffer (Duchefa, M 0255.0001); sucrose 10 g/l; agar 9-12 g/l.
Sterilization solution:

Commercial sodium hypochlorite bleach 0,4 %; Triton X-100 0,05 %; ethanol 95%.

2.3.2. Tobacco BY-2 cell line culture and treatment

BY-2 cell suspension was maintained by weekly subculture made by transferring 3 ml of stationary cells into 100 ml of fresh modified Murashige and Skoog (MS) medium (M 0232.0001 - Basal Salt mixture; Duchefa, Belgium). Cell suspension was cultivated in obscurity on a shaker (130 RPM, IKA KS 250 basic) at 27 °C. BY-2 calls were cultivated on MS plates with modified MS medium supplemented with 7-8 g/l of agar (Duchefa, Belgium) in obscurity at 27°C. Calluses were maintained by transfer of small fraction of cells on fresh MS plates each 3 weeks. BY-2 culture was treated by transferring of cells in exponential phase to the medium with genotoxics in the ratio 1:4.

Modified MS medium for BY-2 (for 1 liter, pH = 5,8):
MS medium Basal salt mixture (Duchefa, M 0232.0001); myo-inositol 100 mg/l; thiamin HCl 0,10 mg/l; 2,4 D 0,2 mg/l; sucrose 30 g/l; KH_2PO_4 200 mg/l.

2.3.3. Arabidopsis PSB-V cell line culture and treatment

PSB-V line was maintained by weekly subculture by transferring 10 ml of stationary cells to 50 ml of the modified MS medium (see below). PSB-V was cultivated on the rotatory shaker (130 RPM, New Brunswick Scientific, Edison, NJ, USA) in obscurity at 26 °C. PSB-V culture was treated by transferring of cells in exponential phase to the culture medium supplemented with genotoxics in the ratio 1:3.

Modified MS medium for PSB-V (for 1 liter, pH = 5,7)

MS medium including vitamins (Duchefa, M 0222.0001); 1 mg/l thiamine; 30 g/l sucrose; 0,5 mg/l NAA; 0,05 mg/l 6-BAP

2.4. Cytological methods

2.4.1. Detection of cell death

2.4.1.1. FDA viability test of BY-2 cells

In living cells, fluorescein diacetate (FDA) is cleaved by esterase activities to a fluorescent product which is analyzed under the epifluorescent microscope using filters with maximal excitation at 493 nm and emission at 520 nm (green light).

Cell suspension was incubated with FDA working solution in ratio 1:1 and incubated 1 min at RT and then directly analyzed under the microscope. The viability of the cells in the culture could be counted as a ratio of number of viable (V) cells to the total number (T = viable + death cells) of counted cells - viability (%) = V/T*100. Cell death rate was presented as a mortality which was counted from the viability by subtraction of viability rate from 100 %. To quantify the viability/mortality of cell suspension, total number of 500 cells was counted. Error bars in the graphs represent the standard deviation of the results of at least 3 independent experiments.

FDA staining solution: 1 ml dH_2O; 1 µl FDA (5 mg/ml in acetone)

2.4.1.2. Evans blue staining of Arabidopsis

Evans blue is a vital dye which is actively exported from the cytoplasm of the living cells with intact plasma membrane whereas the cytoplasm of dead cells remained colored in blue. Arabidopsis cell suspension was incubated with 0,1 % water solution of Evans blue for 10 min. After washing twice in liquid modified MS medium for Arabidopsis without agar (see 2.1.1.) the cells were analyzed under the microscope in bright field.

Staining of detached Arabidopsis leaves was performed by 3x vacuum infiltration of 0,1 % aqueous Evans blue solution. After the last cycle of the infiltration the leaves were incubated in the vacuum for additional 20 min. To distain the leaves from the chlorophyll, the leaves were boiled in alcoholic lactophenol for 2 min and rinsed twice with 50 % ethanol and dH_2O. Dead cells were analyzed using bright field microscope or binocular loupe.

Alcoholic lactophenol: 3 volumes of 95% ethanol: 1 volume of lactophenol

Lactophenol: 17 % (v/v) phenol; 17 % (v/v) acid lactic; 33 % (v/v) glycerin; dH_20

2.4.1.3. Propidium iodide staining

Propidium iodide (PI) is used to identify dead cell in population because this fluorescent dye is excluded from viable cells. When PI penetrates to the cell it binds to DNA by intercalating between the bases. PI staining solution was prepared by addition of 10 µl of PI stock solution (1 mg/ml) in 500

µl of dH_2O. After 3 min of incubation of the Arabidopsis plantlets in the staining solution they were placed on microscopic slide. It was very important to place a piece of tape between the slide and the cover slide to avoid smashing of the roots resulting in artificial PI signal. The analysis was made by confocal microscopy using 488 nm excitation laser and emission filter 575 – 640 nm (red light).

2.4.2. BCECF-AM and Evans blue double staining of BY-2

BCECF-AM (2′,7′-Bis(2-carboxyethyl)-5(6)-carboxyfluorescein acetoxymethyl ester) is nonfluorescent until it is cleaved by intracellular esterases to BCECF which is a green fluorescent fluorescein derivate. Negative charge of this molecule causes that it could be retained in the cells with intact plasma membrane. BCECF-AM and Evans blue double staining permits to analyze the integrity of the vacuole and in consequence of cell death. Five hundred microliters of 0,1 % Evans blue solution (in culture medium) was added to 0,5 ml of BY-2 cell suspension. This mixture was incubated with 6 mM BCECF at RT for 10 min. Afterwards the cells were washed twice with 1 ml of culture medium and then analyzed under the microscope. Evans blue staining was observed in bright field and BCECF fluorescent signal using the same settings of the epifluorescence microscope as for GFP observation. Results represent the mean of 3 independent experiments for each 500 cells in total were counted.

2.4.3. Synchronization of BY-2 cells

Cell synchronization was adjusted according to protocol of Kumagai et al. (2007) with slight modification. Three milliliters of stationary BY-2 (7 days old) were transferred to 28,5 ml of BY-2 culture medium supplemented with 5 mg/l of aphidicolin and cultivated 24 h at standard growth conditions. Aphidicolin block inhibits elongation during DNA elongation and thus blocks the cells at the beginning of S phase. The cells were 4 times washed with sucrose solution (30 g/l) in total volume of 1,5 liter and filtered with the filter equipment Nalgane. At the end of washing the cells were resuspended in 30 ml of MS medium and cultivated again at standard conditions (time 0). Samples for mitotic index progression analysis were taken each hour to determine the efficiency of cell synchronization.

2.4.3.1. Mitotic index evaluation and DAPI staining

Five hundred microliters of cell suspension was mixed with 0,1 % Triton X100 and 0,1 µg/l of DAPI and incubated 10 min at room temperature. DAPI staining allows distinguishing the nuclei of interphasic cells and the nuclei of cells in mitosis. Analysis of the cells was done with epifluorescence microscope equipped with excitation filter 330 – 380 nm (UV light) and emission filter < 420 nm. Total number of the cells counted was 500.

Prior the cell counting it was possible to fix the cells in ethanol : acetic acid (3:1) for 1 h. After twice subsequent wash in 1x PBS, the cells were stained with DAPI as described above.

2.4.4. Flow cytometry analysis

One hundred micrograms of fresh weight of BY-2 cells or Arabidopsis tissue was mixed with 0,1 ml CyStain® UV ploidy (Partec) and chopped with sharp razor blade. After 1 minute of incubation released nuclei were purified by filtering of the mixture through the filter with 30 µm nylon mesh (CellTrics®, Partec). Quantification of nuclear DNA content was directly performed on CyFlow® ploidy analyzer (Partec). For one sample at least 10 000 of nuclei was counted.

2.4.5. Fluorescence in situ hybridization (FISH)

BY-2 cells were fixed in ethanol : acetic acid (3 : 1) for 10 minutes and washed in 1x PBS (2x 10 minutes). The cells were transferred into the drop of 60% acetic acid on the slide coated with poly-L-lysine and squashed under the cover slip. Slides with cover slides were freeze in liquid nitrogen and then the cover slide was removed with razor blade. Next, cells fixed on slides were digested with RNase A (100 µg/ml in 2xSSC, 1h at 37°C) and pepsin (50 µg/ml in 0.01 N HCl, 10 min, RT). The hybridization mix (30 µl per slide) contained 15 ng of custom synthesized peptide nucleic acid (PNA) probe $(C_3TA_3)_2$ labeled with Cy3 (Applied Biosystems), 5% dextran sulphate, and 60% formamide in 2xSSC. Heat-denatured hybridization mixture was applied on slides, covered with plastic cover-slips, and the slides subjected to heat denaturation and gradual lowering of the temperature to 37°C. After hybridization at 37°C for 18 h, slides were washed with 2xSSC at 42°C and 0.1xSSC at 42°C, five minutes each and mounted in Vectashield (Vector Laboratories) with DAPI. Images of BY-2 cell nuclei were registered using Olympus AX 70 fluorescent microscope equipped with filter sets for Cy3 and DAPI and AxioCam MRm camera (Carl Zeiss).

For FISH analysis in Arabidopsis, the inflorescence were treated in aqueous solution of BLM and then fixed in a 3:1 mixture of ethanol and acetic acid. The inflorescence were washed in water and transferred in to 10 nmol/l citrate buffer pH 4,5. Pistils were excised from flower buds and treated with a mixture of 0,5% Ozonuka cellulase (Serva electrophoresis) and 0,5% pectolyase (Sigma) at 37°C at moist chamber for 1 h. Pistils were then transferred into a drop of 60% acetic acid on Poly-L-Lysine-treated slides and squashed under the cover slip. The slides were frozen in liquid nitrogen, the cover slip was removed using a razor blade and then postfixed in 3:1 mixture of ethanol and acid acetic and air dried. After RNAse A (100 µg/ml in 2xSSC, 1h at 37°C) and pepsin (50 µg/ml in 0.01 N HCl, 10 min, RT) treatment the procedure was exactly the same as for BY-2 cells.

2.4.6. Detection of DNA fragmentation

Two different methods could be used for the detection of DNA fragmentation into nucleosome-sized fragments which is typical for apoptosis. It could be visualized as a typical DNA ladder on agarose gel electrophoresis. This method provides only the information if the specific apoptotic DNA fragmentation is present but it's in situ localization or quantification could be done for example by enzymatic in situ labeling of 3´OH free ends of fragmented DNA called TUNEL reaction.

2.4.6.1. DNA laddering assay

The DNA from the cells was extracted using Apoptotic DNA Ladder kit (Roche) according to manufacturer´s instructions. The principle is based on binding of DNA on the glass fibers surface in presence of chaotropic salts. Residual impurities were removed during glass fibers washing step and subsequently DNA was eluted with elution buffer. Finally, the DNA was treated with 1 µl of RNAse 1 (1mg/ml) by incubation at 37 °C for 30 min.

2.4.6.2. TUNEL assay on BY-2 cells

For in situ DNA fragmentation detection we used TUNEL reaction kit from Roche (In situ Cell Death Detection kit, TMR red). The working procedure is based on the work presented by Sgonc et al. (1994). We followed manufacturer instruction with slight modifications which adjusted the method for use on plant tissue because this kit is originally made for the detection of apoptotic nuclei in animal cells. During the whole procedure, cell suspension was transferred from one solution to other in a small basket with a 50 µm nylon mesh at the bottom.

Two hundred microliters of BY-2 suspension was fixed in 1 ml of fixation buffer 1 h at 4 °C. Then the cells were washed twice with 1 ml of 1x PBS for 5 min and placed into the permeabilization buffer for 10 min at 4 °C. After twice wash in 1x PBS for 5 min cells of positive control were treated with DNAse I and served as a positive control. This procedure consists of incubation in DNAse buffer for 1 min and then cells were transferred to DNAse I solution for 20 min at 37 °C. To wash the DNAse I form the sample, the cells were washed again twice in 1x PBS for 5 min.

Prior to the TUNEL reaction all the samples including positive control were incubated in 25 mM Tris-HCL pH 6,6 for 1 min. Twenty microliters of the cells were then transferred into eppendorf tubes with 50 µl of TUNEL reaction or just 45 µl of labeling solution in case of negative control without the enzyme. The cell suspension was placed at 37 °C in the dark for 1,5 h and was mixed each 20 min by gently pipetting with cut tip. To remove the TUNEL reaction the cells were washed twice in 1x PBS for 10 min. The second washing step could be done also overnight in order to decrease the red fluorescence background. Finally, DNA was stained with DAPI by incubation of the cells in the DAPI solution for 10 min and two subsequent wash in 1x PBS for 10 min.

TUNEL stained nuclei were directly analyzed under epifluorescence microscope using excitation filter in range of 520-560 nm (maximum at 540 nm, green light) and detection filter in the range of 570-620 nm (maximum 580 nm, red light). DAPI stained nuclei were analyzed similarly as during mitotic index evaluation (see 2.2.3.1.).

DNase I buffer: 40 mM Tris-HCl pH 7,9; 10 mM NaCl; 6 mM $MgCl_2$; 10 mM $CaCl_2$

DNase I solution: DNAse I buffer; 40 µg/ml DNAse 1

Fixation buffer: 4% paraformaldehyde in 1X PBS

PBS 10X: 80 g/l NaCl; 2 g/l KCl; 14,4 g/l Na_2HPO_4; 2,4 g/l KH_2PO_4; pH 7,4

Permeabilization buffer: 0,1 % (v/v) sodium acetate; 0,1 % (v/v) Triton

TUNEL reaction: labeling solution: TdT enzyme – 9:1 (according to manufacturer instructions, Roche)

2.4.7. Microscope and Image analysis

Confocal microscopy analyses were performed on microscope Zeiss Axiovert 100M with Zeiss LSM 510 Software. Epifluorescence, bright field or differential contrast microscope analyses were made with Nikon E800 or Olympus AX 70 fluorescence microscopes. Acquired images were adjusted with ImageJ software (http://rsbweb.nih.gov/ij/), LSM image browser (Zeiss) or Adobe Photoshop.

2.5. Cloning methods and generation of transgenic material

2.5.1. Extraction of plasmid DNA

Bacterial colony was inoculated in 3 ml of LB (Luria-Bertani) medium with antibiotics depending of the resistance of the plasmid of interest. After overnight cultivation at 37 °C for *E.coli* (28 °C for *A.tumefaciens*) the plasmid DNA was extracted with NucleoSpin Plasmid QuickPure (Macherey-Nagel) according to manufacturer protocol. At the end of the procedure plasmid DNA was eluted from the columns with 50 µl of sterile deionized H_2O (dH_2O).

LB medium: 10 g/l Bacto-Tryptone; 5 g/l Bacto-Yeast extract; 10 g/l NaCl; pH 7,0

2.5.2. DNA sequencing

Between 250-300 ng of plasmid DNA was mixed with 0,5 µl of the primer (10 µM) and filled with sterile dH_2O to total volume of 10 µl. Prepared samples were sent to the IBMP platform of DNA sequencing equipped with an Applied Biosystems 373 DNA sequencer (Perkin Elmer). Technique used for DNA sequencing was according to Sanger et al. (1997).

2.5.3. Polymerase chain reaction (PCR)

Polymerase chain reaction (PCR) permits to amplify DNA fragments determinated by specific oligonucleotides (primers) which are complementary to desired DNA regions. The principle of PCR is repeated cycles of denaturation, hybridization and elongation *in vitro*. The temperature of annealing depends on Tm of primers and time of elongation is adjusted according to the length of amplified PCR fragment. Number of amplification cycles varies according to the amplified sequence and starting concentration of DNA template. As a template, DNA solution in TE buffer or water can be used as well as bacterias during the screening of positive bacterial clones after transformation.

We used GoTaq® Green Master mix (Promega) permitting direct loading of PCR reaction mix on DNA electrophoresis gel. Standard protocol for PCR reaction is shown bellow.

PCR reaction protocol

94 °C	3 min	
94 °C	30 s	n cycles
Tm-5 °C	30 – 60 s	
72 °C	1 min/1kbp	

In general, amplification of PCR fragments during cloning techniques used between 30-45 cycles whereas number of the cycles during RT PCR analysis varied between 20-35 cycles.

Reaction mix for 20 µl

dH_2O	11,7 µl
5x GoTaq® green master mix	4 µl
25 mM $MgCl_2$	1,2 µl
10 µM primer F	0,8 µl
10 µM primer R	0,8 µl
10 mM dNTP mix	0,4 µl
DNA template (5-200 ng)	1 µl
GoTaq DNA pol. (5 u/µl)	0,1 µl

2.5.4. Agarose gel electrophoresis

Agarose gel electrophoresis is used to separate DNA molecules of different length. DNA solution is mixed with loading buffer A (6X) and the mixture is loaded on the agarose gel (0,7 – 2 % in 0,5X TAE buffer depending on desired resolution) which is supplemented with 0,5 µg/ml of ethidium bromide. Ethidium bromide is an intercalating agent which binds between DNA bases. When exposed to UV light it will fluoresce in orange color which intensifies almost 20-fold after binding to DNA. DNA electrophoresis is made in an electrophoretic chamber filled with 0,5X TAE buffer. The power supply is set on the intensity 10V/cm. The DNA in the gel is than visualized under UV light.

Loading buffer A (6X): 50 mM Tris-HCL pH 7,5; 50 % (v/v) glycerol; 0,1 M EDTA; 0,1 % (p/v) SDS; 0,05 % (p/v) bromphenol blue; 0,05 % (p/v) Xylene cyanol

TAE buffer 50X: 40 mM Tris-acetate pH 8,0; 1 mM EDTA

2.5.5. DNA extraction and purification from electrophoretic gel

Parts of agarose gel containing desired DNA fragment could be excised from the gel under UV. After, DNA was extracted and purified using NucleoSpin Extract II kit (Macherey-Nagel)

following the user´s manual. At the end of the procedure, DNA was eluted from the column with 20 – 50 µl of dH_2O or TE buffer.

TE buffer: 30 mM Tris-HCL pH 8,0; 1 mM EDTA

2.5.6. Gateway® cloning technology

Gateway® cloning is a universal cloning technique developed by Invitrogen life technologies. It allows the transfer of different DNA fragments between different cloning vectors by homologous recombination. First, a desired DNA sequence is amplified by PCR with the help of an attB tagged primer pair. Homologous recombination-based BP reaction transfer the attB tagged DNA fragment into the donor vector containing attP recombination sites. The resulting entry clone contains desired DNA sequence flanked by attL sites.

Second LR reaction is also a recombination reaction between attL and attR sites. Entry clone generated during BR reaction includes attL sites and Gateway destination vector contains attR sites. During recombination LR reaction between these two sites results in production of destination vector containing DNA fragment of interest and another DNA molecule as a byproduct.

Cloning via BP and LR reactions was performed exactly according to manufacturer´s protocol (Gateway® cloning technology).

2.5.7. Bacterial transformation by heat shock or electroporation

2.5.7.1. Transformation by heat shock

Between 0,2 µg-1 µg of plasmid DNA (or 5 µl of LP Gateway reaction) was added to 50 µl of DH5α electrocompetent cells previously slowly thawed on ice. After 30 min incubation on ice, the mixture was placed for 30 s at 42 °C. After, 500 µl of liquid LB medium was added and bacterias were cultivated for 1 h at 37 °C. Finally, about 150 µl of the mixture was spread on warmed LB plates (LB medium + 15 g/l agar) containing the appropriate antibiotic and cultivated overnight at 37 °C.

2.5.7.2. Transformation by electroporation

Competent cells of *Agrobacterium tumefaciens* LBA4404 were thawed on ice. One to three microliters of purified plasmid DNA was added to 100 µl of electrocompetent cells and mixed. The mix was incubated 1 min on ice and then transferred on the bottom of an ice cold electroporating cuvette (2 mm between the electrodes). Electrotransformation was made using a BioRad electroporator with following settings: capacitance 25 µF, intensity 2,5 kW/cm and resistance 400 Ω. After the electric pulse, 1 ml of LB medium was added and then the mixture was incubated for 3 h at 28 °C. At the end of the procedure, bacterias were spread on warmed LB plates with appropriate antibiotics added and grown at 28 °C for 3 days.

2.5.8. Construction of etag-E2Fb-GFP, GFP-etag-E2Fb and GFP-etag-NtE2F

ORFs of the genes E2Fb (At5g22220) and NtE2F (AB025347) were amplified from cDNA using attb1-etag and attb2-tagged primers (for sequence see the table of primers). PCR products were cloned by Gateway® technique into the pENTR201 entry vector via BP reaction and positive clones were selected via blue-white screening and PCR. After verification of ORFs by sequencing, genes were cloned into the binary vectors pK7FWG2 and pK7GWF2 via LR reaction. Positives clones were screened again by PCR.

2.5.9. Transformation of BY-2 and Arabidopsis

The method according to An et al. (1985) was used with several modifications. Exponential growing BY-2 cells were filtered and transferred into the same volume of fresh MS medium supplemented with 50 µM of acetosyringone. To stimulate the bacterial infection BY-2 cells were damaged by vigorous pipetting. In small Petri dishes (6 cm in diameter) two milliliters of BY-2 suspension was mixed with different volumes (25 µl, 50 µl and 100 µl) of Agrobacterium suspension grown overnight (OD_{600} < 0,6) in LB medium supplemented with appropriate antibiotics. After 3 days of co-cultivation in standard growth conditions for BY-2 the cells were transferred in 50 ml Falcon tubes, spined by centrifugation (1000 rpm, 25°C, 5 min) and 6 times washed by 50 ml of BY-2 culture medium supplemented with 500 µg/ml of carbenicilin and 100 µg/ml kanamycin. Finally, the cells were selected on BY-2 MS medium agar plates with antibiotics. At least 40 clones were selected at 2-3 weeks after the transformation according to the fluorescence signal of the GFP and growth characteristics.

For Arabidopsis transformation the method of floral dipping was used (Davis et al. 2009). Agrobacterium was inoculated in 3 ml of LB medium supplemented with appropriate antibiotics and cultivated overnight at 28 °C. Five hundred µl of the culture was transferred to 100 ml of LB medium supplemented with 10 mM of $MgSO_4$ and let grow until de OD_{600} = 0,8. Agrobacterium cells were spined down in 2 x 50 ml Falcon tubes (10 min, 4000 rpm, 25 °C) and the supernatant was removed. The cells were resuspended in 25 ml 10 mM $MgSO_4$ with 10 µM acetosyringone and incubated 30 min in room temperature. This suspension was mixed with 250 ml of 5 % sucrose solution supplemented with 150 µl of Silwet L-77. Arabidopsis inflorescences were dipped in this solution, the whole plants were wrapped in cellophane and they were leaved 2 days in obscurity. Finally the plants were placed in the greenhouse until the maturation of the seeds in the siliques. The positive clones were selected on MS plates with appropriate antibiotics.

2.5.10. Genotyping

Before experiments using Arabidopsis T-DNA insertion mutants it was necessary to identify homozygous plants mutated in the gene of interest. This method is based on PCR when specific DNA regions are amplified with primers which sequence was obtained from the seed bank of origin of the

mutant. Genotyping is based on PCR amplification of two specific regions, so two different set of primers are need: first set of primer hybridizes with the region on T-DNA and with the flanking region in the sequence of mutated gene, second pair of primers hybridizes with both flanking regions of each T-DNA side. Because in each plant harbors two copies of each gene we can identify different genotypes according to obtained PCR products visualized by DNA electrophoresis. Homozygous lines show the PCR product only when the first pair of primers is used whereas PCR amplicon using a second pair of primers is not detected because of the big size of T-DNA insertion. Heterozygous plants show the PCR product with both sets of primers. Further information concerning the genotyping of the mutants characterized in this manuscript could be found on the website: http://signal.salk.edu/gabout.html.

DNA for genotyping was extracted from cotyledons, first true leaves or flower bud. The tissue was homogenized in 400 µl of DNA extraction buffer. Twenty µl of 10% SDS was added for protein denaturation and DNA deproteinization and the mixture was vortexed. After 5 min of incubation at room temperature the samples were centrifugated 5 min at 16000 g and the supernatant was transferred to the other tube with the same volume of isopropanol. The DNA was precipitated for 5 min on ice and than centrifugated 10 min at 16000 g. The pellet was washed twice in 70 % ethanol, dried and resuspended in 10 µl of TE buffer. For genotyping, 1 µl of DNA was added as a template for one PCR reaction.

DNA extraction buffer (20 ml): 200 mM Tris-HCl pH 7,5; 250 mM NaCl; EDTA 25 mM

TE buffer: 10 mM Tris-HCl pH 8,0; 1 mM EDTA

2.6. RT qPCR and RT sqPCR

2.6.1. RNA extraction

Extraction of RNA from BY-2 cells or Arabidopsis plants was performed using solution Tri-Reagent (MRC, Gene Company) according to manufacturer´s instruction. Briefly, 50 mg of fresh weight of plant material was mixed with 1 ml of Tri-Reagent solution and 200 µl of glass beads (0,4 mm in diameter). Tubes were placed into the homogenizer (TeSeE PRECESS 24 homogenizer, BioRad) set on maximal speed for 1 min. Samples were incubated at room temperature for 5 min then 200 µl of chloroform was added. Samples were vigorously shaken for 15 s and incubated for 15 min. Tubes were spun at 13000 g for 15 min at 4 °C. The colorless aqueous phase was transferred to another tube and mixed with the same volume of isopropanol. After 15 min of RNA precipitation on ice the samples were centrifuged at 13000 g for 10 min at 4 °C. Supernatant was discarded and RNA pellet was washed twice with 75 % ice cold ethanol for 5 min to eliminate all salts. RNA pellet was air-dried for approximately 10 min. Finally, RNA pellet was resuspended in 30 µl sterile H_20. RNA

dissolution was improved by incubation of the tubes at 65 °C for 10 min and concomitant vigorous vortexing.

2.6.2. Measuring of RNA concentration

RNA concentration was measured using a nanodrop (Thermo Scientific Nanodrop 2000) according to manufacturer instructions. This method is based on measuring of RNA absorbance at 260 nm by spectrometry. The calculation of RNA concentration suggests that one unit of optical density is equivalent to about 0,041 µg/ml of RNA. Protein contamination of RNA samples should be controlled by measuring A_{260}/A_{280} ratio. If the value is near 2 our sample has no high protein contamination.

$$[RNA] = A_{260} \text{ x dilution factor x } 0{,}041$$

2.6.3. DNAse treatment of RNA

DNAse treatment is made on extracted RNA samples and is highly important prior to cDNA synthesis for RT PCR analysis to eliminate genomic DNA. One microgram of RNA was mixed with 1 µl of DNAse I buffer 10x (Fermentas), 1 µl of DNAse I (Fermentas) and sterile dH_2O to final volume 10 µl. After 30 min incubation at 37 °C the DNAse 1 was inactivated by adding of 1 µl of 25 mM EDTA and incubation at 65 °C for 10 min.

2.6.4. Reverse transcriptase

Reverse transcriptase (RT) was used to synthesize complementary DNA (cDNA) from the RNA template. RT requires for cDNA synthesis 3´OH DNA free ends thus DNA primers (e.g. oligo dT or random oligomeres) are needed for initiation of reverse transcription by RT. In our case, we used random deoxyribonucleotide hexamers (dN_6) (Sigma). One microgram of DNAse I-treated RNA was reverse transcribed with Improm-II reverse transcriptase (Promega Corporation, Madison, WI, USA) according to the manufacturer protocol.

2.6.5. qPCR and sqPCR

Quantitative (qPCR) and semiquantitative PCRs (sqPCR) permit the quantification of cDNA in the sample in the relative and absolute level, respectively. These techniques are based on DNA amplification and detection of the product in a real time during qPCR or at the end of PCR amplification during sqPCR when the final amount of the PCR product is usually visualized with DNA electrophoresis. qPCR supposes that PCR amplification rate is proportional to the initial amount of amplified DNA sequence in tested cDNA solution.

Generation of PCR products can be detected by measurement of the SYBR Green I (LighCycler® 480 SYBR Green I Master, Roche) fluorescence signal used during this reaction as a reagent. SYBR green I contains fluorescein as a fluorochrome which is excited at 497 nm and emits at 530 nm. The fluorescein intercalates into the double stranded DNA helix and the fluorescence of unbounded dye has very little fluorescence; however the fluorescence at 530 nm is greatly enhanced

upon DNA-binding (Zipper et al. 2004). Thus during PCR amplification the fluorescence of the sample proportionally increases with the double stranded DNA generated.

To prove that only one PCR product was amplified, melting curve analysis was performed after PCR amplification. During melting curve analysis the sample is slowly heated to 97 °C which causes the melting of DNA helix and thus decrease of SYBR green fluorescence which is monitored and displayed as melting peaks. Amplification of only one particular PCR product is characterized by the presence of one melting peak whereas other amplifications would be represented as additional peaks.

The fluorescence linked to the cDNA amplification is detected at the beginning of exponential phase of the reaction when the efficiency of the amplification of the target could be normalized to another gene of control. This gene is a housekeeping gene having a constant expression during the conditions analyzed in the experiment and permits to standardize the relative quantity of cDNA added into the reaction.

Relative expression analysis of the target gene is counted from the Ct (threshold cycle) value which is a number of cycles when the fluorescence signal overcome the background at the beginning of the exponential phase during the amplification. Ct value of target gene or treated sample is normalized by subtraction of Ct_{gene} by Ct of the reference gene Ct_{18S}. These operations are made at once for treated and untreated samples resulting in ΔCt value: $\Delta Ct_{gene} = Ct_{gene} - Ct_{18S}$ and $\Delta Ct_{control} = Ct_{control} - Ct_{18S}$. Finally, ΔΔCt is counted: $\Delta\Delta Ct = \Delta Ct_{gene} - \Delta Ct_{control}$ and the relative expression Qr is given by the formula $Qr = 2^{-(\Delta\Delta Ct)}$ (Livak and Schmittgen method, Livak et Schmittgen 2001). Qr value represents a difference of gene expression in a genotoxic-treated sample and a sample non-treated and not the level of expression itself. Significant difference in alteration of gene expression is considered when Qr value is higher than 2.

The PCR reaction protocol

50 °C	2 min	
95 °C	10 min	
95 °C	15 s	40 cycles
56 °C	30 s	
72 °C	30 s	

LighCycler® 480 SYBR Green I Master, Roche

Reaction mix for 10 µl

2 x reaction buffer	5 µl
5 µM primers F+R	2 µl
2 µl dH_2O	2 µl
cDNA template	1 µl

Each Qr represent the mean of values from trireplicates each obtained during three independent experiments giving 9 compared Qr values in total. This comparison of results from several independent experiments permitted us to calculate the standard deviations represented as error bars in the graphs.

Results

The aims of this thesis were to characterize genotoxic-induced PCD in plants and to unravel the role of the E2F transcriptional factor which has an important role during animal PCD. For our studies we used an undifferentiated system for investigations at the single cell level and after we worked on highly complex level in Arabidopsis - a multicellular organism with different differentiated tissues. We analyzed the PCD triggered by DNA DBS inducer BLM to make a connection with our previous wide investigation on DNA repair in response to DBS. However to be able to make more general conclusions concerning ATM and ATR signaling and a crosstalk of both pathways we used also other genotoxics inducing replicative stress such as hydroxurea (HU) and aphidicolin (APC). These drugs were used mainly in the analyses of several Arabidopsis T-DNA insertion mutants such as *atm* and *atr*. It is important to note that DSBs are more deleterious lesions compared to SSB that is why we used BLM a DSB inducer to study cell death induction in plants.

3.1. Chapter 1: BLM-induced PCD in tobacco BY-2 cells

3.1.1. Characterization of BLM-induced PCD at the cellular level

3.1.1.1. Growth conditions of BY-2 cell line

Tobacco BY-2 cell line is a widely used cell suspension in plant biology because of its high homogeneity and high growth rate since it is able to multiply up to 100-fold during one week after subculturing. The cell suspension growth is characterized by 3 phases (Figure 3.1A): a first lag phase corresponding to reentry into S phase (day 1-2 of subculturing), followed by an exponential phase (day 3-4 of subculturing), when cells undergo several rounds of cell division. During this phase the cells are characterized by dense cytoplasm, high number of small vacuoles and presence of cytoplasmatic strands (Figure 3.1B). At the end of subculturing (after 5 days) the cells stopped to divide and reached the stationary phase when most of the cells in the culture were arrested in G1 phase. These cells harbored many vacuoles fused to the large one filling most of the inner space and pushing the nucleus from the cell center close to the cell membrane. For most of the experiments we used cells in exponential phase (mid-log phase cells) in order to study the effect of genotoxics on cell division and cell morphology.

3.1.1.2. Kinetics of PCD induction with BLM

Mid-log phase cells were transferred into the medium containing different doses of bleomycin (BLM) and maintained in standard growth conditions. We used increasing concentrations of BLM (10^{-5} M and 10^{-4} M) as well as increasing time of treatment. We have evaluated cell viability using FDA staining after different BLM treatments after 3 days (Figure 3.2). To analyze the kinetics of BLM-induced cell death, we determined cell mortality as the number of dead cells to the total cell number in

the culture. As shown in Figure 3.3 the cells treated with 10^{-4} M BLM progressively increased the mortality to 60%. The mortality of cells treated with 10^{-5} M BLM increased to 5% at day 1 and 2 and finally reach 20 % at day 3 compared to the control non-treated cells which had a very low mortality level (not more than 1%). These results showed the ability of BLM to induce cell death in BY-2 cell culture in a time- and dose-dependent manner.

3.1.1.3. Morphological characterization of BLM-induced PCD

When BY-2 cells were exposed to BLM, besides cell death induction, we observed also apparent changes in cell morphology (Figure 3.4). The cells begun to increase in size, elongate and the most of the cellular content fulfilled one central vacuole as seen on the example after 2 days of 10^{-4} M BLM treatment. These changes started to be apparent from 24 hours of the treatment and they were more pronounced for the cells treated with higher BLM concentration. These observations prompt us to focus on the vacuole dynamics.

We used BCECF-AM and Evans blue double staining which permits to monitor integrity of the tonoplast and cell viability after BLM treatment, respectively. As previously described by Higaki et al. (2007) in living cells, BCECF-AM is cleaved by intracellular esterase activity into the fluorogenic product BCECF with typical localization pattern in the vacuole, whereas Evans blue is actively exported out of the cell. Conversely in dead cells, BCECF-AM is no more visible whereas Evans blue is accumulated in cells. According to the staining patterns we can distinguish three different cell categories (Figure 3.5A): Type I – intact viable cells with intact plasma membrane and tonoplast harbored Evans blue-negative and BCECF-vacuolar specific-positive staining; Type II – viable cells with intact plasma membrane and disintegrated tonoplast had Evans blue-negative and BCECF-positive staining, with a BCECF signal in the cytosol and nucleus; and Type III – with dead cells having disintegrated plasma membrane presenting Evans blue-positive and BCECF-negative staining. After 10^{-5} M BLM treatment, type II and III cell patterns increased progressively from day 2 to day 3 of genotoxic treatment with a 15% maximum (Figure 3.5B). When 10^{-4} M BLM was applied, type II and type III cell patterns appears earlier at day 1 of genotoxic treatment, and increased with a maximum at day 2 for type II cells (40%), and a maximum for type III cells at day 3 (60 %) (Figure 3.5B). In non-treated control culture, 99 % of cells harbored the type I pattern during all 3 days of cultivation (data not shown). These results indicate that vacuolar collapse precedes cell death induction upon BLM treatment.

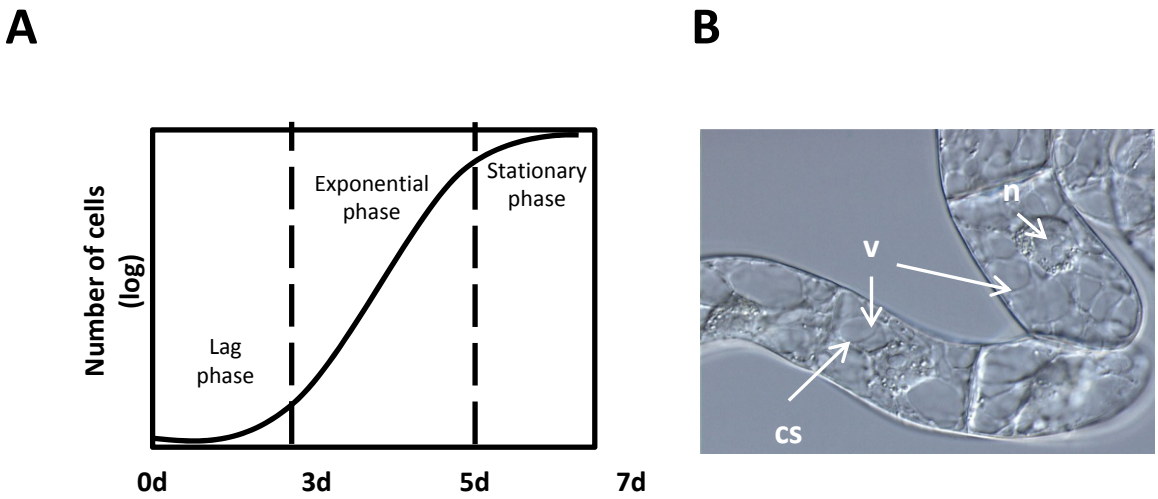


Figure 3.1: Tobacco cel line BY-2. **A:** Growth curve of tobacco cell line BY-2. **B:** Morphology of mid-log BY-2 cells (v – vacuole; n – nucleus; cs – cytoplasmatic strands)

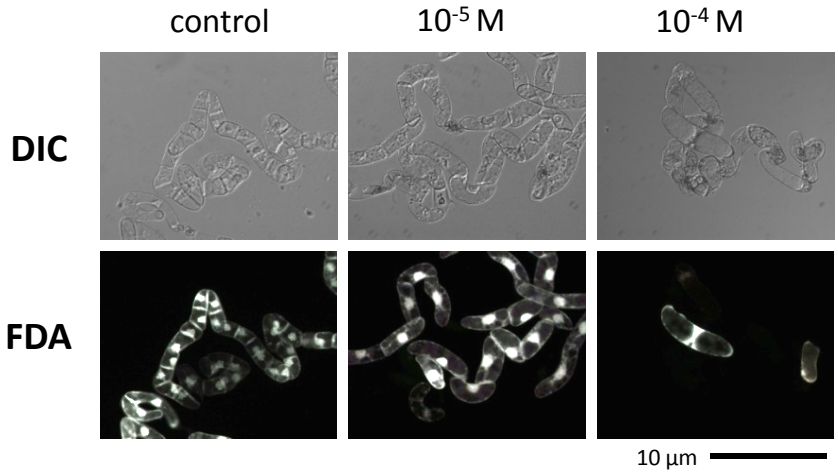


Figure 3.2: FDA viability test of BY-2 cells after 3 days of BLM treatment (10^{-5} M, 10^{-4} M). FDA – fluorecent signal, DIC – differential interference contrast.

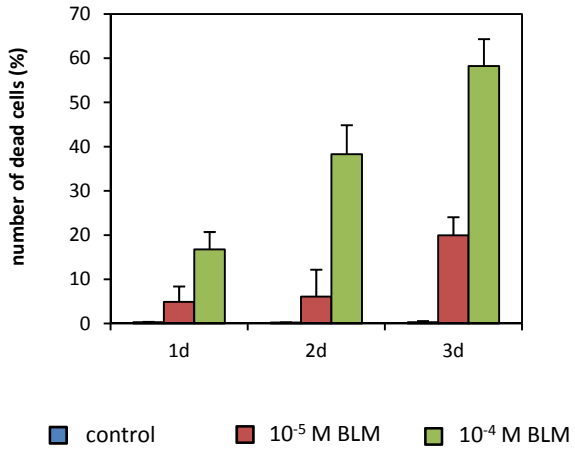


Figure 3.3: Kinetics of cell death induction during 3 days of BLM treatment (10^{-5} M, 10^{-4} M).

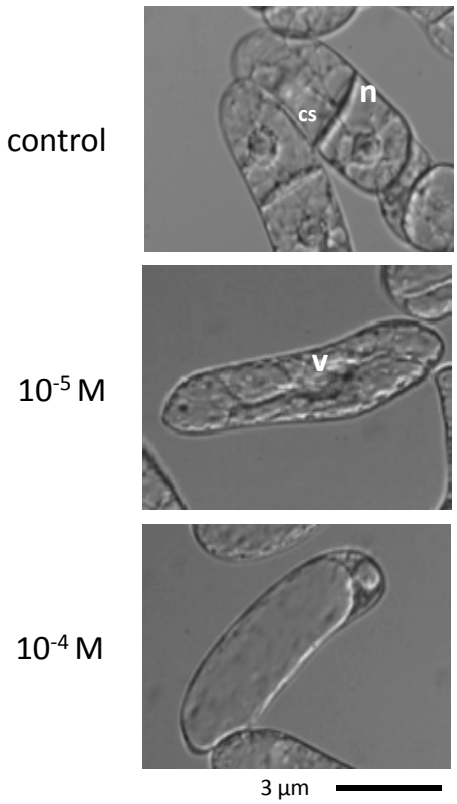


Figure 3.4: DIC images of BLM (10^{-5} M, 10^{-4} M)-treated BY-2 cells during 2 days. (n – nucleus; v – vacuole; cs – cytoplasmatic strands)

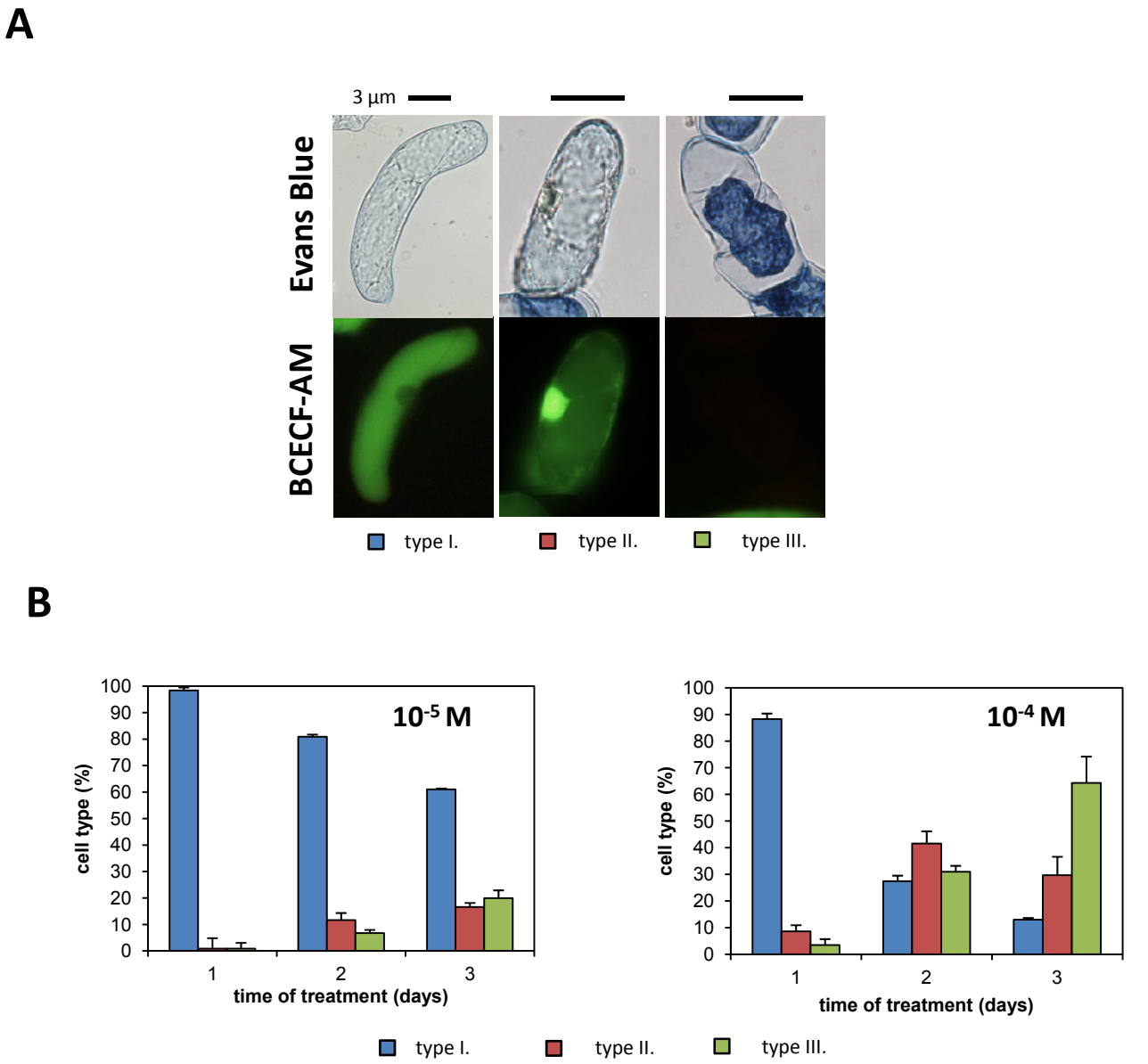


Figure 3.5: Analysis of vacuolar integrity after BLM treatment (10^{-5} M, 10^{-4} M). **A:** Different cell types distinguished by different pattern of Evans blue and BCECF-AM double staining. Type I – intact viable cells, type II – viable cells with disintegrated vacuole, type III – dead cells; **B:**.Quantification of cell categories after 3 days of BLM treatment.

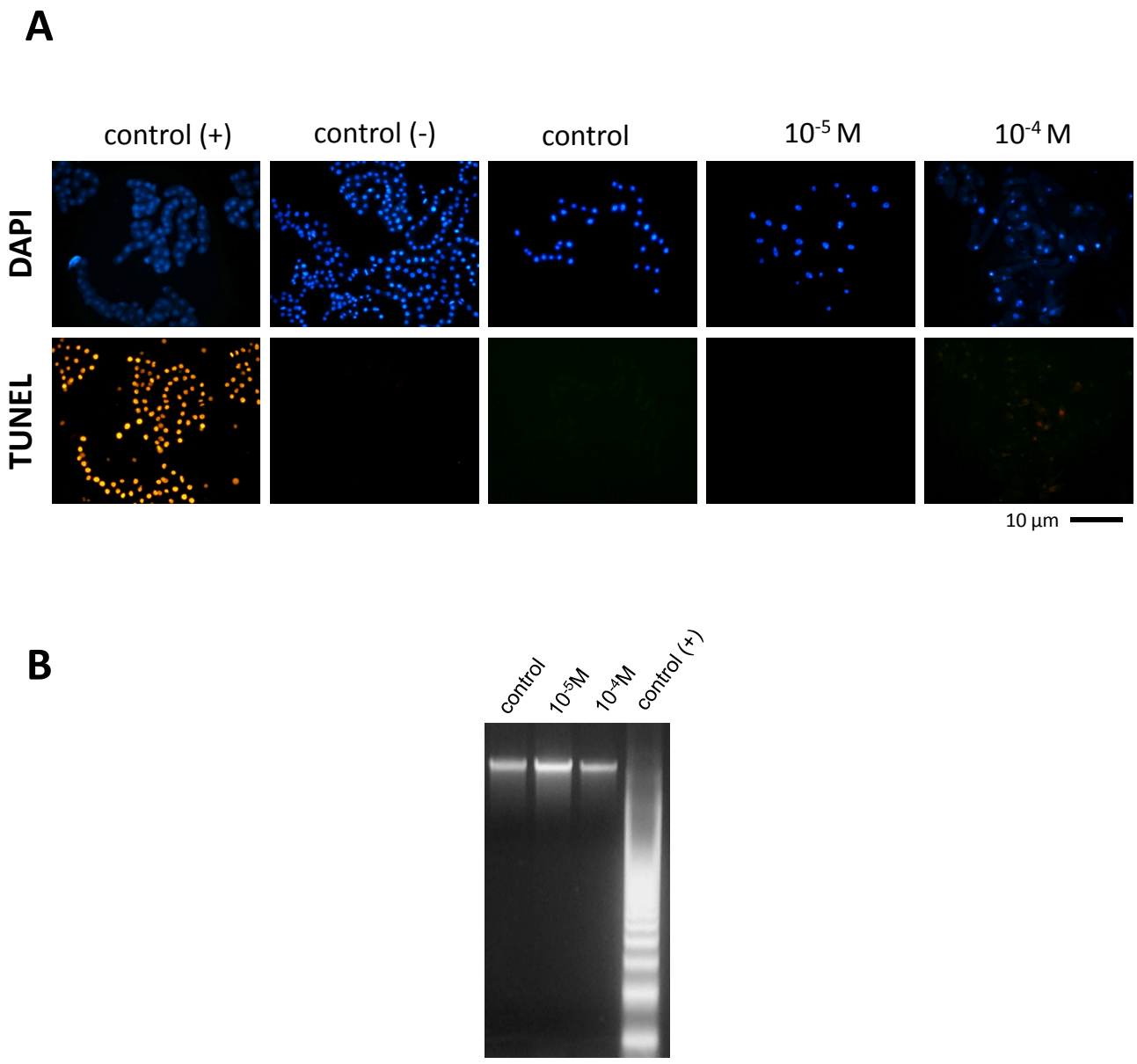


Figure 3.6: Detection of specific DNA fragmentation in BY-2 after BLM treatment (10^{-5} M, 10^{-4} M). **A:** DAPI and TUNEL TMR red staining after 3 days of BLM treatment. control (+) – positive control treated with DNAse I (20 µg/ml) prior to TUNEL reaction, control (-) – negative control without TdT enzyme in the reaction mix; **B:**. „DNA laddering" assay after 7 days of BLM treatment. control (+) – positive control containing DNA from apoptotic cells.

3.1.2. Characterization of BLM-induced PCD at the molecular level

3.1.2.1. DNA fragmentation test

To assess if BLM-induced cell death is linked with specific DNA laddering typical for apoptotic-like PCD, we used two available methods. DNA fragmentation could be detected by direct visualization of fragmented DNA on electrophoresis or by *in situ* TUNEL reaction, which labels free 3´OH ends of fragmented DNA by adding modified nucleotides (e.g., biotin-dUTP, DIG-dUTP, fluorescein-dUTP) to the 3´-OH termini. The enzyme Terminal deoxynucleotidyl transferase (TdT) catalyzes the template independent polymerization of deoxyribonucleotides to single- or double-stranded DNA.

After 3 days of BLM treatment of BY-2 cells, we performed TUNEL reaction (*In situ* cell death detection kit, Roche) (Figure 3.6A). Cells treated with 20 µg/ml of DNAse I prior to TUNEL reaction served as a positive control whereas negative control cells were used in the reaction without TdT. Even if a strong signal was obtained in the positive control we were not able to detect positive signal in BLM-treated cells. For DNA laddering assay, total DNA was extracted using DNA apoptotic ladder kit (Roche) and as a positive control we used DNA from apoptotic U937 cells included in the kit. No DNA laddering was observed after 7 days of BLM treatment when almost all the cells in the culture were dead; indeed the genomic DNA remained still intact (Figure 3.6B). These results suggest that during BLM-induced cell death no specific oligosomal DNA fragmentation occurs.

3.1.2.2. Expression level of PCD-related genes

For further characterization of BLM-induced cell death, we analyzed the expression levels of several PCD-related genes using Real time qPCR – *Pathogenesis related proteins 1* (*PR1a*, *PR1b*), *Vacuolar processing enzymes* (*VPE1a*/*VPE1b, VPE2*, *VPE3*), *metacaspase II* (*MCA II*) and *Bax inhibitor-1* (*BI-1*). Samples for RNA extraction were taken 24 hours after the transfer of exponential cells into the medium containing 10^{-4} M BLM. We tested this BLM concentration because of significant cell death induction in BY-2 cells (18%; Figure 3.3) at 1 day of treatment which preceded the huge induction of vacuolar collapse observed at 2 days of BLM exposure (40%, Figure 3.5B). For normalization of the results we used as an internal standard ribosomal *18S* gene. All the results of RT qPCR are presented in Figure 3.7. Results were presented as Qr related to delta of Ct from samples treated with BLM minus Ct of control samples.

Tobacco PR1 proteins are divided on acidic and basal forms, so for expression analysis we choose genes encoding one member of each group. *PR1a* (X06930) encodes a member of acidic forms accumulating rather in the extracellular matrix during host-pathogen interaction and *PR1b* (X66942) encodes a basic form targeted to the vacuole (van Loon and van Strien, 1999). RT-qPCR analysis

revealed that after 24 hours of 10^{-4} M BLM treatment *PR1a* expression was only 2-fold higher than in untreated samples. Substantially higher expression level was observed for vacuolar *PR1b* – after 24 hours the Qr value reached 27. These results show that vacuolar *PR1b* is highly expressed upon BLM treatment compared to *PR1a*.

VPEs are plant proteases that exhibit caspase 1-like activity and are crucial for hypersensitive response or during embryogenesis (Hara-Nishimura et al. 2005, Hatsugai et al. 2006). In tobacco, 4 different VPEs were identified till now – VPE1a, VPE1b, VPE2 and VPE3. Due to the high homology of *VPE1a* and *VPE1b* we could not analyze the Qr values separately and thus our results represent an expression obtained for both genes using common pair of primers. After 24 hours of 10^{-4} M BLM treatment, *VPE1a/VPE1b* were upregulated up to 17-fold, whereas *VPE2* and *VPE3* were upregulated to a lesser extent (around 6-7-fold). Thus, these expression profiles show that *VPE1a/VPE1b* are highly upregulated upon DNA damage compared to *VPE2* and *VPE3*.

Metacaspases are orthologs of animal caspases and were described in various Eukaryotes including plants (Uren et al. 2000). In *Nicotiana tabaccum* only a partial CDS for one metacaspase was identified and designated as a member of type II metacaspase (*MCA II*). We performed RT qPCR analyses in the same conditions as for previous PCD-related genes. Our data showed no significant upregulation of *MCA II* in response to BLM suggesting that this gene is not regulated in the DBS response in BY-2 cells in our experimental conditions (Figure 3.7).

Another marker of PCD, *BI-1* was described to be upregulated under different stress conditions leading to cell death (Watanabe et Lam 2006; Bolduc et Brisson 2002). Furthermore, *BI-1* is able to suppress elicitor-induced PCD in rice or PCD induced by hydrogen peroxide or salicylic acid in tobacco BY-2 cells (Kawai-Yamada 2004). After 24 hours of BLM treatment we observed 5-fold increased of Qr when using 10^{-4} M BLM (Figure 3.7).

After having characterized the PCD induced by BLM in BY-2 cells we have investigated its relationships with DNA damage signaling and cell cycle checkpoints.

3.1.3. Link between BLM-induced PCD and cell cycle checkpoints

We treated mid-log phase cells with BLM after transfer to fresh medium containing BLM (10^{-5} M, 10^{-4} M). First, the mitotic index (MI, percentage of dividing cells) was monitored during 3 days of BLM treatment to see how BLM affects cell division (Figure 3.8A). In the control cells, MI reached around 7 % and progressively decreased to 2 % at day 3. These values are in agreement with normal parameters of BY-2 cells division kinetics (Nagata et al. 1992). When the cells were transferred to the medium with BLM, MI decreased considerably, to less than 1% after 1 day of BLM treatment at both concentrations (Figure 3.8A). These data show that cell divisions were completely blocked in response to BLM, suggesting an arrest of cell cycle.

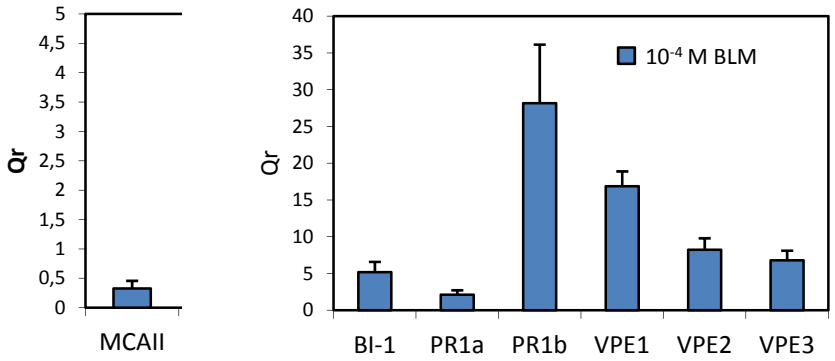
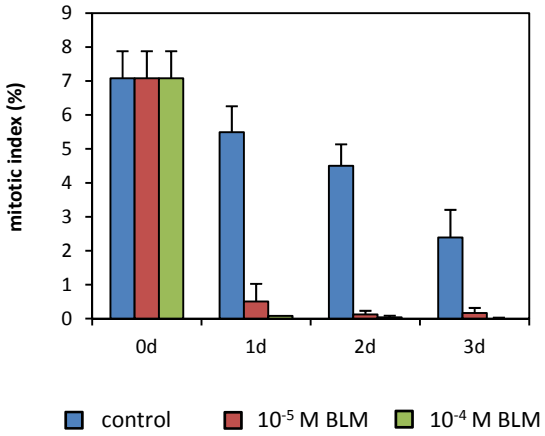


Figure 3.7: Real time qPCR analysis of the expression of PCD-related genes *MCAII, VPE1ab, VPE2, VPE3, PR1a, PR1b* and *BI-1* after 10^{-4} M BLM treatment during 24 hours. As a reference gene 18S was used.

A



B

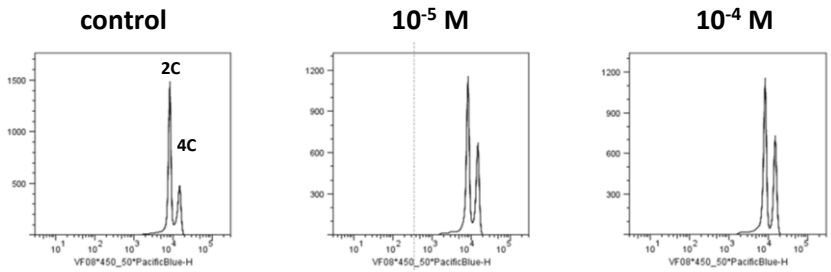


Figure 3.8: Effect of BLM (10^{-5} M, 10^{-4} M) on the cell cycle. **A:** Mitotic index progression during 3 days; **B:**. Flow cytometry analysis after 1 day of BLM treatment.

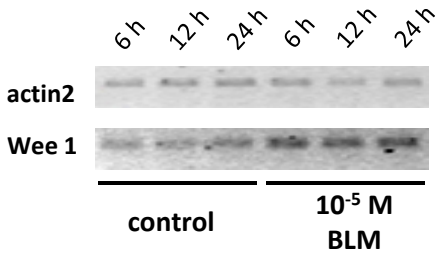
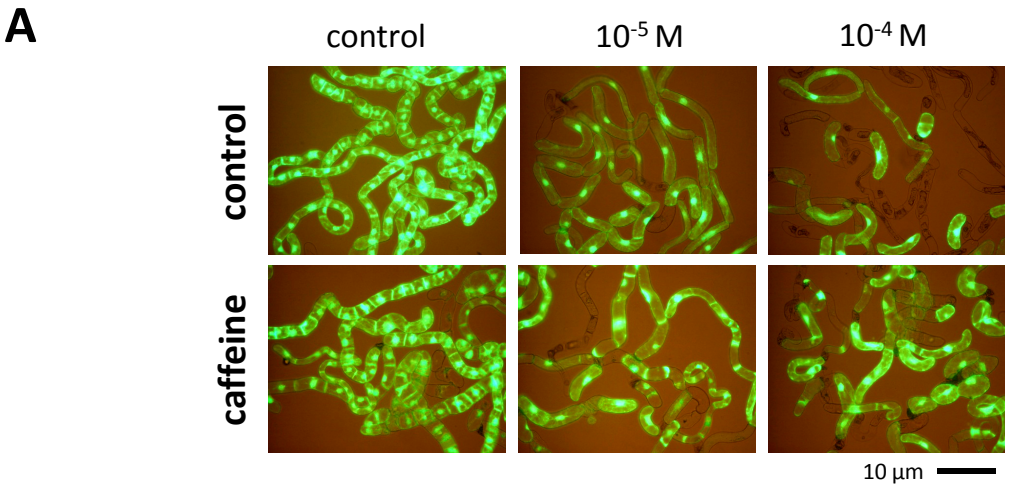


Figure 3.9: Semiquantitative RT PCR performed to analyze the expression of *Wee1* after 6, 12 and 24 hours of 10^{-5} M BLM treatment. *Actin2* gene used as an internal standard.



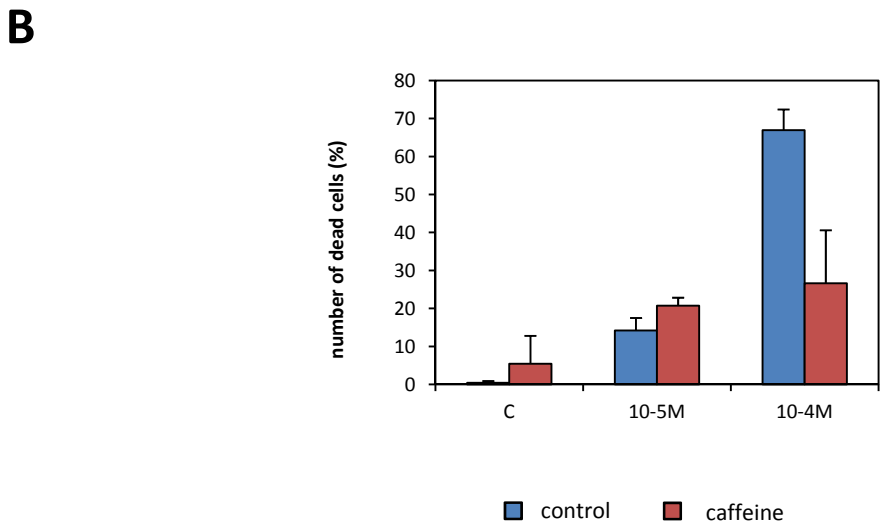


Figure 3.10: Effect of caffeine (5 mM) on cell death induced after 3 days of BLM treatment (10^{-5} M, 10^{-4} M). **A:** FDA staining of BY-2 cells; **B:** Cell death quantification.

In order to precise which cell cycle checkpoint is activated, we used flow cytometry approach. After 1 day of 10^{-5} M and 10^{-4} M BLM treatment, more cells had a 4C DNA content corresponding to G2 phase cells compared to control cells (Figure 3.8B). Consequently, BLM induced G2/M cell cycle checkpoint in BY-2 cells.

3.1.4. Link between PCD and DNA damage response

In Arabidopsis during DSB cellular response, both ATM and ATR contributes to the induction of a CYCB1;1:GUS fusion after γ irradiation, but only ATR is required for the persistence of this response (Culligan et al. 2006) reflecting a new role of cyclin B1 in the DNA damage response. ATM (Ricaud et al. 2007) seems also to control stem cell maintenance and more recently to act in concert with ATR for the genotoxic-PCD induction in stem cell (Fulcher et Sablowski 2009).

In plants, one of the well documented downstream effector of ATM is WEE1 kinase and is responsible for G2 cell cycle arrest. It is documented that its expression is induced after zeocin treatment or γ irradiation in Arabidopsis (de Schutter et al. 2006). Thus we tested if in our conditions the tobacco homologue of *Wee1* gene is transcriptionaly induced upon BLM treatment. RNAs for semiquantitative RT PCR analysis were extracted after 6, 12 and 24 hours after the transfer of exponentially growing BY-2 cells in the medium with 10^{-5} M BLM. We detected *Wee1* upregulation that suggests its implication in the G2 checkpoint activation after BLM exposure in tobacco BY-2 cells (Figure 3.9).

We further analyzed if BLM-induced cell cycle arrest and subsequent cell death, is ATM-dependent. As no *atm* mutant is available in tobacco, we have investigated the effect of inhibitors of ATM activity using caffeine. Caffeine was described as a drug which is able to specifically inhibit kinase ATM in animal cells (Blasina et al. 1999). In plants, caffeine bypasses G2 cell cycle checkpoint upon DNA damage (González-Fernández et al. 1985) but fail to inhibit ATR-dependent replicative stress checkpoint (Pelayo et al. 2001). Taken these data together we can suggest that in plants the caffeine is a specific inhibitor of ATM as well.

Therefore we treated BY-2 cells with BLM in presence of 5 mM caffeine which was added at least 1 hour prior to BLM treatment to ensure high inhibitory effect of this drug. FDA viability test revealed that caffeine affected BLM-induced cell death (Figure 3.10A). After 3 days, caffeine reduced cell death in the samples treated with 10^{-4} M BLM by 40 % (Figure 3.10B). Interestingly, the cell death rate was not reduced upon 10^{-5} M BLM in the presence of caffeine. Caffeine-mediated cell death inhibition in BY-2 was only seen at the higher concentration of BLM 10^{-4} M and in addition, the level of cell death between 10^{-5} M (-) caffeine, 10^{-5} M (+) caffeine and 10^{-4} M BLM (+) caffeine was comparable. In the control cells treated with caffeine the cell death induction reached around 5% suggesting that caffeine alone induces cell death in a small extent. These results indicate that at least

one fraction of BY-2 cells dies in an ATM-dependent manner in the presence of high BLM concentration.

The caffeine inhibitory effect was also evaluated at the molecular level analyzing the expression of PCD-related genes. RTqPCR analysis revealed that the transcriptional response of all tested PCD-related genes was strongly inhibited by caffeine upon 10^{-4} M BLM treatment except for PR1a with a Qr value 12 times higher than the control (Figure 3.11). The expression of control housekeeping gene *EF1α* was not modified by caffeine treatment showing us that caffeine do not modify the transcriptional response (data not shown). These results indicate that expression of PCD-related genes is ATM-dependent except for PR1a.

3.1.5. Caspase-like activities

We characterized BLM-induced PCD by cellular and molecular approaches including FDA test, BCECF-AM staining, DNA fragmentation or inhibition of DNA damage response by caffeine. Next, we asked if any caspase-like activities are necessary for cell death induction in BY-2 cells. Several reports have shown the implication of caspase-like activities during various types of PCD including genotoxics-induced e.g., after γ irradiation or *cis*-platine treatment (Danon et al. 2004; de Jong et al. 1999). From RT qPCR analyses of the expression of PCD-related genes we observed a high up-regulation of genes encoding VPEs which exhibit animal caspase-1 activity.

The presence of caspase-like activities have been already extensively studied using two available methods. First one is a direct detection of caspase-like activity using specific caspase substrates based on fluorogenic or chromomeric reaction. The second method uses the specific reversible or irreversible inhibitors which bind to the active site of the enzyme and thus block the enzyme activity. For our analysis, we used Ac-YVAD-CHO and Ac-DEVD-CHO (Bachem) irreversibly binding to animal caspase-1 and -3 respectively and which were previously used to block PCD after cytokinin treatment in BY-2 cells (Mlejnek et Procházka 2002).

Exponentially growing BY-2 cells were transferred to MS medium containing 100 μM caspase inhibitors to achieve high inhibitory effect and the BLM (10^{-5} M and 10^{-4} M) was added in the medium after 1 hour of preincubation. We evaluated cell death induction after 3 days of the culture (figure 3.12A). In our experimental conditions, caspase-1 (Ac-YVAD-CHO) and -3 inhibitors (Ac-DEVD-CHO) do not modify the rate of cell death after BLM-treatment – the number of cell death was 10 - 15 % in response to 10^{-5} M BLM and 59 - 65 % in response to 10^{-4} M BLM with or without caspase inhibitors.

To confirm these negative data we set up another experiment using the cytokinin 6-benzylaminopurie (6-BAP) as a positive control of PCD inducer. Cytokinins are known to be potent

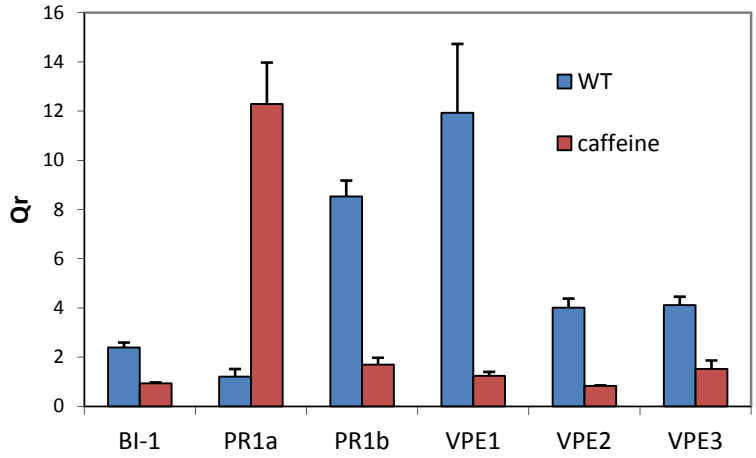


Figure 3.11: Real time qPCR analysis of the expression of PCD-related genes *VPE1ab, VPE2, VPE3, PR1a, PR1b* and *BI-1* after 10^{-4} M BLM treatment during 24 hours. The effect of caffeine (5 mM) was tested in the same experimental conditions.

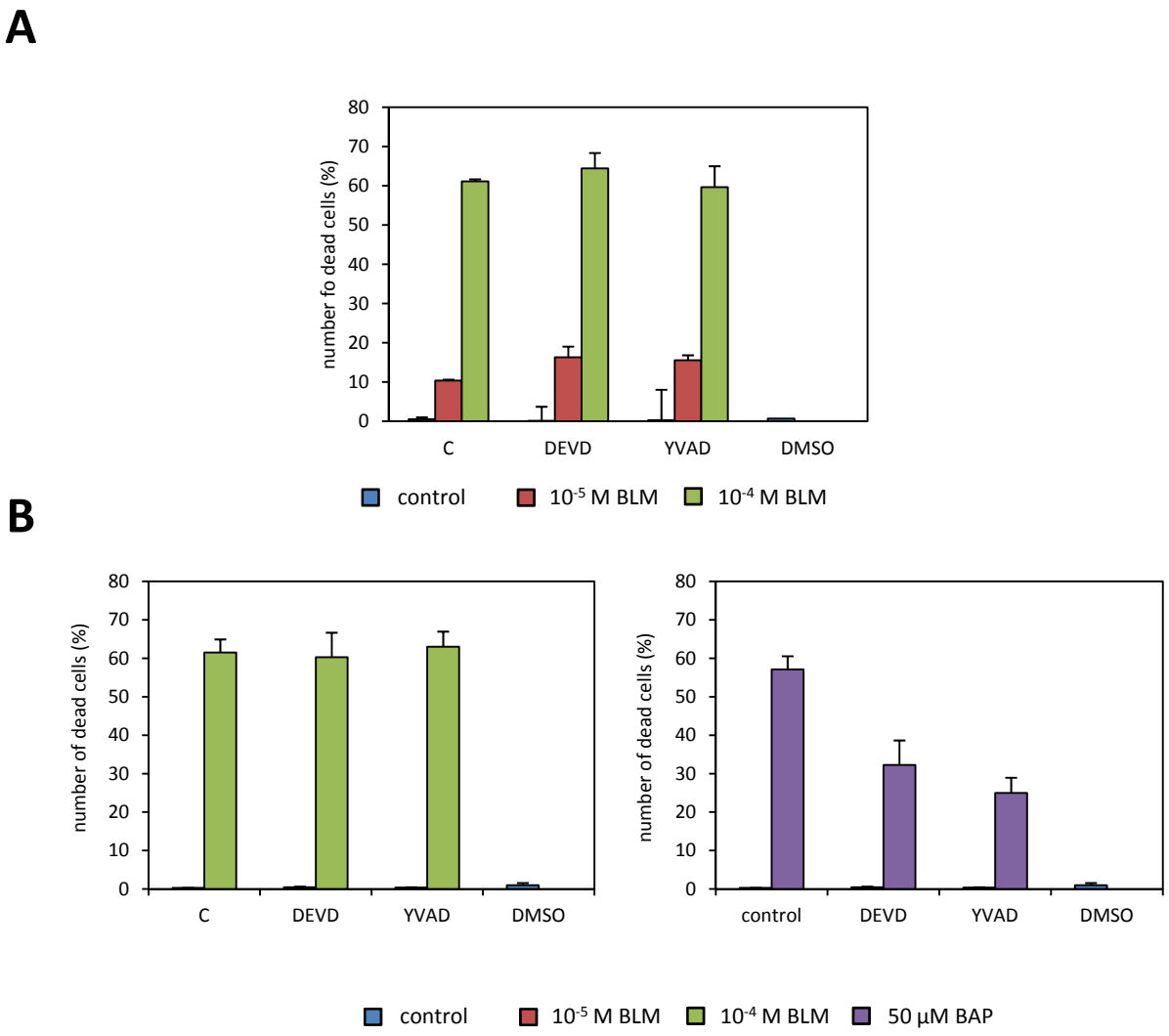


Figure 3.12: Effect of caspase inhibitors (DEVD and YVAD) on cell death induction after 3 days BLM treatment. **A:** 100 µM caspase inhibitors (in DMSO) were added 1 hour before 3 days BLM treatment (10^{-5} M, 10^{-4} M); **B:** 200 µM (in DMSO) caspase inhibitors were added 1 hour before BLM treatment (10^{-4} M). BAP (50 µM) was taken as a positive control.

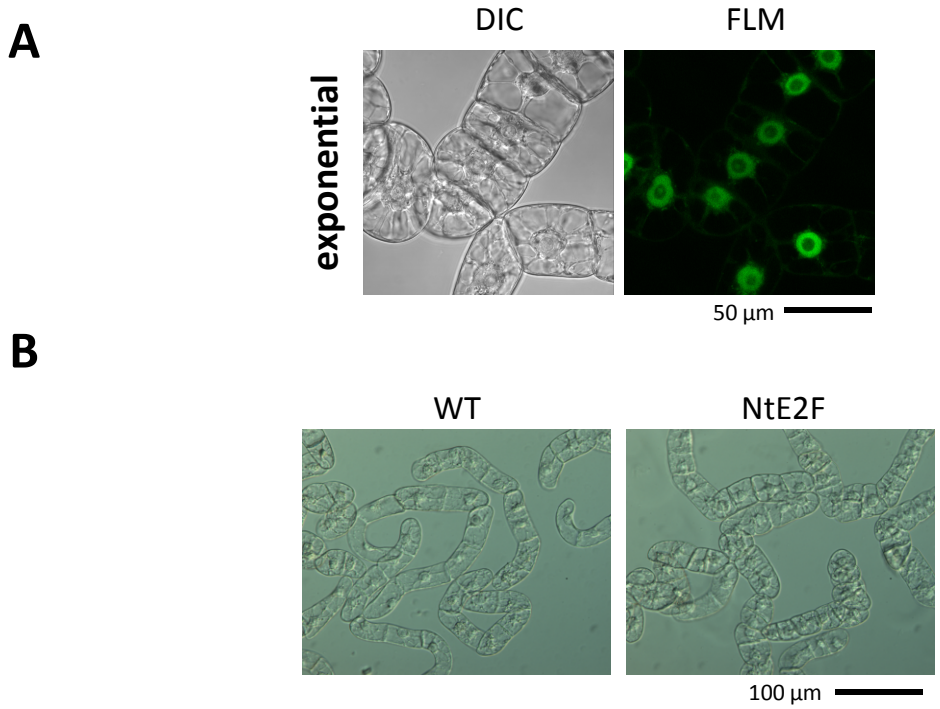


Figure 3.13: Ectopic expression of GFP-NtE2F in BY-2 cells. **A:** Exponential cells under differential contrast microscopy (DIC) and fluorescent GFP signal (FLM). **B:** DIC images of WT and NtE2F cells in exponential growth.

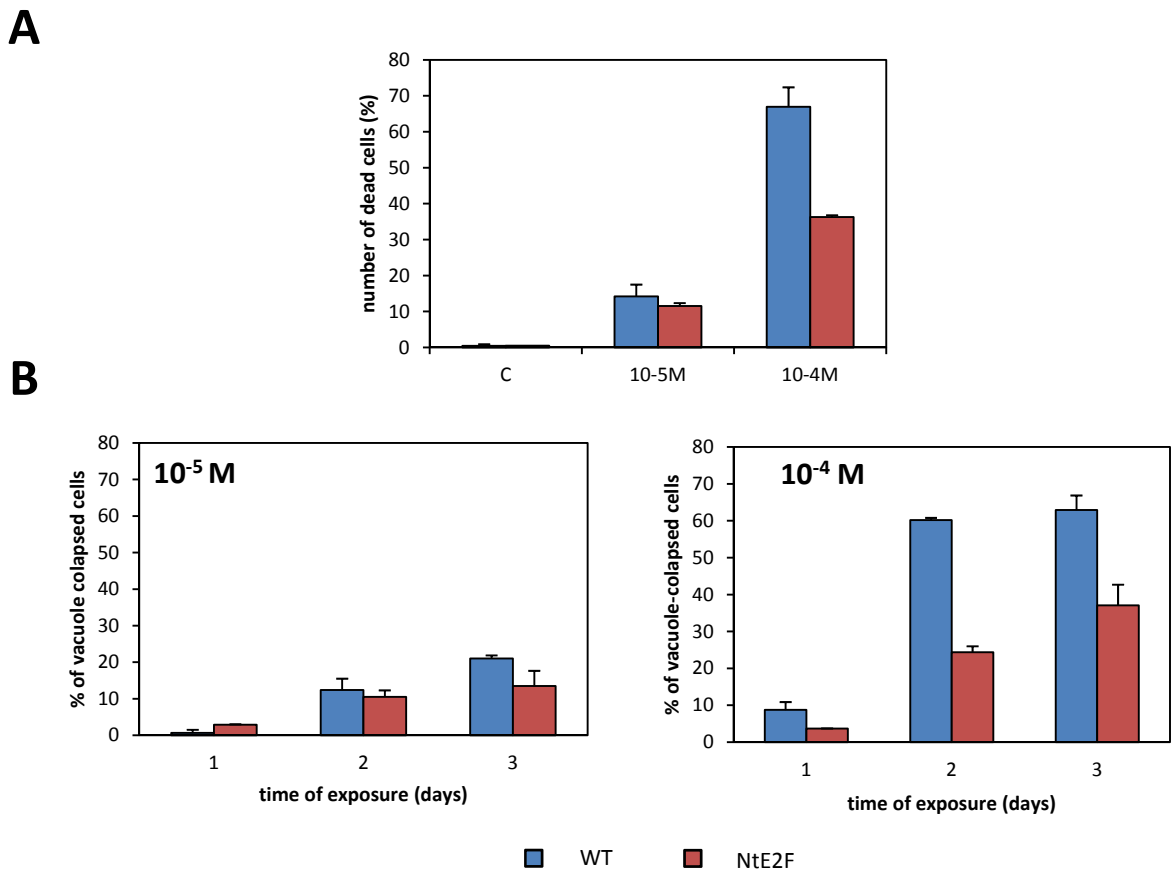


Figure 3.14: Vacuole-mediated cell death in WT and NtE2F cells after 3 days of BLM treatment (10^{-5} M or 10^{-4} M). **A:** Quantification of cell death using FDA test. **B:** Quantification of type II cells (living cells with disintegrated vacuole) during 3 days of BLM treatment. Untreated control showed less than 1% (data not shown).

activators of plant PCD which is characterized by specific DNA fragmentation and the activation of caspase-like proteases (Mlejnek et al.2004; our unpublished results). Even in the presence of high concentration of caspase inhibitors (200 μM), the rate of cell death was not modified in BLM-treated cells whereas the cells of positive control were affected with a decrease of cell death reaching 27 % for Ac-DEVD-CHO and 36 % for Ac-YVAD-CHO (Figure 3.12B). These results suggest that even *VPE* genes are activated, caspase-like activities are not involved in the execution of BLM-induced cell death at least during our experimental conditions.

3.2. Chapter 2: Effect of NtE2F overexpression on PCD induction in BY2

In tobacco, it has been previously shown that NtE2F protein level is increased upon DNA damage induced by UV-C (Lincker et al. 2004). UV-C is also able to induce cell death in Arabidopsis (Danon et al. 2004). Thus we analyzed what could be the effect of ectopic expression of NtE2F in BY-2 on DNA damage-induced cell death.

3.2.1. Characterization of NtE2F OE line

In our laboratory we have previously studied the role of E2F in DSB response in BY-2 cells and Arabidopsis (Roa et al. 2009; Lang et al., submitted). To investigate the role of E2F transcriptional activator in plant PCD we overexpressed N-terminal GFP fusion to NtE2F in BY-2 cells under constitutive *CaMV 35*S promoter (Figure 3.13A) because N-terminal fusion seemed to be more stable compared to the C-terminal fusion (Lang, personal communication). Previously, we showed that GFP:NtE2F fusion protein is able to activate luciferase gene under the control of *NtRNR1b* promoter when co-expressed with its NtDP transactivating partner. So we deduce that N-terminal GFP fusion does not abolish proper NtE2F function (Lang et al., submitted).

GFP signal was mainly nuclear with only a minor signal from the cytoplasm (Figure 3.13A). Concerning the cell morphology, NtE2F cells were smaller in diameter and contained higher number of cells in the chain (Figure 3.13B).We suggest that this phenotype is probably due to increased activity of E2F responsive genes since similar results were observed in BY-2 cells co-expressing AtE2Fb and DPa but at a higher degree (Magyar et al. 2005). However further experiments are needed to confirm this arguing.

3.2.2. Effect of NtE2F OE on BLM-induced PCD – cellular level

In order to analyze the effect of *NtE2F* overexpression on BLM-induced PCD, exponential WT and *NtE2F* overexpressing BY-2 cells were transferred to MS medium containing 10^{-5} M and 10^{-4} M BLM. At 10^{-5} M BLM cells, both lines had the same mortality that slightly overlapped 10 % (Figure 3.14A). Significant difference in the number of cells undergoing PCD was observed at 10^{-4} M BLM, when *NtE2F* overexpression decreased the cell mortality by 30 % compared to WT cells. This experiment shows that NtE2F overexpression inhibits BLM-induced cell death only at high BLM concentration similarly to caffeine inhibitory effect.

Next, as we observed the inhibition of PCD induction by NtE2F overexpression, we were interested if vacuolar collapse was affected. For this purpose we used BCECF-AM and Evans blue double staining as described previously. The progression of cell disintegration strongly differed between WT and NtE2F cells (Figure 3.14B). At a 10^{-5} M BLM treatment, *NtE2F* overexpression slightly decreased the percentage of cells with disintegrated vacuole (type II cells) to 5% compared to

WT at day 3. Such effect was considerably increased upon a 10^{-4} M BLM treatment, where the percentage of type II cells decreased to about one half compared to WT. The number of type II cells in both untreated lines did not exceed 3 % during the whole cultivation period. Thus, these data indicate that *NtE2F* overexpression affects the control of vacuolar collapse leading to decreased level of cell death in response to BLM.

3.2.3. Effect of NtE2F overexpression - molecular level

To further characterize the effect of NtE2F OE we analyzed the response at the transcriptional level for PCD-related genes. RNAs for RT qPCR were extracted from the cells at 24 hours after 10^{-4} M BLM treatment because at this concentration we saw a high difference in cell death induction between WT and NtE2F cells. The expression of PCD-related genes was affected by *NtE2F* in different manners (Figure 3.15) – transcript levels of *VPE*s, which expression was strongly induced after 24 hours of 10^{-4} M BLM treatment in WT, decreased approximately to one half in *NtE2F*-overexpressing cells. The expression of *BI-1* decreased after 10^{-4} M BLM treatment in NtE2F cells compared to WT, thus in similar way as VPEs genes. The expression of PR1s genes were not affected by NtE2F overexpression. The only upregulated gene by NtE2F overexpression was *MCAII*. In WT cells, this gene was not upregulated after 10^{-4} M BLM treatment compared to NtE2F cells. These results show that *NtE2F* overexpression mainly inhibits the transcriptional activation of *VPEs* and *BI-1*, increases the expression of *MCAII*, but does not affect the expression of *PR1s*. That means that NtE2F modulates PCD-related signaling also at the transcriptional level.

3.2.4. Effect of NtE2F overexpression on cell cycle regulation upon DNA damage

3.2.4.1. NtE2F increases genomic instability

In animals, deregulated *E2F1* expression through Rb inactivation leads to increased genomic instability as well as deregulation of DNA damage checkpoints (Pickering et Kowalik, 2006). To assess the effects of *NtE2F* overexpression on BLM-treated cells, we observed DAPI staining of the cells upon BLM treatments of WT or NtE2F cells (Figure 3.16A). After 3 days of a 10^{-5} M BLM treatment, 27 % of the nuclei of NtE2F cells harbored extranuclear DAPI staining DNA particles. These micronuclei were not observed in the control cells or in NtE2F treated with 10^{-4} M BLM (Figure 3.16B).

To determine the nature of the extranuclear DNA particles, we set up Fluorescence *in situ* hybridization (FISH) experiment. After cell fixation and hybridization with a probe specific to telomeric sequences we detected a positive signal not only in the nucleus, but also in the micronuclei showing at least a genomic instability linked to telomeric sequences (Figure 3.16C). BLM treated NtE2F cells also displayed formation of anaphase bridges that were not observed in NtE2F untreated cells as well as in WT cells after any BLM treatment. These results indicate that ectopic *NtE2F*

expression leads to genomic instability in BY-2 cells. Such response was only observed using 10^{-5} M BLM and therefore appears to be BLM-dose dependent.

3.2.4.2. NtE2F overexpression bypassed the G2/M cell cycle checkpoint

E2F transcriptional factors belong to the key regulators of eukaryotic cell cycle by regulating G1/S transition (Francis, 2007). To understand how *NtE2F* overexpression inhibits BLM-induced PCD at the level of the cell cycle checkpoints, we compared cell cycle progression of synchronized WT and NtE2F cells treated with BLM at different phases of the cell cycle as described in Kuthanová et al. (2008). First, we used 10^{-5} M BLM because at this concentration we previously observed increased genomic instability in NtE2F cells suggesting some cell cycle checkpoint deregulation. A specific inhibitor of DNA polymerase α and δ, aphidicolin (APC) was used to reversibly block BY-2 cells in early S phase. After APC removal (time 0 h) the cells were treated with 10^{-5} M BLM at 1, 5 and 9 hours that correspond to different phases of the cell cycle S, G2 and M phase, respectively (Figure 3.17A).The efficiency of cell synchronization is given by the peak of MI which was of 25 % for WT cells and occurred at time 9 hours, whereas the peak of MI for NtE2F cells reached 18 % and was delayed by 1 hour occurring at 10 hours after APC release (Figure 3.17B). When we analyzed cell cycle progression of WT cells after BLM addition during S phase (time 1 h) the MI did not exceed 2 %. In parallel, NtE2F cells showed slight increase of MI to 5 % starting from 10 hours. Clear difference in cell cycle progression was detected when BLM was added during G2 phase (time 5 h). NtE2F cells bypassed the complete G2/M block of cell cycle progression (observed in WT cells) and went through mitosis with a MI around 13 %. MI progression was similar in both WT and NtE2F cell lines when BLM was added during M phase (time 9h). WT cells only presented faster MI drop than NtE2F cells indicating that NtE2F cells are somehow more resistant to BLM induced block of mitosis initiation. These results indicate that in response to BLM, NtE2F overexpression is able to bypass the G2/M checkpoint arrest which was observed in WT cells.

To complete our studies, we have evaluated the DNA content in the cells by flow cytometry. Synchronized WT and NtE2F cells were submitted to a 10^{-5} M BLM treatment at different time points of the cell cycle (Figure 3.17A) - during S (time 1 h), G2 (time 5 h), M (time 9 h) and G1 phase (time 12 h) and Flow cytometry analyses were performed at 24 hours after BLM treatment. In control cells (without BLM treatment), WT and NtE2F cells had mainly 2C DNA content that corresponds to G1 phase (Figure 3.18). When BLM was applied during S and G2 phases, a 4C DNA content was observed in WT cells that correspond to G2 phase cells, suggesting a G2/M cell cycle arrest induced by BLM leading to cell division arrest observed in figure 3.17B. On the contrary, NtE2F cells treated with BLM during S and G2 phase had mainly 2C DNA content, showing the absence of a G2/M checkpoint arrest as observed in WT, but subsequent activation of a G1/S checkpoint leading to the arrest of cell division with 2C DNA content. WT and NtE2F cells treated with BLM during M and

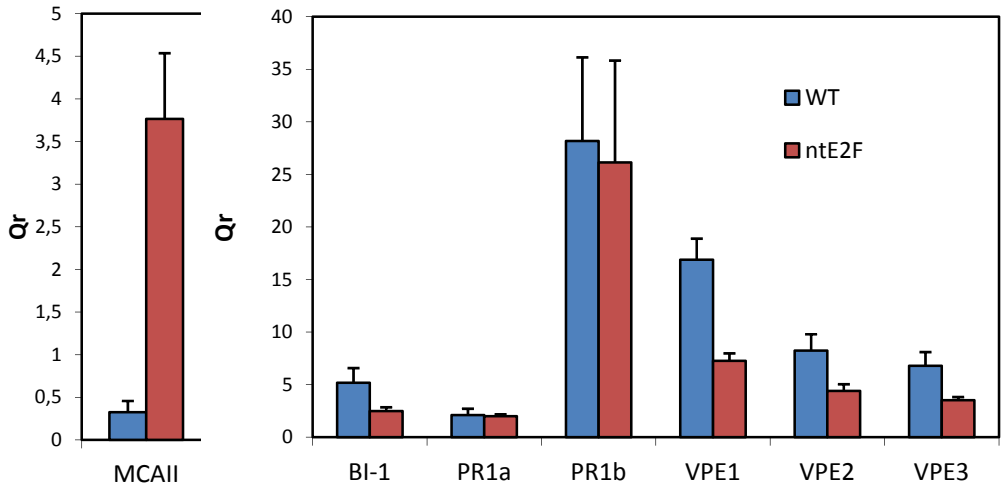


Figure 3.15: Real time qPCR analysis of the expression of PCD-related genes *VPE1ab, VPE2, VPE3, PR1a, PR1b* and *BI-1* in WT and NtE2F cells after 24 hours of 10^{-4} M BLM treatment.

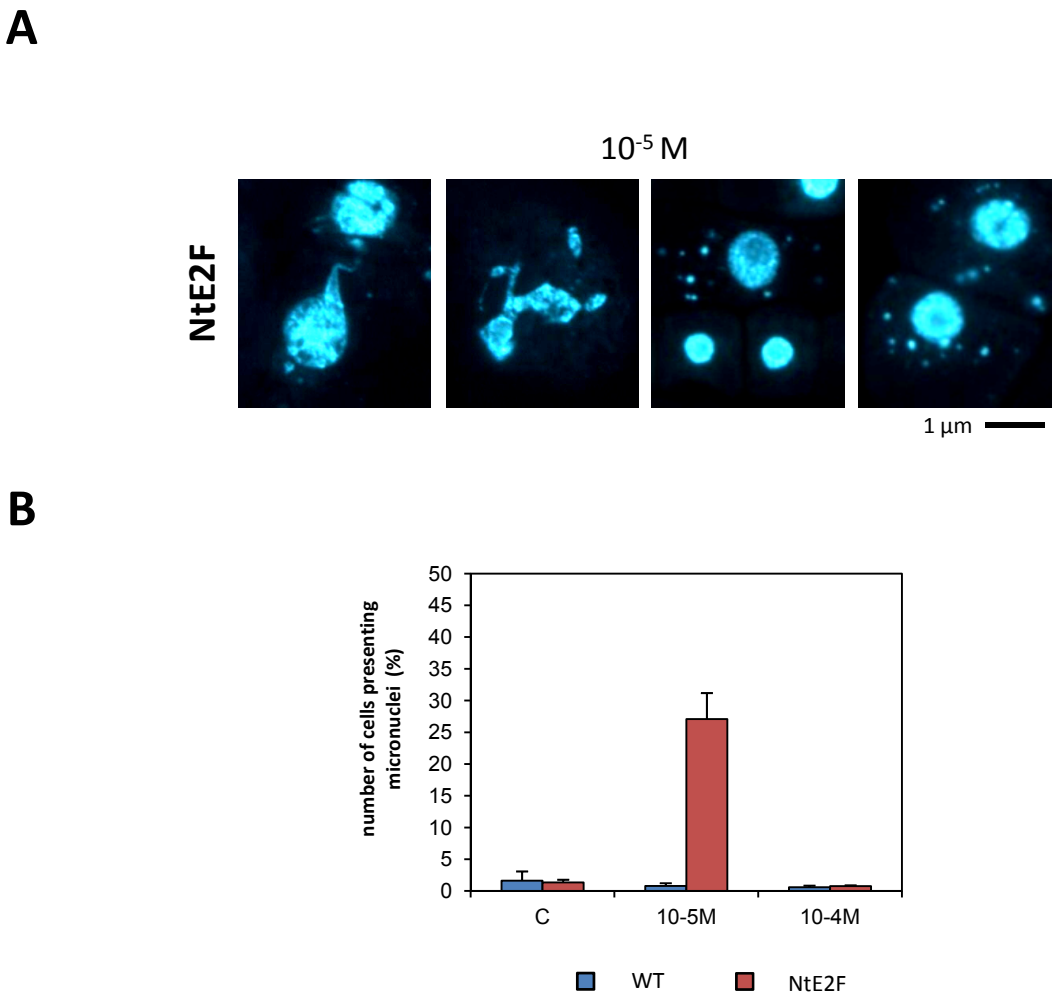


Figure 3.16: Genomic instability after 3 days of BLM treatment (10^{-5} M or 10^{-4} M) in WT and NtE2F cells. **A:** Anaphase bridges, nuclear alterations or micronuclei formation (arrows) in NtE2F cells; **B:** Quantification of cells presenting micronuclei; **C:** FISH using a telomere-specific probe (red) and DAPI staining (blue).

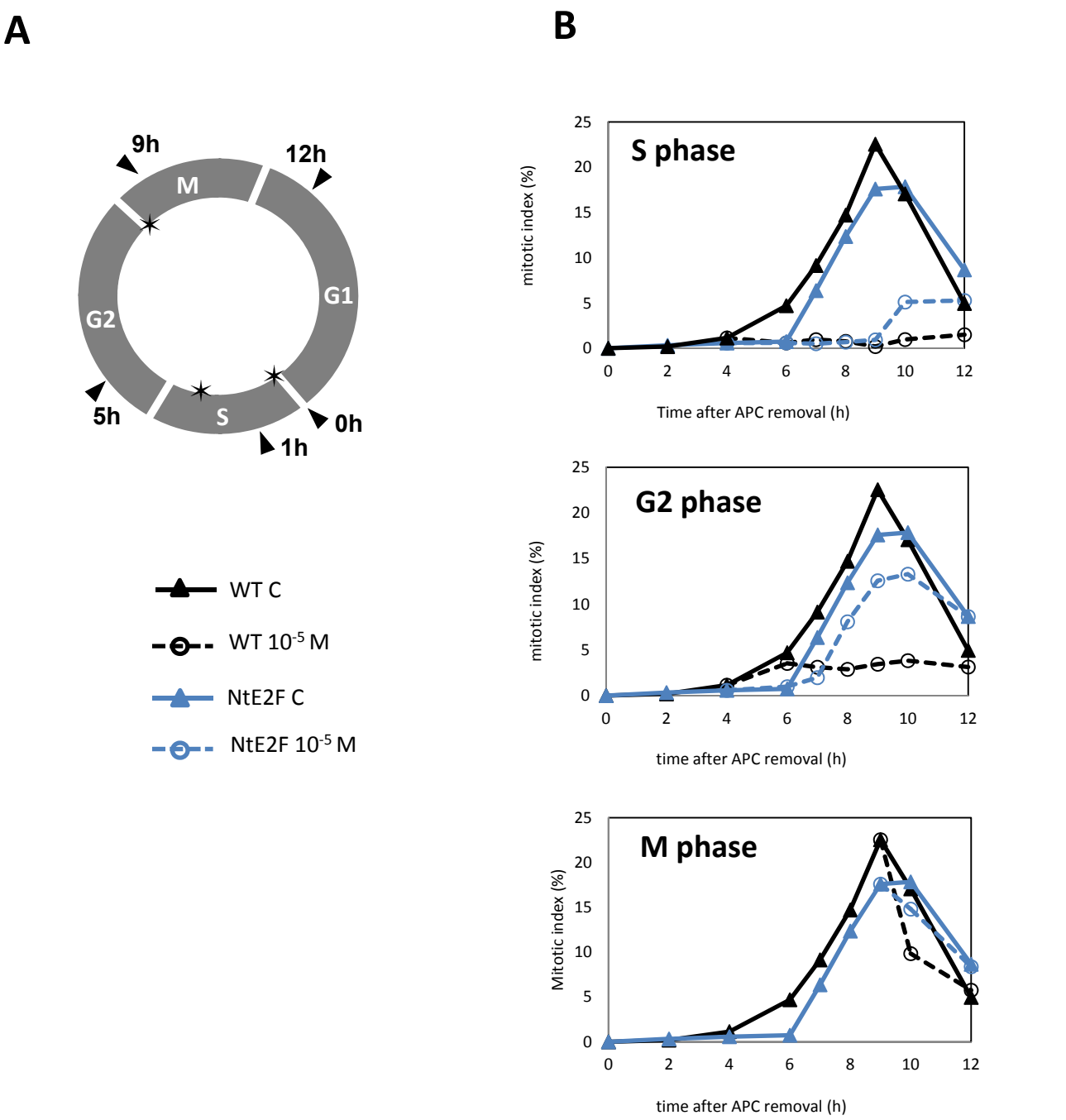


Figure 3.17: NtE2F overexpression bypass G2/M checkpoint. **A:** BLM was added at different time points of the cell cycle after APC removal (time 0h), during S phase (time 1h), G2 phase (time 5h)M phase (time 9h). Stars (*) indicate cell cycle checkpoint G1/S, intra S and G2/M. **B:** Mitotic index progression after 10^{-5} BLM treatment added at S, G2 and M phase after the release of APC.

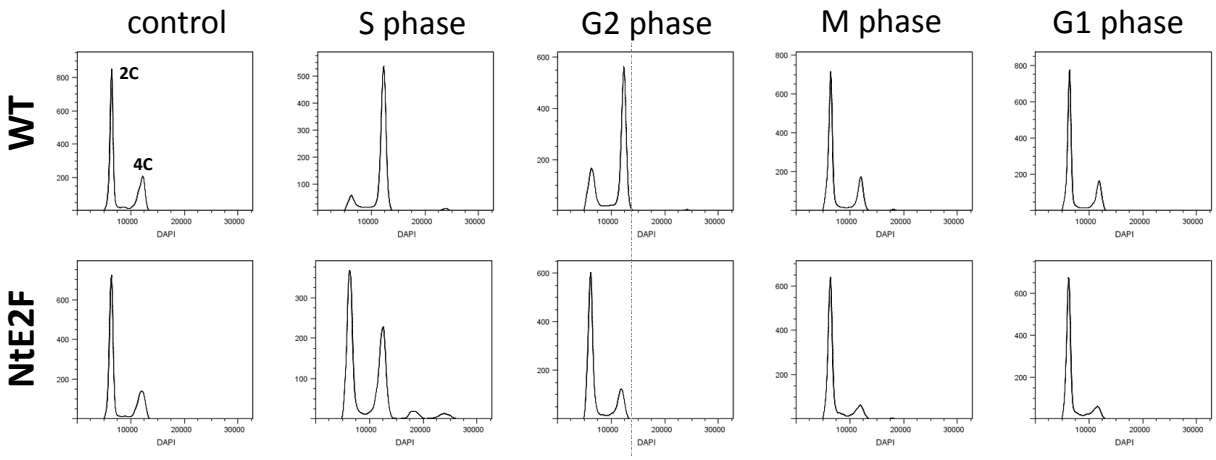


Figure 3.18: Flow cytometry analysis 24 hours after APC release. Cells were treated with 10^{-5} M BLM at different time points of the cell cycle (S, G2, M, G1 phase).

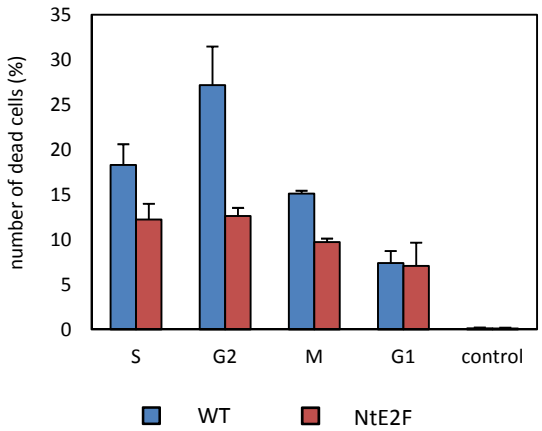


Figure 3.19: Cell death quantification 3 days after APC release. Cells were treated with 10^{-5} M BLM at different time points of the cell cycle (S, G2, M, G1 phase).

G1 phase did not show any difference in their DNA content. As shown in Figure 18, the majority of the cells were in G1 phase with 2C DNA content. These results indicate that after a 10^{-5} M BLM treatment, G2/M checkpoint is activated in WT cells whereas NtE2F overexpressing cells go through mitosis and are arrested in G1.

3. 2.4.3. PCD-induction is cell cycle regulated in response to low BLM concentration

Previous studies demonstrated that according to cell cycle phase, PCD induction may be influenced (Kuthanová et al. 2008). Therefore, mortality was evaluated in synchronized cells treated with BLM at different phases of the cell cycle and the possible role of NtE2F was evaluated in this process. Mortality was determined after 3 days of BLM treatment as describes previously. A 10^{-5} M BLM treatment of WT cells at G2 phase transition induced the highest mortality rate (up to 25 %) compared to the other cell cycle phases (Figure 3.19). At the same time points, overexpression of *NtE2F* decreased the mortality to about 12 %. BLM applied during S and M phase caused mortality of 16-18 % and 10-13 % in WT and NtE2F cells, respectively. This difference of PCD induction between WT and NtE2F cells was not observed when BLM was added in G1 phase, when the mortality of both lines was around 7 %. We conclude that WT cells treated with 10^{-5} M BLM are the most susceptible for PCD induction during G2 phase. NtE2F overexpression inhibits G2/M checkpoint leading to decreased cell death and increased genomic instability as observed previously.

To check if *NtE2F* overexpression can overcome the G2/M checkpoint using higher concentrations of BLM, we treated synchronized BY-2 cells with 10^{-4} M BLM at G2 phase, where a clear discrepancy was observed between WT and NtE2F cells using a 10^{-5} M BLM treatment. Eleven hours after APC release, we did not observe any significant difference between WT and NtE2F cells compared to our previous results using 10^{-5} M BLM (Figure 3.20A). Flow cytometry analyses did not show any difference in DNA content as well (Figure 3.20B). After 24 hours after APC release both lines had the majority of the cells in G2. However, cell death induction was decreased in NtE2F cell line compared to WT as observed with 10^{-5} M BLM (Figure 3.20C). These data indicate that the NtE2F overexpression do not bypass G2/M checkpoint at high level of BLM, however cell death induction is reduced compared to WT cells.

3.2.5. Summary of the results obtained with BY-2

Among the main results obtained with BY-2 cell line, we can highlight several features of BLM-induced PCD. First, BLM induces autophagic cell death which is mediated by vacuolar rupture and characterized by the absence of DNA fragmentation but with impaired expression of PCD-related genes. Low BLM concentration induces PCD in lesser extent and this cell death seems to be ATM-independent suggesting from our observation that ATM inhibitor caffeine does not modify the PCD response in these conditions. PCD induced with higher doses of BLM (10^{-4} M) is likely ATM-dependent due to the inhibitory effect of caffeine on BLM-induced cell death.

Next, the effect of NtE2F ectopic expression also differs according to the severity of the damage. At low BLM concentrations, NtE2F overexpression bypasses G2/M checkpoint when the cells are the most sensitive to PCD induction. Simultaneously, this leads to increased genomic instability and low cell death induction when the cells are treated during G2 phase. However the difference of cell death in non synchronized culture was not observed. At high BLM concentration, NtE2F overexpression somehow prevents BLM-induced cell death induction (which is cell cycle phase independent) probably via inhibition of vacuolar rupture. Thus in general, ectopic expression of NtE2F has rather inhibitory effect on BLM-mediated cell death.

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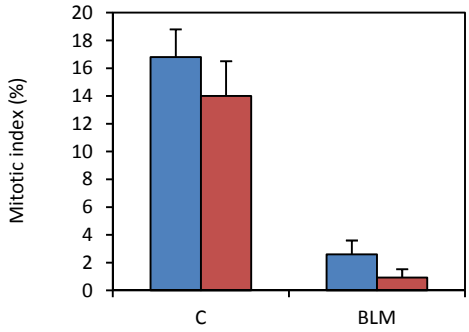

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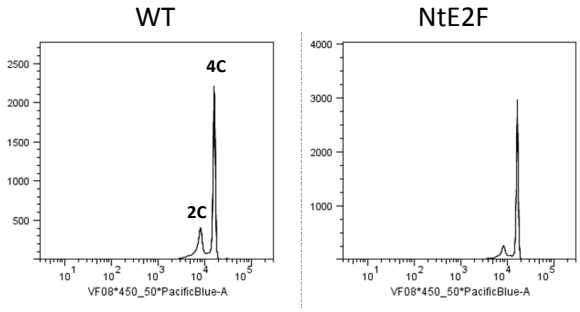

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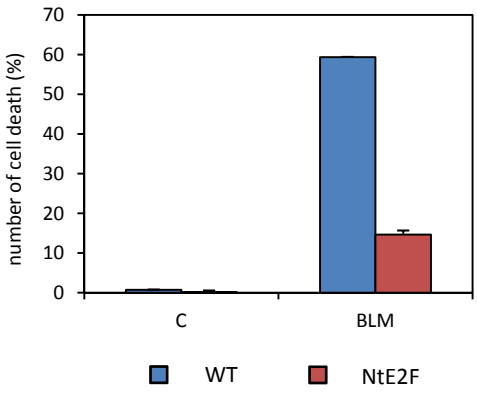


Figure 3.20: Effect of 10^{-4} M BLM treatment during G2 phase on synchronized WT and NtE2F cells. **A:** Mitotic index 11 hours after APC release. C – untreated control. BLM – cells treated during G2 phase. **B:** Flow cytometry analysis 24 hours after APC release. **C:** Quantification of cell death 3 days after APC release.

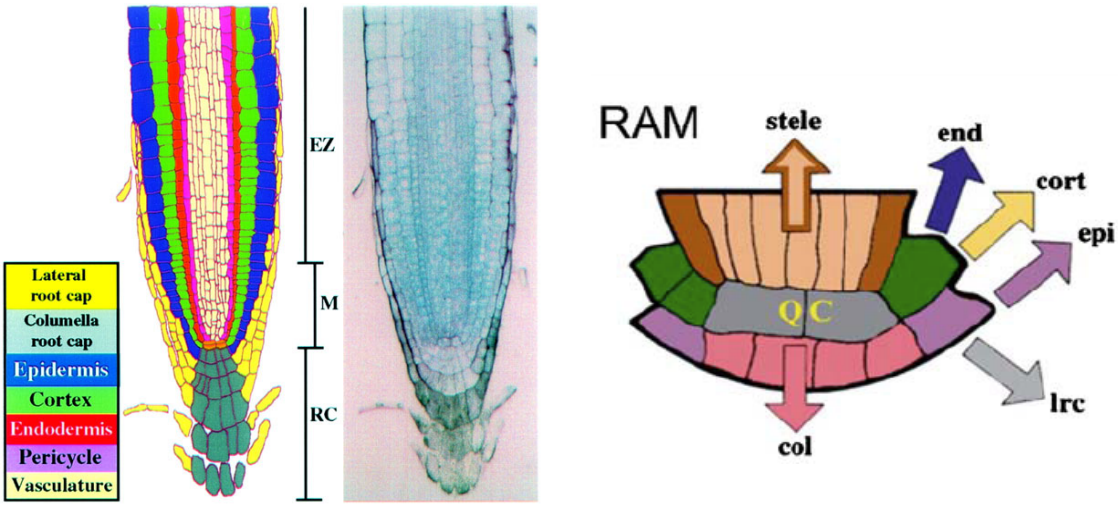


Figure 3.21: Schematic illustration of root apical tissues delineating root cap (RC), meristem (M) end elongation zone (EZ) accoding to Marchant et al. (1999) and quiescent centre enclosed by stem cells giving rise to stele, endodermis (end), cortex (cort), epidermis (epi), lateral root cap (lrc), and columella (col). RAM – root apical meristem.

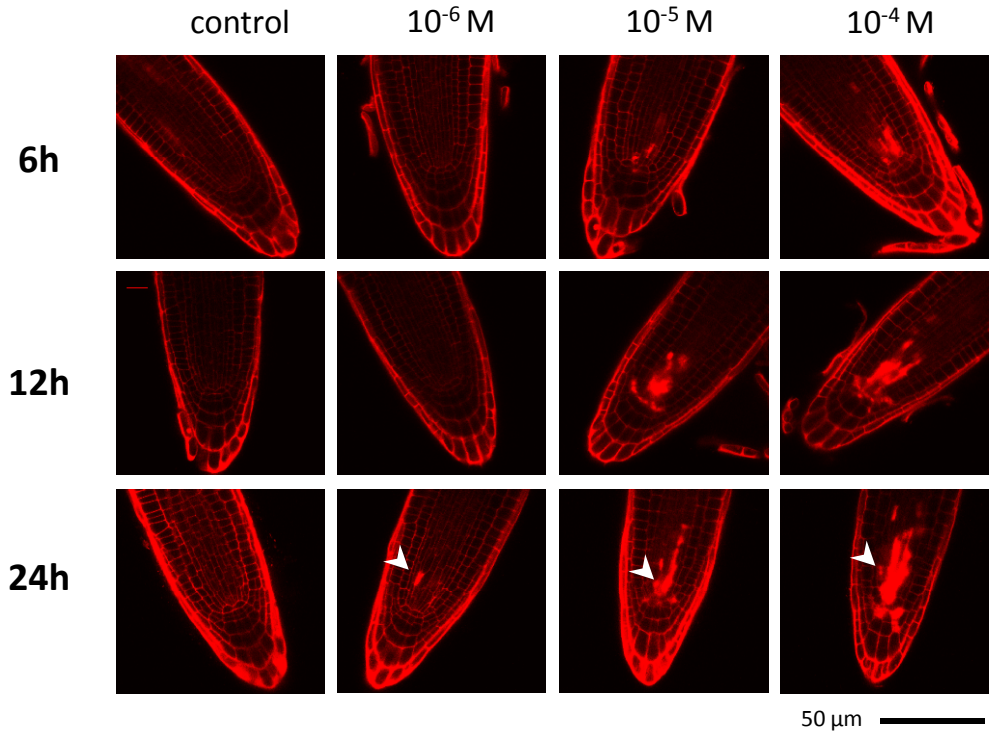


Figure 3.22: Kinetics of cell death induction in Arabidopsis root stem cells detected by propidium iodide (PI) after 6, 12 and 24 hours of BLM treatment (10^{-6} M, 10^{-5} M, 10^{-4} M). According to Fulcher and Sablowski (2009). Arrows indicate cell death detected with PI.

3.3. Chapter 3: Characterization of genotoxic-induced PCD in Arabidopsis

Genotoxic-induced PCD is still poorly understood in plants especially in the context of plant development. Arabidopsis root system is a powerful tool to investigate physiological processes linked to development due to its well defined structure (Figure 3.21). Genotoxic-induced cell death in Arabidopsis roots was only poorly described at the moment of the beginning of my work on my thesis. During last years, several reports have shown cell death induction upon DNA damage such as γ irradiation (Ricaud et al. 2007; Hefner et al. 2006), APC, HU (Culligan et al. 2004) or recently zeocin and γ irradiation (Fulcher et Sablowski 2009; Fukurawa et al. 2010) (see introduction). Nevertheless we used Arabidopsis plants to verify our results obtained with BY-2 cells in the more complex tissue system of Arabidopsis roots.

At the time, when I have started to work on this project, our team was focused on DNA repair signaling in various genetic backgrounds in Arabidopsis using T-DNA insertion mutants or plants overexpressing genes implicated in DNA repair or cell cycle regulation. In addition, genotoxic effects were mainly tested at the level of root growth. Therefore we have mainly focused the studies of genotoxic-induced PCD analyzing the root apical meristem.

3.3.1. Short term-BLM treatment

We have investigated the response using different concentrations of BLM after 6, 12 and 24 hours of genotoxic treatment. Five days-old-plantlets were treated with (10^{-6} M, 10^{-5} M, and 10^{-4} M) and we analyzed cell death induction using iodide propidium (PI) staining of the root tips similarly as described in Fulcher et Sablowski (2009) (Figure 3.22). Cell death induction occurs mainly in the meristem and was time- and dose-dependent. At 10^{-6} M BLM, cell death occurred only in a few cells and was detected only after 24 hours of the treatment. Using 10^{-5} M BLM, cell death is already detected at 6h of BLM treatment and was increasing until 24 hours. Using higher doses of BLM, such as 10^{-4} M, we induced massive cell death in the root meristem, especially at the level of the stele as well as in some cells of the stem cell niche (see arrows, Figure 3.22). These results show that BLM induces cell death in Arabidopsis meristematic cells in a time- and dose-dependent manner.

3.3.1.1. Alterations in meristem organization

To further characterize the changes coupled with cell death in stem cells in response to BLM, we have used the line p_{WOX5}:GFP-NLS expressing GFP, with a nuclear localization signal (NLS), under the control of the promoter WOX5 (Wuschel-related homeobox 5) which is specifically expressed in the quiescent center (QC) (Figure 3.23) (Sarkar et al. 2007). At low concentration of BLM (10^{-6}M; 24h), when only little cell death was observed, the pattern of WOX5 expression seems to be unchanged compared to untreated cells (control). In addition we showed that when massive cell death occurs after a 10^{-4} M BLM treatment, of p_{WOX5}:GFP-NLS plantlets, the GFP expression pattern that was initially restricted to QC switched to pericycle and endodermis (Figure 3.23).

As auxin signaling is also highly regulated in stem cells (Marchant et al. 1999), we have investigated the auxin response using the marker line p_{DR5}:GFP, where an increased GFP signal correlates with a higher auxin level in the cell. The patterning of root apical meristem involves two auxin maxima in the QC and mature columella. Roots exposed to increasing doses of BLM had decreased level of auxin at QC but maintained high auxin level in mature columella. In addition, we observed also increased GFP expression in the central zone of the stele at 10^{-6} M, 10^{-5} M and 10^{-4} M BLM in a dose-dependent manner (see arrows, Figure 3.23). We conclude that BLM induces changes in the patterning of WOX5 and auxin-signaling in the root apical meristem as a result of massive cell death in stem cells.

3.3.1.2. Expression of PCD-related genes

As in BY-2 cells, BLM-induced PCD was linked to up-regulation of several specific PCD-related genes, we have investigated such transcriptional response in Arabidopsis. We have first chosen genes that were previously described to be induced in Arabidopsis mutants, such as *rnr* where increased cell death was observed (Wang et al., 2006). Such genes play also a role during oxidative stress response - *Glutathion-S-transferase* (*GST11*) and *Peroxidase cb* (*PRXcb*) or during interaction of a plant with a pathogen - *Enhanced disease susceptibility 1* (*EDS1*) and *Pathogenesis protein 1* (*PR1).* Plantlets were treated with 10^{-5} M BLM during 2 and 6 hours, and RNA was extracted from whole plant. Using specific primers, these genes were significantly up-regulated at 6 hours as observed by semi-quantitative RT-PCR analysis (Figure 3.24A). The level of induction of these different genes seemed also dependent on the concentration of BLM (10^{-6} M, 10^{-5} M, 10^{-4} M) that we used during a 3 hours BLM treatment (Figure 3.24B). These results indicate that BLM response in Arabidopsis involves a time- and dose-dependent transcriptional activation of PCD-related genes. That suggest also, that ROS and also stress defense signaling may be activated in response to BLM.

3.3.2. Long term BLM-treatment

BLM induced increased PCD in BY-2 cells after long term BLM treatment (up to 60% after 10^{-4} M BLM). Therefore, we performed similar long term continuous exposure to genotoxics on Arabidopsis plantlets, notably to know if some other cell files in the root do not undergo cell death later on. After 3 days of BLM treatment at concentrations of 10^{-5} M and 10^{-4} M, we observed complete arrest of root growth and cell division as analyzed by root growth test and MI evaluation in the root meristem (Figure 3.25A and B). Dead cells were mainly restricted to the root meristem, in stem cells as well in stele and endodermis (see arrows, Figure 3.26A). The most apparent were morphological changes of the root tip including the increase in cell size mainly of the epidermal and cortex cells and the differentiation of epidermal cells to root hairs. Long treatment up to 14 days on solid or in liquid MS medium supplemented with 10^{-6} M, 10^{-5} M and 10^{-4} M BLM induced leave chlorosis suggesting that at these conditions Arabidopsis plants exhibit marks of senescence (Figure 3.26B).

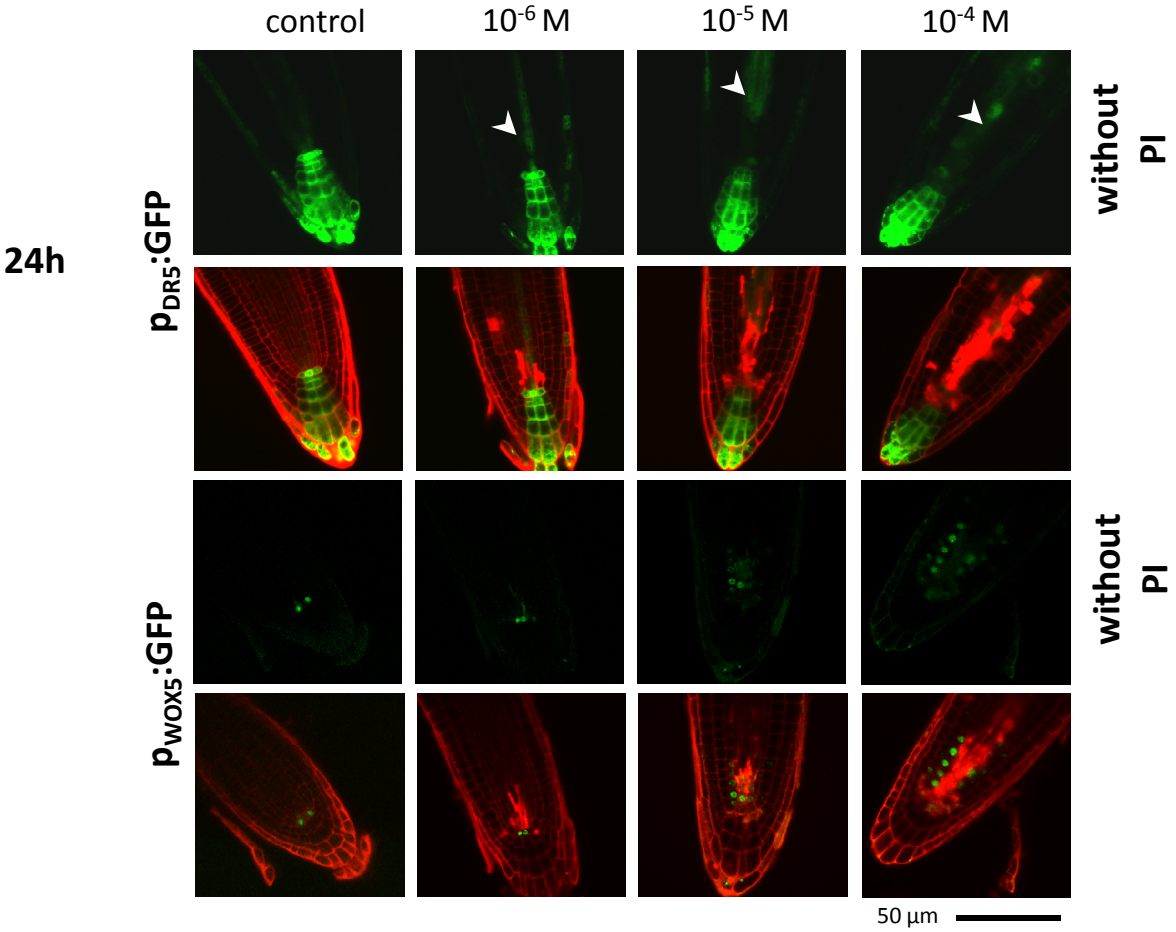


Figure 3.23: Changes in GFP expression pattern in WOX5:GFP and DR5:GFP marker lines after 24 hours of BLM treatment (10^{-6} M, 10^{-5} M, 10^{-4} M). Arrows show increase of GFP signal in the stele of DR5:GFP line. In red PI staining.

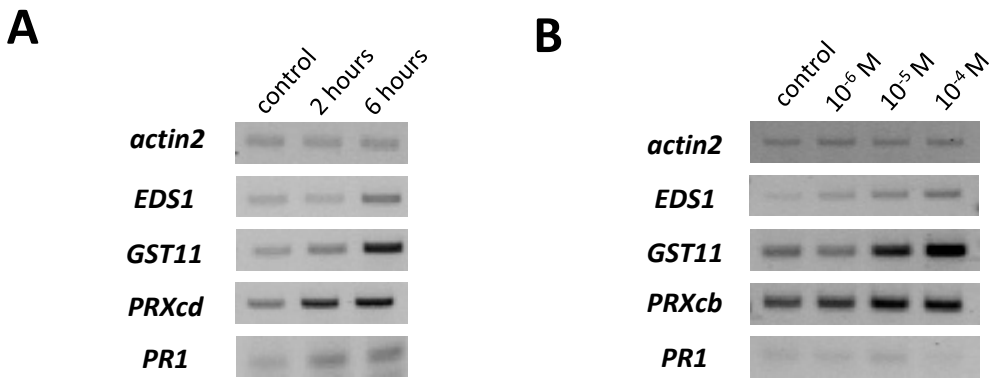


Figure 3.24: Semi-quantitative RT PCR analysis of the expression of PCD marker genes (*EDS1*, *GST11*, *PRXcb*, *PR1*). *Actin2* gene was used as a standard. **A:** Time-dependent expression after 2 and 6 hours of 10^{-5} M BLM treatment; **B:** Dose-dependent expression after 3 hours of 10^{-6}M, 10^{-5} M and 10^{-4} M BLM treatment.

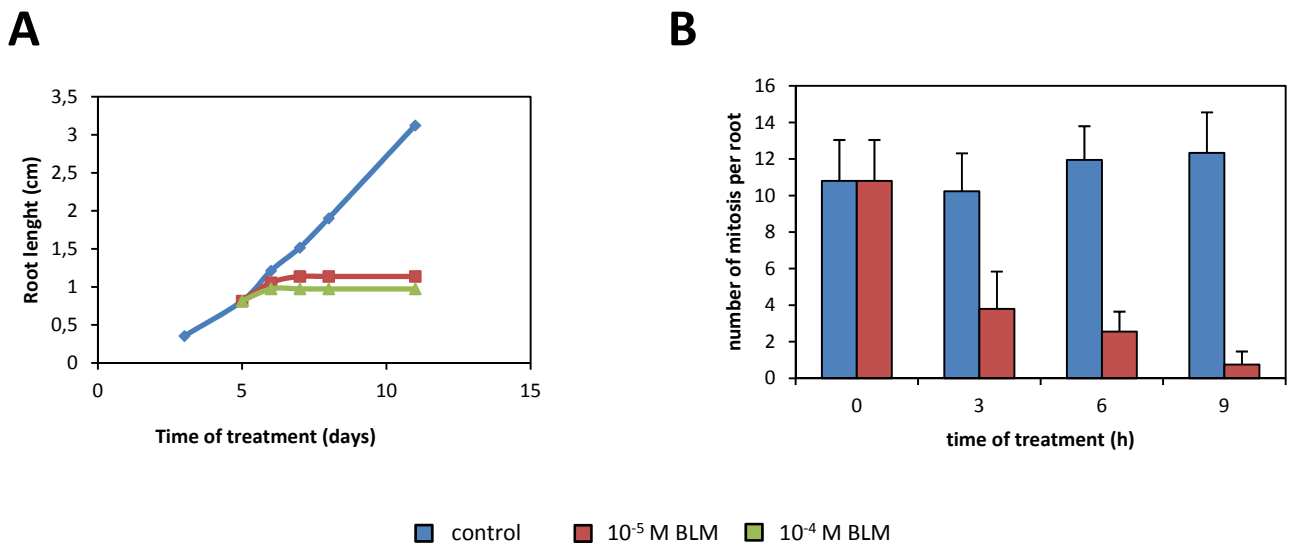


Figure 3.25: Effect of BLM on root growth and cell division in Arabidopsis. **A:** Growth test of Arabidopsis roots after BLM treatment (10^{-5} M, 10^{-4} M); **B:** Number of mitosis in the root tip upon 10^{-5} M BLM treatment.

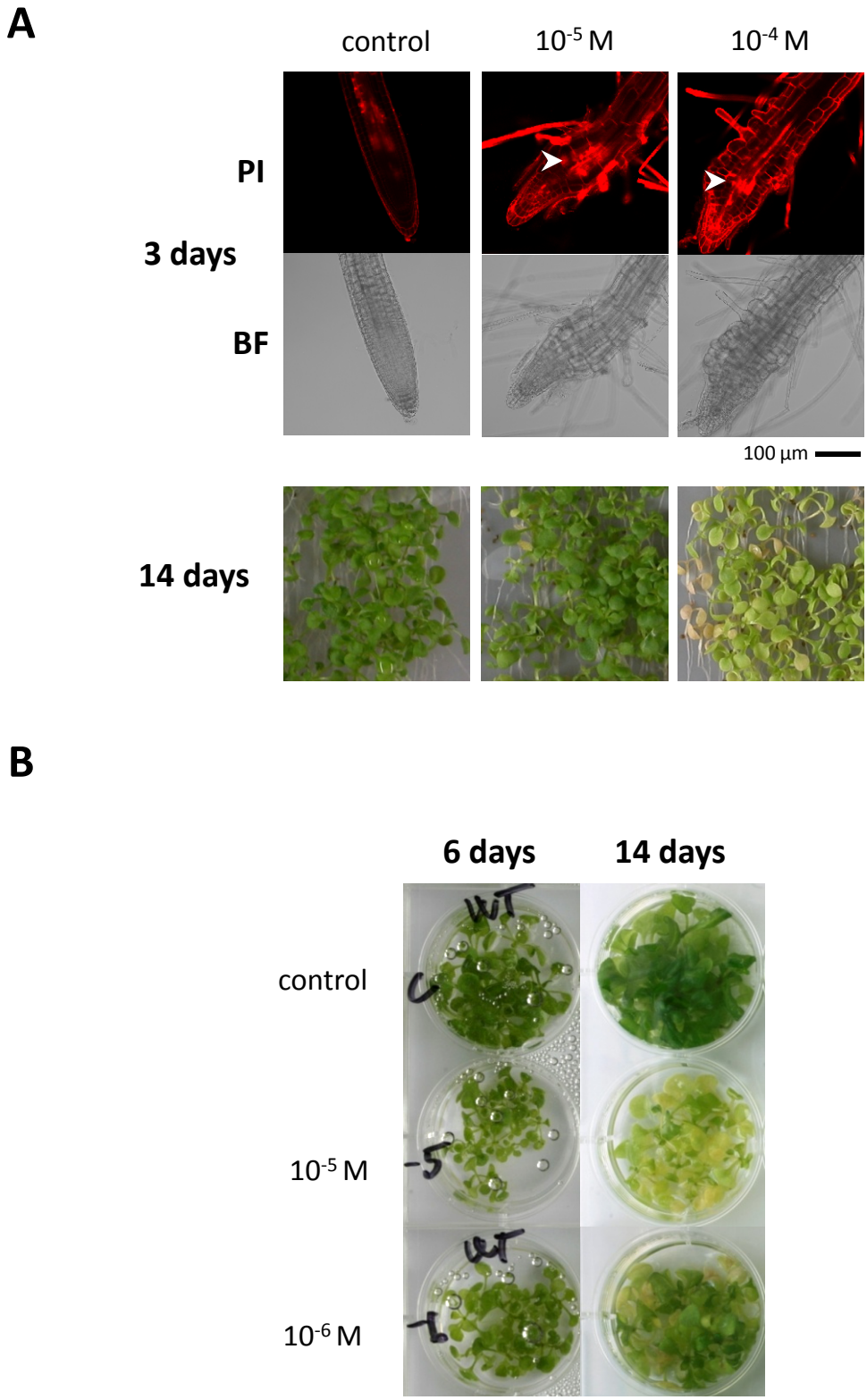


Figure 3.26: Long-term BLM treatment, 5 DAG plantlets were transferred on media with genotoxics. **A:** Changes of root morphology and chlorosis induction after BLM treatment (10^{-5} M and 10^{-4} M) on solid MS medium after 3 and 14 days. PI – propidium iodide staining, BF – bright field; arrows indicate cell death. **B:** Arabidopsis plantlets after BLM treatment (10^{-6} M and 10^{-5} M) in liquid MS medium during 6 and 14 days.

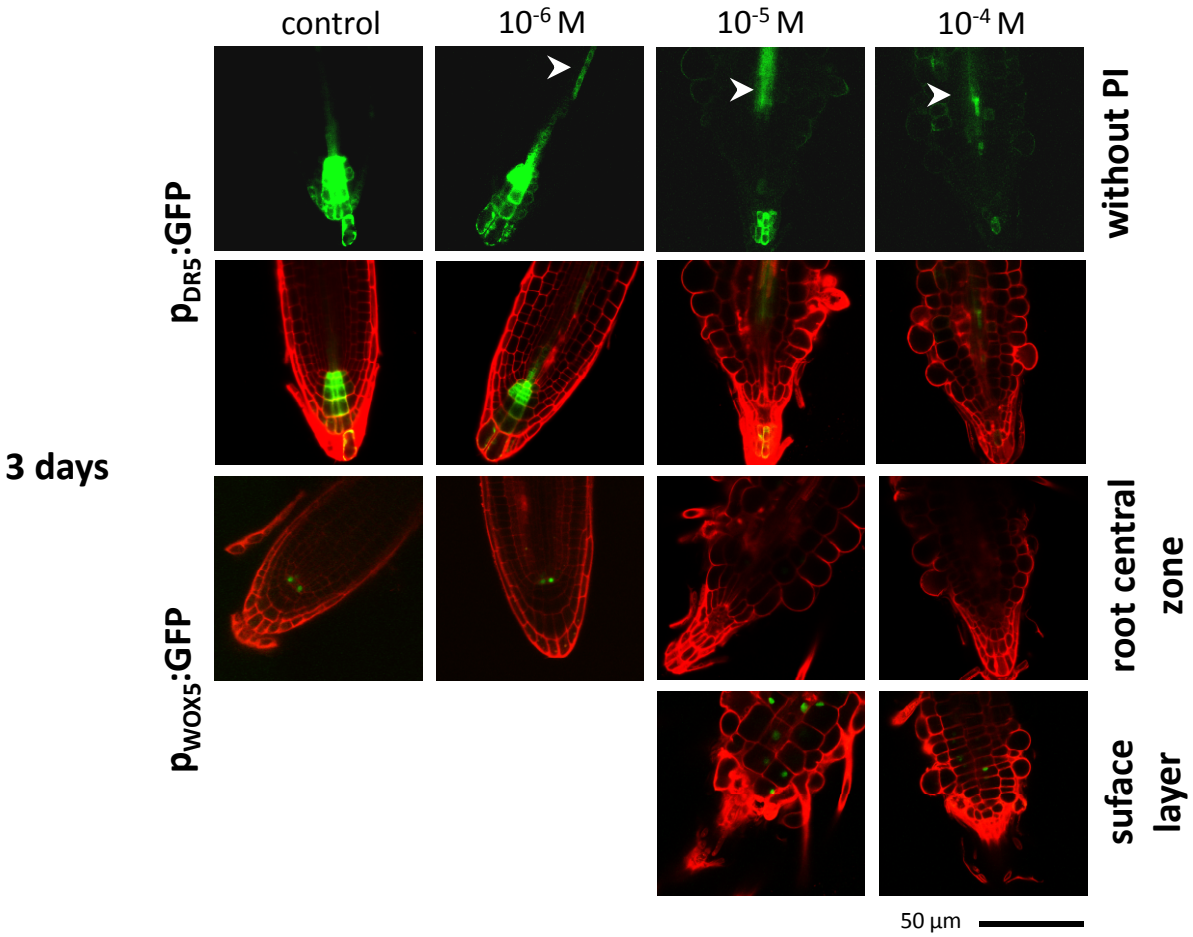


Figure 3.27: Changes of GFP expression pattern in WOX5:GFP and DR5:GFP marker lines after 3 days of BLM treatment (10^{-6} M, 10^{-5} M, 10^{-4} M).

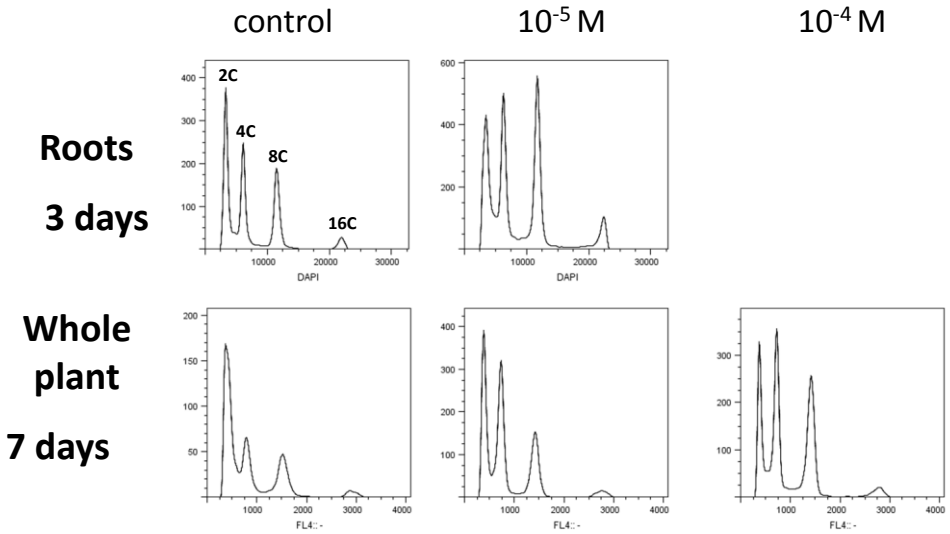


Figure 3.28: Flow cytometry analysis of DNA content in the cells of Arabidopsis roots or the whole plantlets after 3 or 7 days of BLM treatment (10^{-5} M, 10^{-6} M).

3.3.2.1. Alteration in meristem organization

We also investigated the pattern of WOX5 expression and auxin (Figure 3.27) using specific GFP lines as previously described. Whereas WOX5 expression pattern was unchanged compared to control cells after a 10^{-6} M-BLM treatment, strong WOX5 expression pattern changes were observed using 10^{-5} M and 10^{-4} M BLM. WOX5 expression disappeared from the QC of the root meristem but the GFP signal became apparent in the cells of cortex or epidermis. At the level of DR5 expression, we observed a decrease of the signal in QC at low concentration of BLM (10^{-6} M; 3 days) as for a 24 hours BLM treatment. Strong morphological alterations of the root tip architecture after 10^{-5} M and 10^{-4} M BLM were accompanied by reduction of the DR5 signal in the columella that switched to maxima in the stele as already observed after 24 hours BLM treatment.

Taken all together, our results indicate that the primary BLM-induced cell death in stem cells leads subsequently to strong disturbance the normal organization of the root apical meristem leading to changes in the root morphology.

3.3.2.2. BLM-induced endoreduplication in the root

Taking into account our findings in Arabidopsis BLM response, where root meristematic cells continued to grow by swelling and epidermal cells continued to differentiate to the root hairs indicate that BLM treatment induces arrest of cell division but likely does not inhibit cell growth and differentiation in Arabidopsis root. Similar results were described after γ irradiation (Culligan et al. 2004; Hefner et al. 2006; Ricaud et al. 2007). The cell swelling and differentiation is often linked to activation of endocycle leading to increased nuclear ploidy (Kondorosi et al. 2000), thus we carried out an analysis of ploidy levels after BLM treatment in roots.

We found that 3 days 10^{-5} M BLM treatment induced a switch to endocycle in the root cells with a significant increase of the cell fraction with 8C and 16C DNA content (Figure 3.28). Flow cytometry measurements also showed that BLM treatment increased the number of the cells with 4C. Similar behavior was found at the whole plant level when treated with 10^{-5} M and 10^{-4} M BLM for 7 days, showing increased population of C4 and C8 cells but only a slight increase of 16C cells. Increased populations of C4 and C8 + C16 nuclei suggest that BLM in Arabidopsis plants activates the G2 checkpoint and subsequent endoreduplication through inhibition of the entry into mitosis.

3.3.3. Effect of other genotoxics on Arabidopsis roots

BLM or zeocin was shown to induce endoreduplication in Arabidopsis roots (our results, Ramirez-Parra et al. 2007). To determine if other genotoxics then BLM induces the switch to endocycle, we used camptothecin or hydroxyurea. Simultaneously we focused on the morphological changes such as cell swelling or root hairs formation. To study the effect of other genotoxic treatments

on Arabidopsis, we analyzed the root growth as well as morphological alterations and possible changes in the level of endoreduplication.

An anticancer alkaloid camptothecin (CPT) targets topoisomerase I and transforms this enzyme into a DNA-damaging agent resulting in DNA double strand breaks, through conversion of SSBs in DSBs during DNA replication (Wang et al. 1996). In general CPT treatment had a similar effect on Arabidopsis roots as BLM or zeocin. After 7 days of CPT exposure at concentrations higher than 10 μM, cell swelling and differentiation of epidermal cells to root hairs were observed (Figure 3.29A). Evaluation of DNA content with flow cytometry showed increased frequency of 4C nuclei, suggesting the activation of the G2 DNA damage checkpoint as during BLM response. Similarly to BLM treatment, fractions of 8C and 16C nuclei were increased suggesting that BLM and CPT have a similar effect on the induction of endoreduplication.

Hydroxyurea is a specific inhibitor of Ribonucleotide reductase (RNR) catalyzing the final step of deoxyribonucleotide synthesis. Similarly to CPT and BLM, we analyze the effect of HU on plant root growth (Figure 3.29B). Upon 1 mM HU exposure, root growth of the plants was not changed compared to the control whereas at 2, 4 and 8 mM HU root growth was completely blocked. However, even root growth is blocked, no altered morphology of the roots were observed compared to BLM or CPT treatment. In parallel, the flow cytometry analysis showed increased level of endoreduplication in HU-treated roots but to a lesser extent than BLM or CPT. The majority of the cells after HU treatment had 2C DNA content similarly to untreated control. Slight increase was detected in 4C and 8C fraction whereas 16C remained unchanged in all HU-treated variants.

Simultaneously, to see whether the changes in root morphology are related to cell death in the meristem, we used PI staining to detect cell death in the meristem as previously. HU (1 mM and 4 mM) and APC (12 μm/ml) did not show any cell death induction suggesting that changes in root morphology might be not related to cell death in the meristem (Figure 3.29C).

Taken these data together, we conclude that in our experimental conditions DNA double strand break damage induces root arrest, cell enlargement and differentiation whereas DNA replicative stress inhibits root growth without changes in root morphology but with a slight induction of endoreduplication levels. Thus there are distinct reactions of Arabidopsis roots leading to different morphological changes after DSB induced DNA damage and replicative stress.

3.3.4. Effect of BLM on Arabidopsis cell suspension

As mentioned previously, the role of endoreduplication during the maintenance of genomic integrity is under debate. According to one hypothesis, endoreduplication could buffer the mutation accumulated during the development and thereby protects the genetic information and prevents cell death (Inzé et de Veylder 2006). We have shown that BLM induces cell death in BY-2 cell suspension

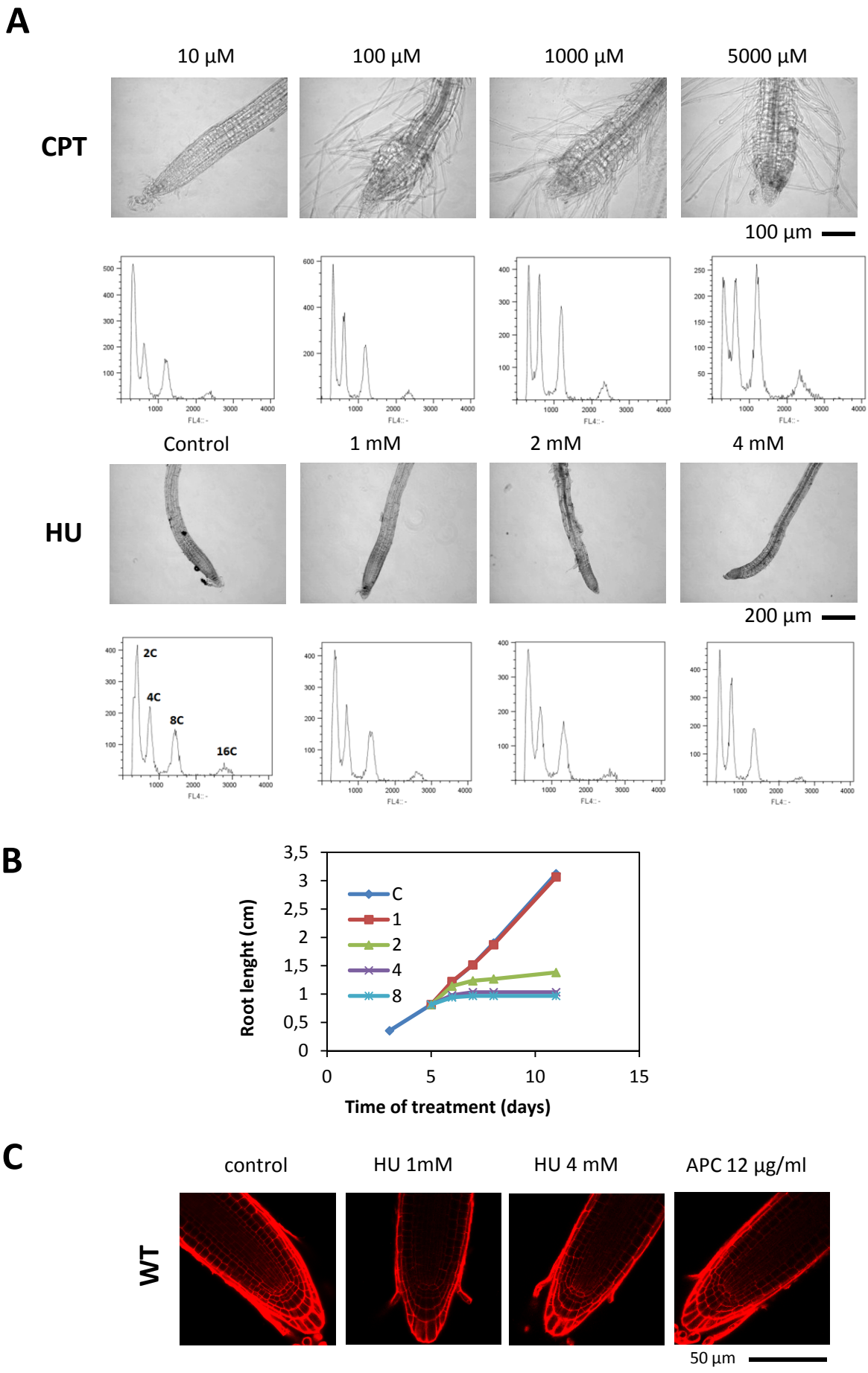


Figure 3.29: Effect of HU, APC and CPT on Arabidopsis. **A:** Changes in root morphology and DNA content and Flow cytometry analysis after 3 days of HU and CPT treatment; **B:** Growth test after HU treatment (1, 2, 4 and 8 mM). **C:** PI detection of cell death after 1 day of HU (1, 4 mM) and APC (12 µg/ml) treatment.

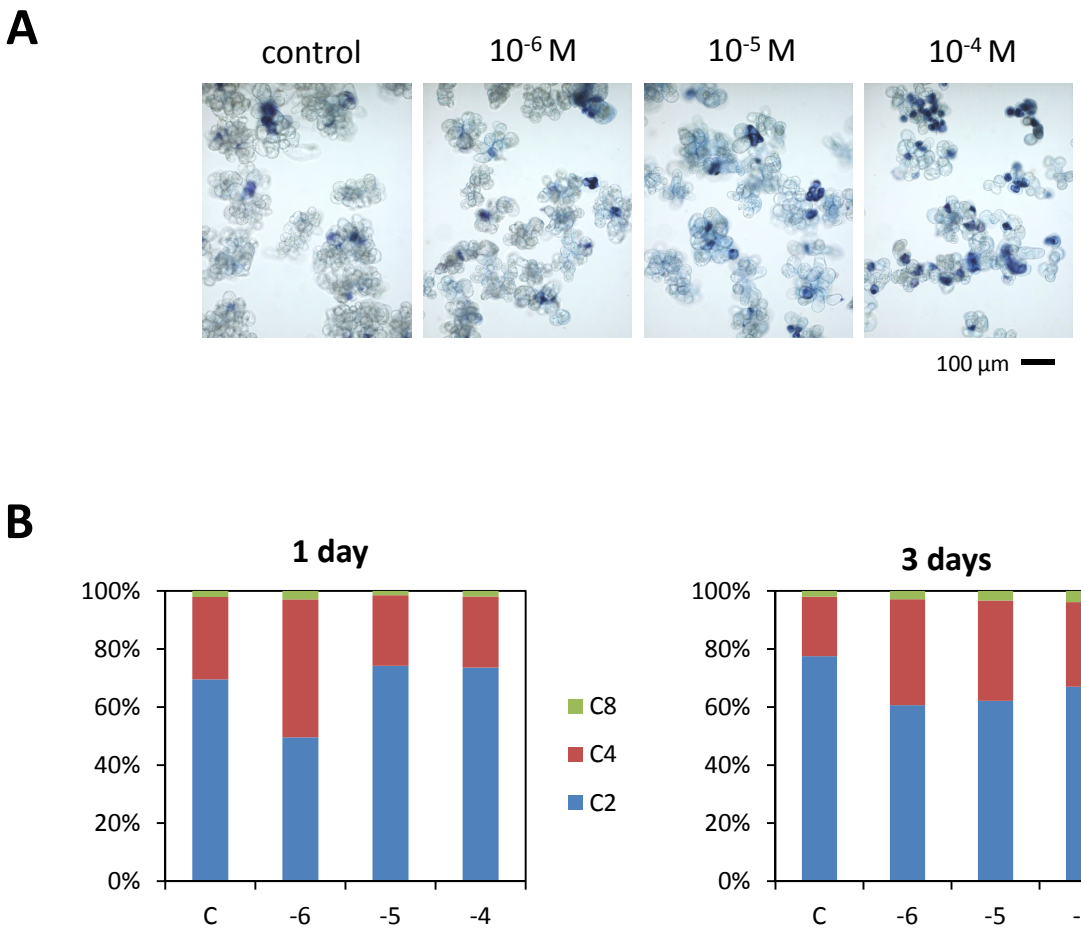


Figure 3.30: Effect of BLM (10^{-6} M, 10^{-5} M, 10^{-4} M) treatment on Arabidopsis cell culture PSB-V. **A:** Cell death was detected using Evans blue staining; **B:** Flow cytometry analysis after 1 and 3 days of genotoxic treatment.

but without increased of ploidy levels. In Arabidopsis, BLM-induced cell death is limited only to a small population of root meristematic cells whereas other cells undergo differentiation and increased in endoreduplication.

In order to avoid plasticity linked to plant development, we used Arabidopsis cell suspension culture to analyze the link between cell death and endoreduplication induction. The PSB-V cell suspension line that we used does not exhibit such cell homogeneity and harbors cell clusters enabled efficient counting and quantification of dead cells as performed with BY-2 cells. However, we tested if Arabidopsis PSB-V cells undergo cell death or endoreduplication in response to BLM. Similarly to BY-2 cells, PSB-V cells were treated during mid-log phase growth with different doses of BLM (10^{-6} M, 10^{-5} M, 10^{-4} M) and cell death was evaluated after 3 days of cultivation using Evans blue vital dye (Figure 3.30A). In the control a few dead cells were present but this number progressively increased upon BLM treatment.

Flow cytometry analysis showed that after 1 day of genotoxic treatment the number of 4C nuclei only increased significantly in response to 10^{-6} M BLM compared to WT or other BLM treatments probably due to the (Figure 3.30B). When longer BLM treatments (3 days) were applied, we observed increased level of 4C nuclei for all tested BLM concentrations. The number of endoreduplicated nuclei 8C remained low in all variants and was comparable to the control.

These data demonstrate that Arabidopsis cells do not enter into the endocycle but arrest their cell cycle in G2 phase in response to long BLM treatments. Similarly to BY-2 cells, PSB-V cells showed induced cell death in a dose-dependent manner in response to BLM. Subsequently, we can suggest that the control of cell death induction in response to BLM is not species-specific but rather depends on cellular context.

3.3.5. Implication of caspase-like proteases in BLM-induced PCD

For further characterization of BLM-induced cell death we analyzed the implication of caspase-like proteins similarly to our experiments with BY-2. Caspases are the main executors of animal apoptosis, but their homologues were not identified in plants (Uren et al. 2000). However, metacaspases belong to caspase-like protein family which regroups proteins with similar characteristics e.g. homology, substrate specificity or presence of conserved domains. Metacaspases were identified as closest orthologs of animal caspases and their implication was suggested during several types of plant PCD (Boshkov et al. 2000, He et al. 2008). Besides metacaspases, the second group of caspase-like genes encoding Vacuolar processing enzymes (VPEs) which have the ability to recognize and cleave animal caspase 1 specific sequence YVAD (Hatsugai et al. 2006).

3.3.5.1 Metacaspases

We first investigated the transcriptional response of genes encoding metacaspases (*MCA1 – MCA9)* in response to BLM. We performed semi-quantitative RT PCR using RNA extracted from whole plantlets (8 DAG) treated with BLM (10^{-6} M, 10^{-5} M, 10^{-4} M) during 3 or 12 hours (Figure 3.31). At 3 hours of treatment, only *MCA8* was strongly upregulated by 10^{-4} M BLM. The expression of other metacaspases remained unchanged. At 12 h of treatment with 10^{-4} M BLM, the expression of *MCA8* was not anymore detected that means its transitory transcriptional activation but *MCA2* showed strong up-regulation. These results indicate that in our experimental conditions, only two metacaspases genes were induced by high doses of genotoxics.

In order to investigate the role of *MCA2* and *MCA8* in BLM-induced cell death, we used T-DNA insertion mutants characterized in our laboratory. After genotyping with LP/RP pair of primers, using RT PCR we verified that the transcripts are not present in *mca2* and *mca8* mutants with primers specific to N-terminus and C-terminus of the gene, respectively (Figure 3.32A). No transcripts were detected in our mutants which are probably null-allele.

We therefore, investigated the induction of cell death in response of BLM in these mutants compared to WT (Figure 32B). No significant differences were observed, suggesting that *mca2* and *mca8* mutants are not directly implicated in cell death induction. However, we cannot exclude overlapping function of MCA8 and MCA2 in cell death induction upon BLM treatment.

3.3.5.2. Vacuolar processing enzymes

In Arabidopsis 4 genes encoding VPE α – δ were identified. Eight DAG plantlets were treated with different concentration of BLM (10^{-6} M, 10^{-5} M, 10^{-4} M) during 3 hours and expression analyses were performed by semi-quantitative RT PCR using specific primers. Only *VPE α* is transcriptionaly activated (Figure 3.33A). To demonstrate that *VPE α* is specifically expressed upon BLM treatment, we made a kinetic analysis (3, 6, 12, 24 and 36 hours) of VPE expression upon 10^{-4} M BLM treatment (figure 33B). This concentration of BLM induced higher expression level of *VPE α* after 3 hours of treatment. During the kinetic, *VPE α* remained highly induced with a peak of expression at 6 hours compared to *VPE β*, *VPE γ* and *VPE δ* that were down regulated at 24 and 36 hours upon BLM compared to untreated plants. Therefore, it seems that *VPE α* is specifically expressed when Arabidopsis plants are exposed to high doses of BLM compared to others *VPEs* in our experimental conditions.

As mentioned previously, VPEs are functional homologues of animal caspases and exhibit caspase-1 activity (Hatsugai et al. 2006). To test the role of VPEs during BLM-induced cell death in Arabidopsis roots we have chosen two approaches. First a reverse genetic approach using *vpe* mutants,

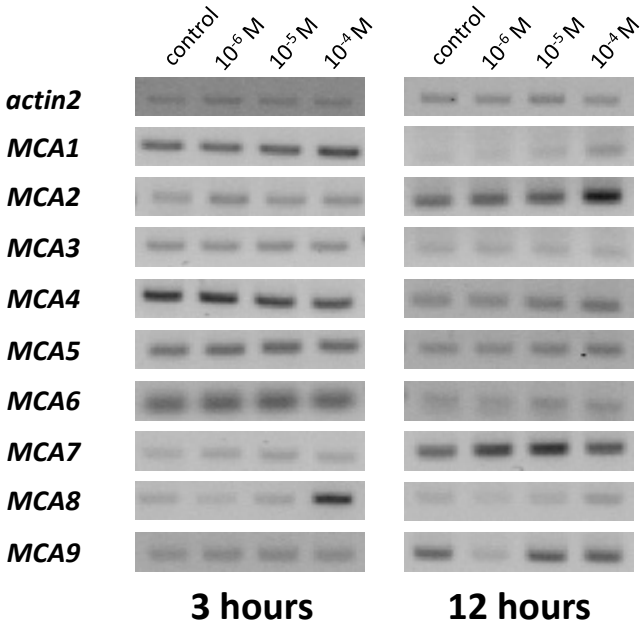


Figure 3.31: Semiquantitative RT PCR analysis of metacaspase genes (*MCA1* – *MCA9*) expression after BLM treatment (10^{-6}M, 10^{-5}M and 10^{-4}M) during 3 and 12 hours.

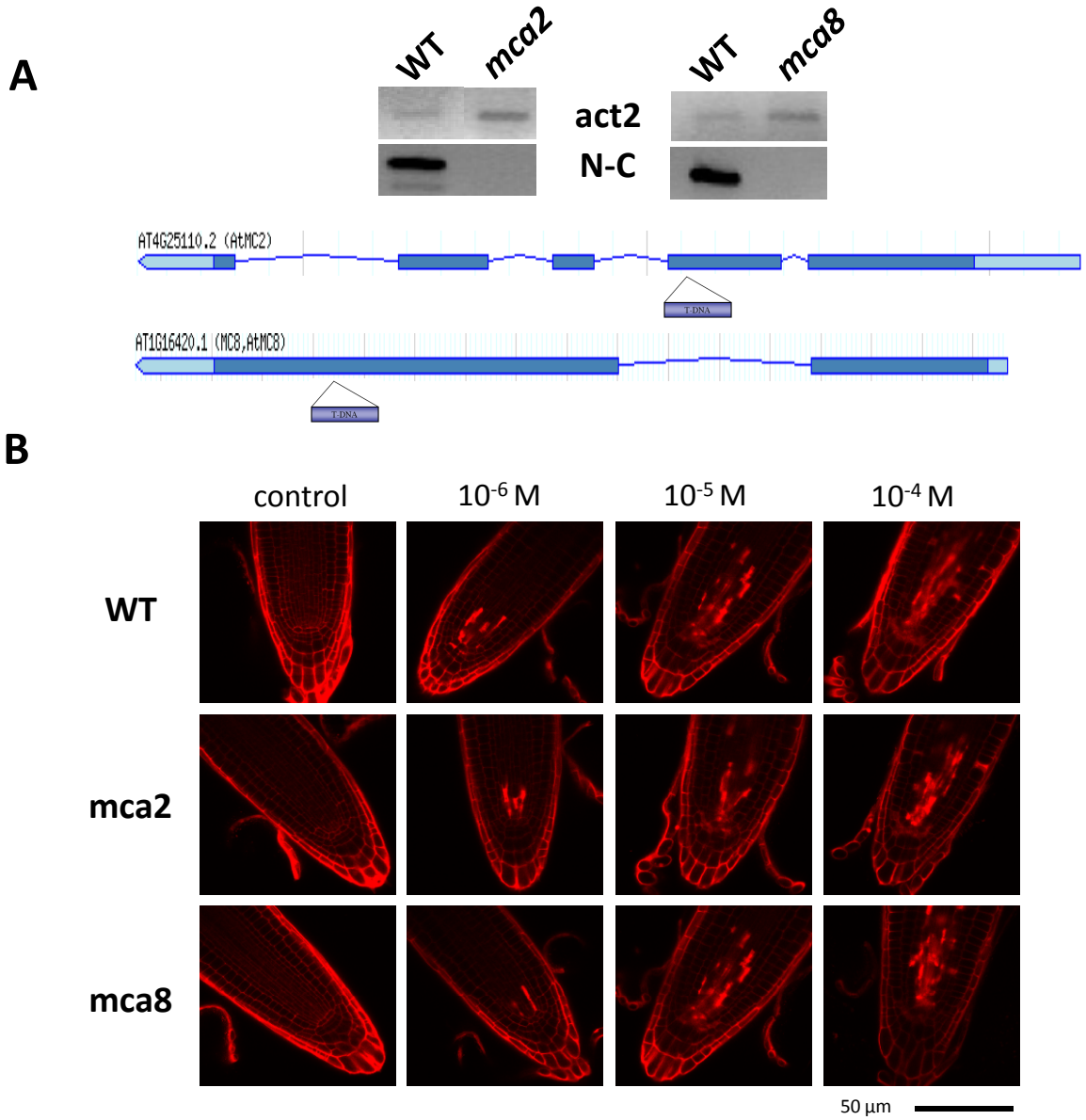
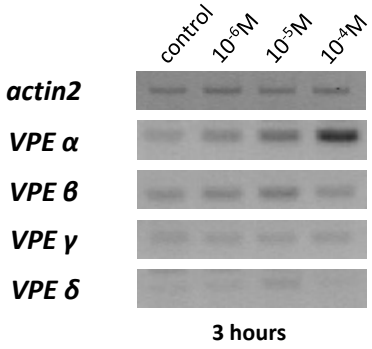


Figure 3.32: Metacaspase mutants *mca2* and *mca8*. **A:** Loss of *MCA2* and *MCA8* expression in *mca2* and *mca8* mutants was detected by semiquantitative RT-PCR; **B:** Comparison of cell death induction upon 10^{-6} M, 10^{-5} M, 10^{-4} M BLM treatment in mutants *mca2* and *mca8* compared to WT. Propidium iodide staining.

A



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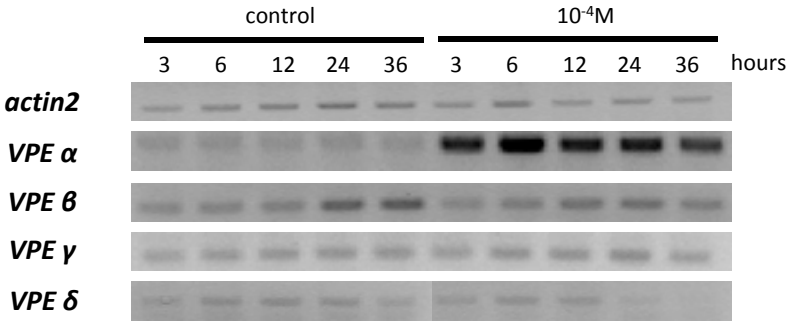


Figure 3.33: Semiquantitative RT PCR analysis of the expression of 4 Arabidopsis *VPE* genes (*VPE α-δ*) upon BLM treatment; **A:** *VPEs* expression at 3 hours after 10^{-6} M, 10^{-5} M, 10^{-4} M BLM treatments; **B:** Kinetics of *VPEs* induction after 3, 6, 12, 24 and 36 hours of 10^{-4} M BLM treatment.

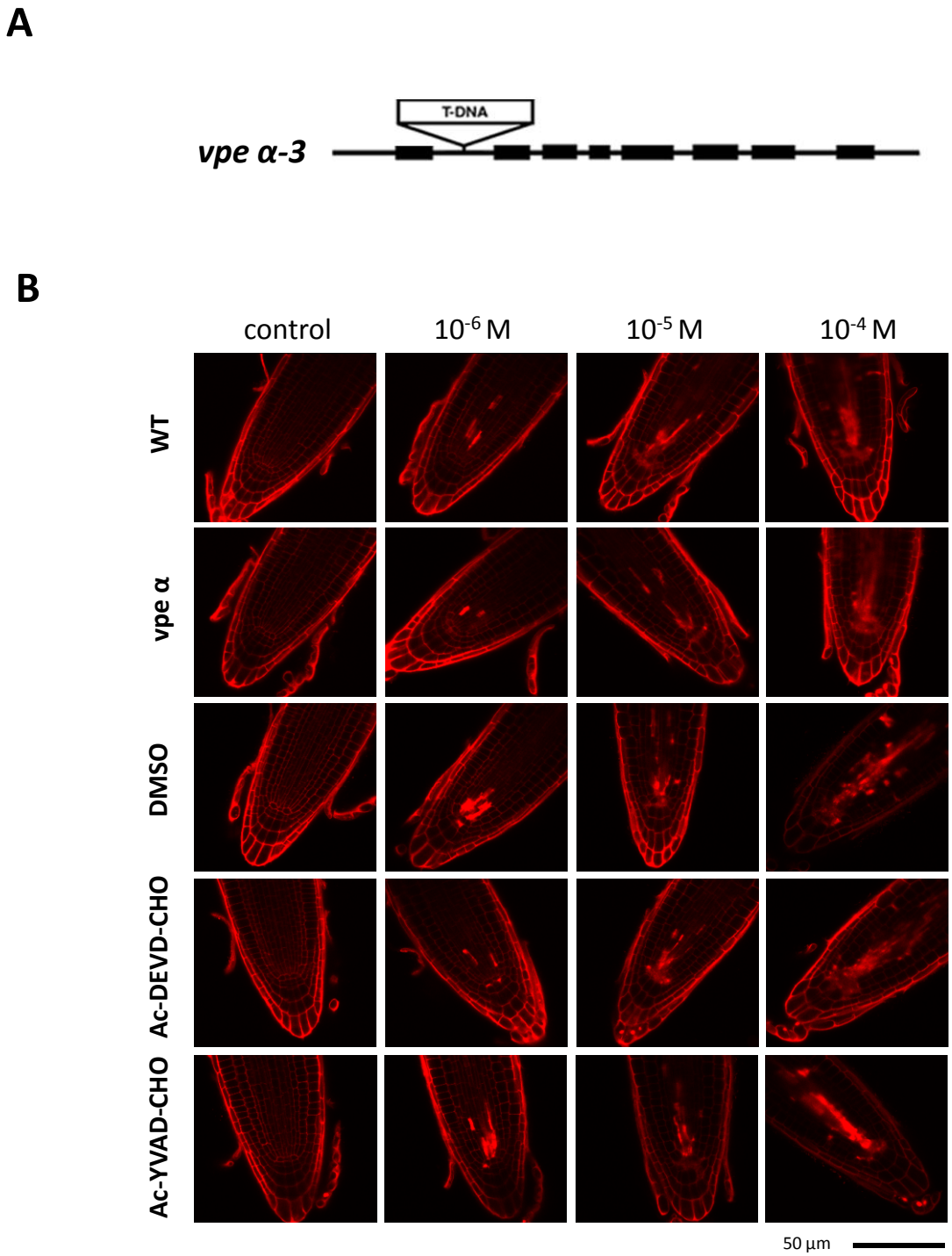


Figure 3.34: The role of VPEs during BLM-induced cell death. **A:** Scheme of vpeα-3 insertion mutant (according to Kuroyanagi et al. 2003); **B:** Comparison of cell death induction upon BLM (10^{-6} M, 10^{-5} M, 10^{-4} M) treatment in WT and *vpe α* or in presence of caspase-1 inhibitor (YVAD, 100 µM in DMSO), caspase-3 inhibitor (DEVD, 100 µM in DMSO) and DMSO alone.

secondly by using specific caspase inhibitors which have been described functional in plant cells for inhibiting caspase-like activity dependent cell death.

vpeα (αvpe-3) mutant (Syngenta Biotechnology) was characterized previously as an insertion mutant with T-DNA in the N-terminus of the gene (Figure 3.34A) (Kuroyanagi et al. 2003). WT and *vpeα* plants were transferred 5 DAG on a medium with BLM (10^{-6} M, 10^{-5} M, 10^{-4} M) and let grown in standard growth conditions for 24 hours. Despite of a transcriptional upregulation of *VPE α* observed in our previous experiment, we did not detect any changes in BLM-induced PCD in root stem cells. We concluded that possibly other VPEs (VPE β – δ) could overlap the function of VPE α resulting in unchanged cell death sensitivity of the *vpeα* mutant, or alternatively other components of the PCD machinery.

In plants, animal caspase inhibitors were successfully used as a proof of implication of caspase-like activities in different types of PCD (Mlejnek et al 2002, Lam et del Pozo 2000). Although we had negative results using caspase inhibitors on BY-2 cells, we set up the same experiment in Arabidopsis to investigate the effects of these drugs.

Five days old plants were transferred on MS plates with caspase inhibitors Ac-YVAD-CHO and Ac-DEVD-CHO (100 μM). One hour after preincubation of plants on MS plates with caspase inhibitors, we put them on MS plates supplemented with caspase inhibitors plus BLM (10^{-6} M, 10^{-5} M, 10^{-4} M). Because caspase inhibitors were dissolved in DMSO and the final concentration was 0,1 % we used DMSO containing MS plates as a control. After 1 day of cultivation in standard growth conditions we evaluated cell death by propidium iodide staining in the root tips. DMSO treated plants showed similar cell death induction as WT plants in response to BLM (Figure 3.34B). When using inhibitors, neither Ac-DEVD-CHO nor Ac-YVAD-CHO suppressed cell death induction in roots. Thus, caspase-like activities are likely not involved during BLM-induced cell death in Arabidopsis.

3.3.6. Implication of DNA damage signaling during BLM-induced cell death

3.3.6.1. Role of upstream actors: ATM/ATR and SOG1

In BY-2 cells, we used caffeine to block DNA repair pathways via specific inhibition of ATM kinase (Figure 3.10). To validate these results in Arabidopsis we analyzed the effect of caffeine on cell death induction in the root tips and we compared the response to that observed in the *atm* and *atr* mutants defective in sensing of DSB DNA damage and replicative stress, respectively. Arabidopsis mutants *atm* and *atr* were already characterized and showed normal growth phenotype at standard conditions except of partial sterility of mutant *atm* and slightly accelerated growth after germination (Garcia et al., 2003). Recently, it was shown that DNA damage induced cell death by zeocin or BLM in Arabidopsis stem cells depends on kinase ATM, but not on ATR. The mutation in ATR change the localization of dead cells to more dispersed pattern within the root tip (Fulcher and Sablowski 2009).

To complete this study and to validate our observations that caffeine blocks BLM cell death in BY-2, we compared cell death induction after BLM treatment in WT, *atm* and *atr* genetic backgrounds or in the presence of caffeine.

To obtain high inhibitory effect of caffeine, plants were treated with caffeine (5mM) 1 hour before addition of BLM (10^{-6} M, 10^{-5} M, and 10^{-4} M). After 24 hours, cell death induction followed by propidium iodide staining was completely blocked by caffeine in meristematic cells in response to 10^{-6} M and 10^{-5} M (Figure 3.35). However, the massive cell death induction in stele by 10^{-4} M BLM was blocked only partially since some dead cells remained scattered in the stele and endodermis. Analyzing the response in *atm*, cell death occurring in meristem is less suppressed compared to caffeine-treated WT plants in response to BLM.

Suppressor of gamma response 1 (Sog1-1) was identified as a suppressor of radiosensitive phenotype of seeds defective in the repair endonuclease XPF (Preuss et Britt 2003). Later, it was suggested that the signals related to genotoxics stress and processed via kinases ATM and ATR are integrated through the transcription factor SOG1-1 (Yoshiyama et al. 2009). Gamma irradiation does not have a positive effect on SOG1-1 transcription, although the mutation in this gene is able to suppress cell death in γ irradiated roots (Furukawa et al. 2010). It means that this factor might be regulated posttranscriptionaly. To test if SOG1-1 plays a role also during BLM-induced cell death, we compared cell death induction between WT and *sog1-1* mutant (Figure 3.35). Indeed, cell death induction was strongly inhibited similarly to caffeine effect in response to BLM.

Our data indicate the autophagic character of BLM-induced cell death in Arabidopsis. Our transcriptional analyses of caspase-like enzymes revealed a high upregulation of *VPE α* upon BLM treatment. Thus, we analyzed *VPE α* expression in *sog1-1* and *atm2* which exhibit increased resistance to cell death induction. Semi-quantitative RT PCR analyses showed the absence of *VPE α* induction in response to 3 hours treatment of 10^{-6} M, 10^{-5} M, 10^{-4} M BLM in *atm* or *sog1-1* compared to WT (Figure 3.36). These findings demonstrate that *VPE α* expression is ATM- and SOG1-1-dependent.

3.3.6.2. Role of downstream actors: BRCT-domain proteins

To identify other actors involved in the genotoxic-induced PCD we tested several candidates which are implicated in DBS response. One feature of all tested candidates is the presence of BRCT domains in their sequences, which is a characteristic, at least in mammals, to mediate protein/protein interaction during DNA repair or cell cycle regulation through recognition of specific phosphorylated residues (Scully et al. 1997). Among these is BRCA1 (breast cancer susceptibility gene 1) which in animals is phosphorylated by ATM leading to BRCA1 foci formation within the nucleus and enhancement of DNA repair-related transcription (Scully et al. 1997). In plants, *BRCA1* is strongly up-regulated in γ irradiated Arabidopsis plants or by BLM (Lafarge et Montane 2003; Reidt et al. 2006).

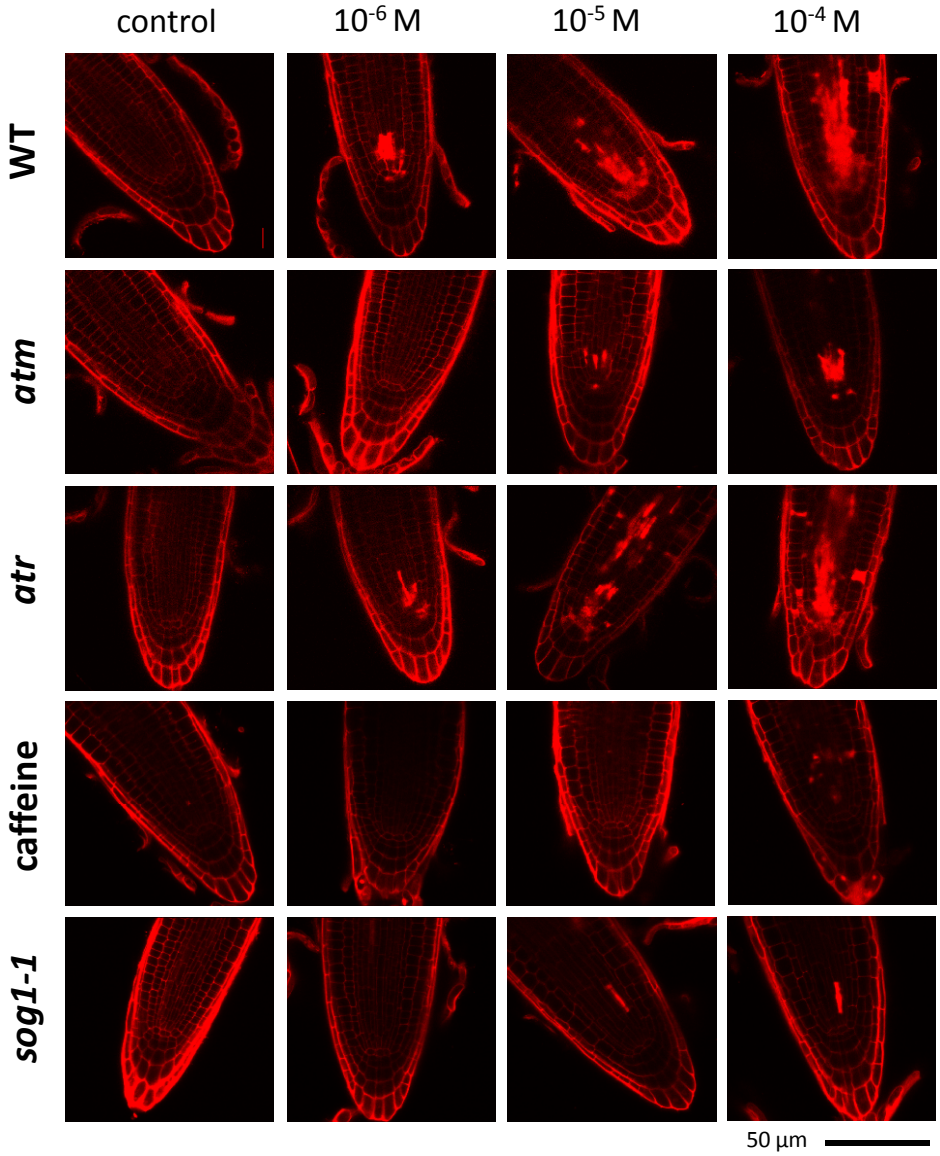


Figure 3.35.: Comparison of cell death induction after 24 hours of 10^{-6} M, 10^{-5} M, 10^{-4} M BLM treatment in WT, *atm*, *atr* and *sog1-1* or in the presence of caffeine (5 mM).

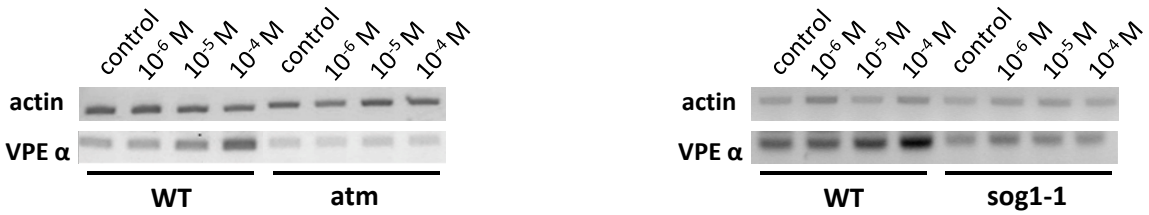
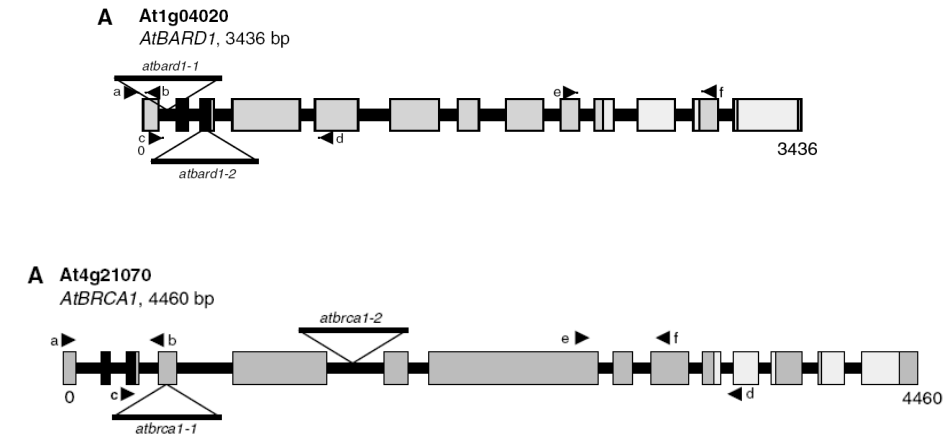


Figure 3.36.: Semi-quantitative RT PCR analysis of *VPE α* expression in WT, *atm* and *sog1-1* after 3 hours of 10^{-6} M, 10^{-5} M, 10^{-4} M BLM treatment.

A



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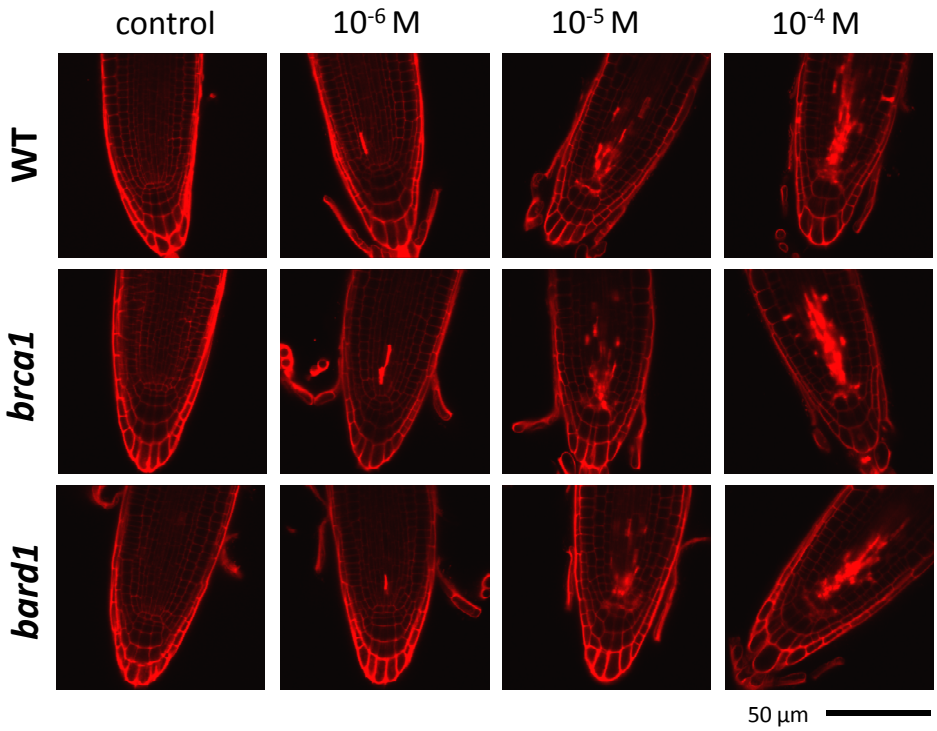


Figure 3.37.: Response of *BRCA1* and *BARD1* T-DNA insertion mutants to BLM; **A:** Scheme of Arabidopsis *BARD1* and *BRCA1* gene with the position of T-DNA insertions in appropriate mutants *atbard1-2* (SALK 031862) and *atbrca1-1* (SALK 014731) according to Reidt et al. (2006); **B:** Cell death detection in Arabidopsis root meristem after 24 hours of BLM treatment (10^{-6} M, 10^{-5} M, 10^{-4} M) in different genetic background *atbard1-2* and *atbrca1-1*.

BARD1 (breast cancer associated gene 1), can interact with BRCA1, but its corresponding gene is not strongly induced in response to DSB, but is supposed to play a role in the same signaling pathway than BRCA1 (Reidt et al. 2006).

Bard1 and *brca1* mutants are lethal in animals (Goven et al. 2003; McCarthy et al. 2003) but in plants, *brca1* is fully viable without any developmental defects whereas a *bard1-3* mutant shows altered morphology, notably at the level of SAM (Han et al. 2008). Moreover both mutants are hypersensitive to crosslinking agent mytomicin C (MMC) and have altered regulation of homologous recombination. We used two mutants (kindly provided by H. Puchta) *brca1-1 and bard1-2* mutant (figure 3.37A) that were characterized as null mutants, however recent findings have shown that *bard1-2* mutant is producing a C-terminal part of the protein (Han et al. 2008).

As performed with *atm* and *atr* mutants, *brca1* and *bard1* mutants we treated with BLM for 24 hours and cell death analyses revealed no difference compared to WT (Figure 3.37B).

Another BRCT-domain protein related to BRCA1 and BARD1 was identified in our laboratory by the screen of unknown function protein containing BRCT-domain in Arabidopsis. Two independent T-DNA insertion mutants (*to12* and *to24*) were identified; they display a little sensitivity to BLM but high sensitivity to CPT, an inducer of DSB during replication (Sanchez-Calderon, manuscript in preparation). In addition, mutated plants presented genomic instability (Sanchez-Calderon, manuscript in preparation). *To* was shown to be up-regulated upon DSB DNA damage in an ATM-dependent manner (Ricaud et al. 2007). As for *bard1* and *brca1* mutants, *to* mutants are not affected in cell death induction compared to WT in response to BLM (Figure 3.38).

To summarize, we tested 3 different BRCT family proteins – BRCA1, BARD1 and TO. Our results using T-DNA insertion mutants did not show any effect on cell death response of Arabidopsis plants after BLM treatment.

3.3.7. Link between DNA damage signaling, endocycle regulation and PCD

BLM treatment of Arabidopsis induces cell differentiation which was coupled with increased endoreduplication levels in roots. In addition, this differentiation was not observed upon HU treatment in WT but only in *atr* mutant (Culligan et al. 2004). To test the connection between endoreduplication induction, cell differentiation and cell death, we quantified DNA content in *atm* and *atr* mutant after BLM and HU long-term treatment. The experiments were performed 3 days after the transfer of 5 DAG plants on MS medium with genotoxics (Figure 3.39A). DNA content was evaluated in roots and data from 4 independent experiments are presented as a ratio of number of endoreduplicated cells with DNA content higher than 4C versus the number of cells with 2C or 4C DNA content. When we compare only *atm* and *atr* mutants to WT without genotoxics we saw an increased endoreduplication level in both mutants with the more pronounced difference in *atr*. In WT, both BLM and HU

treatments induced endoreduplication with a more apparent effect of BLM. However, no significant changes were observed in *atm* and *atr* mutants, suggesting that ATM and ATR are not controlling the modification of endoreduplication level in response to BLM or HU.

In parallel to flow cytometric analyses, we analyzed the cell death induced by 3 days of genotoxic treatment. We transferred 5 DAG plants on MS plates supplemented with 10^{-5} M BLM or 1 mM HU and we let them to growth for 6 additional days in standard growth conditions. Longer genotoxics exposure permitted us to see induced morphologic changes of Arabidopsis roots. Roots of *atm* and *atr* mutants treated with BLM had comparable phenotypes to WT plants – such as increased cell volume of differentiation of epidermal cells to the root hairs (Figure 3.39B). On the contrary, in HU response *atr* phenotype was similar to phenotypes of plants treated with BLM. Such phenotype is characterized by enlargement of the cells and root hair formation. As our previous results showed that this change in root morphology is coupled to cell death in meristematic cells, we analyzed root cell death upon genotoxics in *atr* plants using propidium iodide. Two inducers of replicative stress were used, HU which induce G1/S arrest (Culligan, 2004) in young plantlets and APC which induces arrest later on than HU (Reichheld et al., 1998) .

To have an overall view on the cell death induction in root cells we exposed previously characterized mutants WT, *atm*, *atr* and *sog1-1* having a role in sensing of DNA damage to different concentrations of HU (1 mM and 4 mM) and APC (12 µg/ml) and the cell death was analyzed after 24 hours. WT and *sog1-1* plants did not show any cell death induction (Figure 3.40). Using 1 mM of HU, *atr* showed cell death in the meristem compared to WT and *atm*. Such results are similar to those obtained in WT with 10^{-5} M BLM treatment demonstrating that HU mimics BLM treatment in *atr* mutant. However APC was not able to induce any cell death in *atr*, suggesting that different checkpoints are activated under replicative stress. Interestingly, a few dead cells were observed in the initials or stele in response to APC and HU 4mM. These results indicates that cell death induction strongly depends on DNA damage pathways governed by ATM and ATR and also depends on which cell cycle checkpoint is activated in response to different DNA damage inducers. Thus we focused on the E2F transcriptional factors modulating cell cycle control according to our results obtained in BY-2 and the results of others.

3.3.8. Analysis of the role of E2F during DNA damage response

One of the aims of my thesis was to study the role of the E2F transcription factor during genotoxic-induced cell death. This process was extensively investigated in animals and it was shown that E2F1 overexpression leads to cell death in a p53-dependent or a p53-independent manner (Qui et al. 1995; Kowalik et al. 1994) After DNA damage, levels of E2F1 is increased and stabilized by its ATM-dependent phosphorylation (Lin et al. 2001). Moreover E2F1 -/- mice develop thymic hyperplasia as a result of a defect in thymocite apoptosis (Field et al. 1996). Thus, in animals the

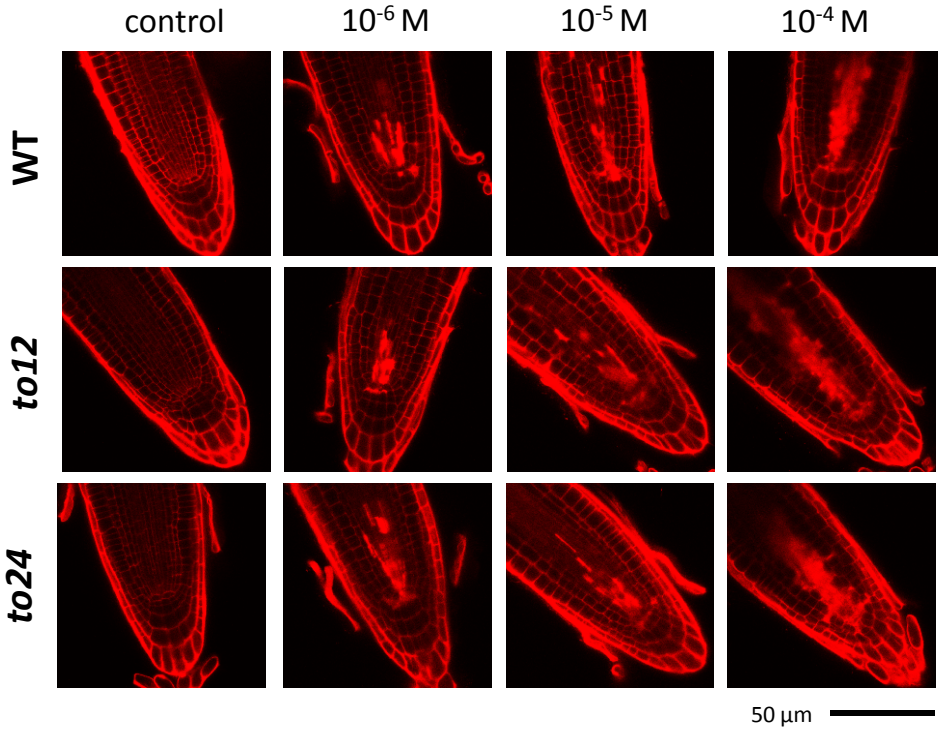
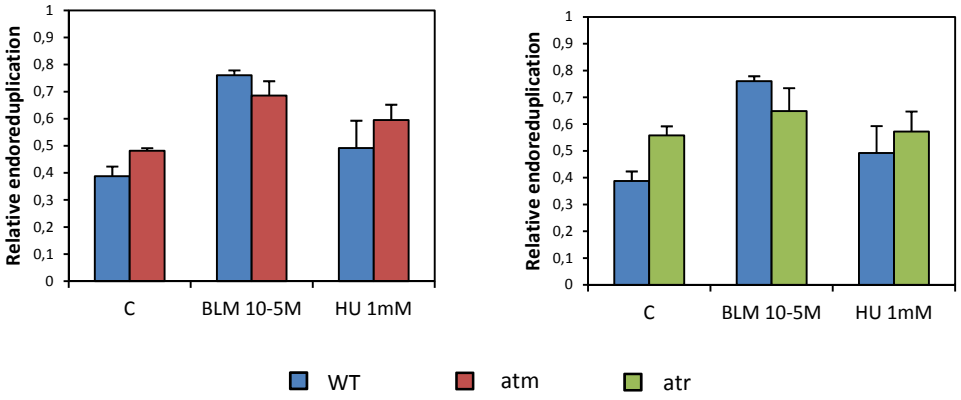


Figure 3.38.: Cell death detection in Arabidopsis *to12* and *to24* root meristem after 24 hours of BLM treatments (10^{-6} M, 10^{-5} M, 10^{-4} M).

A



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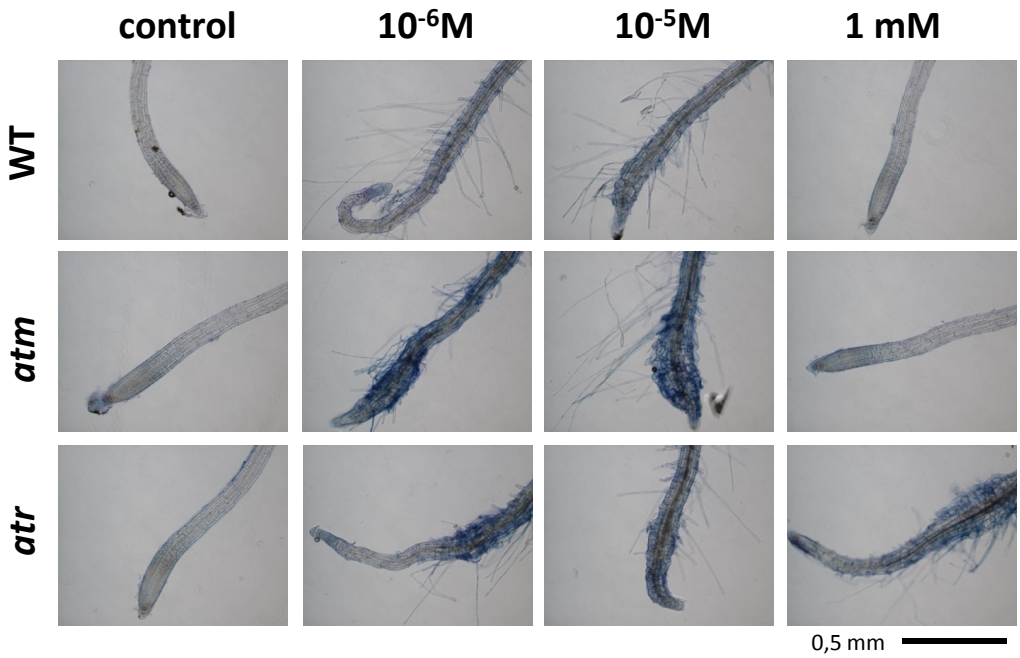


Figure 3.39.: Effect of HU (1 mM) or BLM (10^{-6} M, 10^{-5} M) on endoreduplication and root architecture in different genetic background WT, *atm* and *atr* during 3 days. **A:** Flow cytometry analysis performed on the roots treated with genotoxics. Columns represent relative endoreduplication (see in the text); **B:** *atm* and *atr* mutants upon BLM or HU treatment during 6 days.

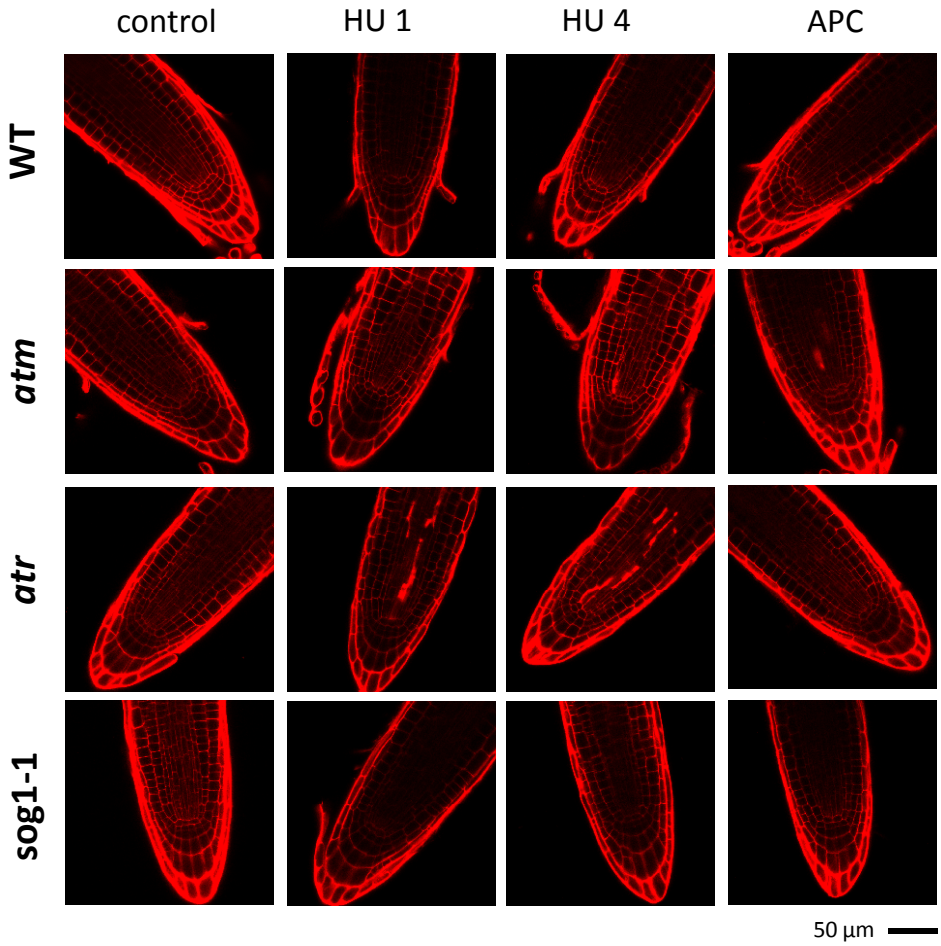


Figure 3.40.: Comparison of cell death induction during replicative stress using HU (1 mM, 4 mM) and APC (12 µg/ml) treatment in WT, *atm*, *atr* and *sog1-1* after 24 hours treatment.

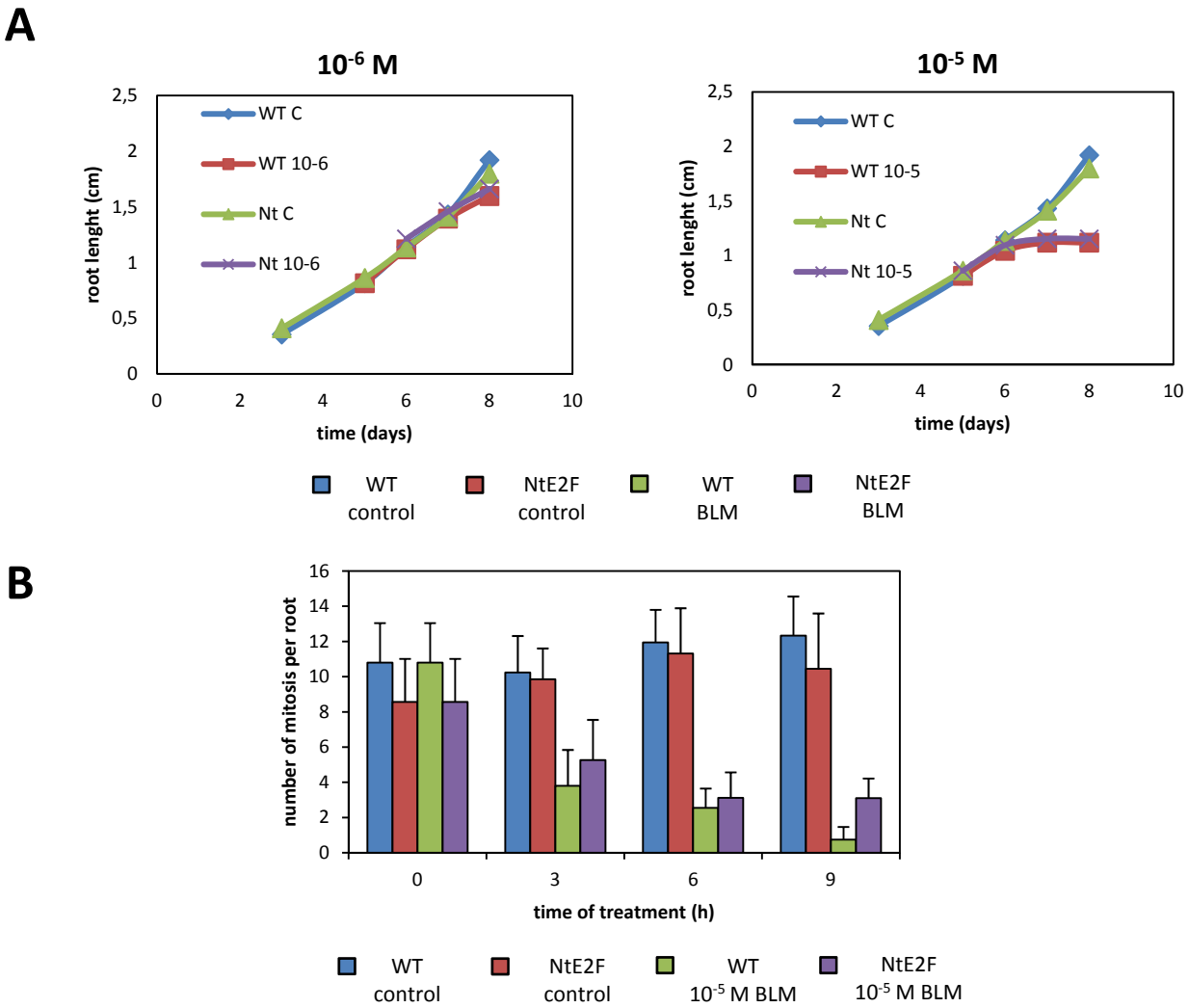


Figure 3.41: Sensitivity of NtE2F plants to 10^{-5} M and 10^{-6} M BLM treatment **A:** Root growth test. 5 DAG plants were transferred on the MS medium with BLM. **B:** Mitotic index in the root tip upon 3, 6 and 9 hours of BLM treatment in WT and NtE2F 5 DAG plants.

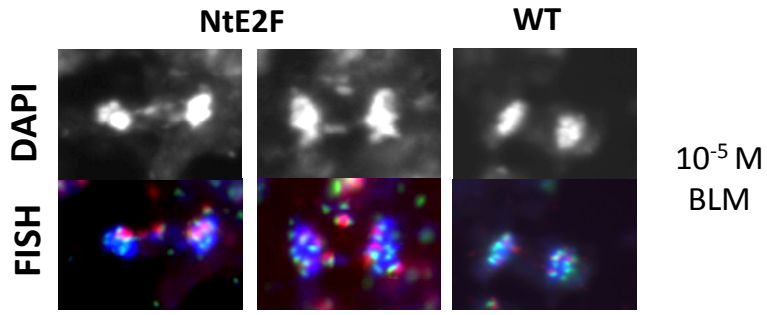


Figure 3.42: FISH stained mitosis in NtE2F and WT pistils upon 2 days of BLM treatment (10^{-5} M). blue – DAPI stained nuclei; green – centromere-specific probe; red – rDNA-specific probe.

implication of E2F transcriptional factors during DNA damage signaling and apoptosis is well documented.

Concerning the PCD response in BY-2 cells, we have shown that NtE2F overexpression abrogates BLM-induced G2/M checkpoint and at high concentration of genotoxics its overexpression inhibits cell death in BY-2 cells. Thus we investigated if similar effect could be observed in Arabidopsis plants.

3.3.8.1. Cellular response of Arabidopsis plants overexpressing NtE2F

To reveal the effect of NtE2F overexpression on cell division and growth of Arabidopsis, we analyze sensitivity of WT plants treated Arabidopsis roots with various concentrations of genotoxics. Arabidopsis plants were grown on MS plates and 5 days after germination they were transferred on plates with BLM (10^{-6} M, 10^{-5} M). In presence of BLM root growth was arrested after 1 day of treatment and we did not observe any difference between WT and NtE2F plants (Figure 3.41A). To see the effect of BLM on cell cycle, we counted the number of mitosis in the root tips of plantlets treated with BLM. The mean number of mitosis in non treated roots was around 10 in WT or 8 in NtE2F (Figure 3.41B). In the presence of 10^{-5} M BLM the number of mitosis decreased progressively to 0 within 9 hours of the treatment in WT roots whereas the number of mitosis decreased more slowly in NtE2F roots. Thus these data suggested that NtE2F overexpression inhibits cell cycle arrest upon BLM treatment in Arabidopsis as shown for BY2 cells.

Based on these observations, we evaluated if the progression of mitosis was affected during BLM treatment. Due to the difficulty to analyze divisions at the cellular level due to the small size of nuclei, we analyzed nuclei in pistils of non maturated flowers where the rate of division is higher. Visualization of mitotic figures was performed using DAPI staining. Immature inflorescences were dipped in 10^{-5} M BLM for 2 days. After fixation, we performed FISH analyses using centromere -(green signal) and rRNA-specific (red signal) probes (Figure 3.42). We detected interchromosomal anaphase bridges (DAPI staining) in NtE2F nuclei compared to WT in response to BLM. These anaphase bridges may suggest the fusion of two chromosomes. We conclude that NtE2F overexpression impaired mitosis leading to genomic instability in Arabidopsis in response to BLM; however it would be necessary to make supplementary experiments to determine in which extent the deregulation of cell cycle checkpoint by *NtE2F* overexpression occurs.

3.3.8.2. Does E2F affect cell death induction in root stem cells?

To study the role of E2F during BLM-induced cell death, we used different genetic background – T-DNA insertion mutants *AtE2Fa* and *AtE2Fb* and also lines overexpressing NtE2F and E2Fb characterized previously.

Arabidopsis mutant line *e2fa* (GABI-Kat, line 348E09) was already characterized in the laboratory. For E2Fb we isolated T-DNA insertion mutant (SALK, line N631064) as well (Figure 3.43A). After genotyping of homozygous lines, we verified the lack of *E2Fb* expression in *e2fb* using primers located in the N- and C- terminus of the gene (Figure 3.43B).

Previously it was reported that *E2Fa* is transcriptionaly upregulated upon BLM or Mitomycin C treatment in both cellular suspension and Arabidopsis plants (Roa et al, 2009; Chen et al. 2003). We also tested the expression of *E2Fb* after a 10^{-5} M-BLM treatment at 2 and 6 hours and we did not find any upregulation using semiquantitative RT PCR (Figure 3.43B). However these results suggest that at least E2Fa is implicated in DNA damage response that was already demonstrated in our laboratory (Roa et al. 2009).

To study the role of Arabidopsis E2Fa and E2Fb in BLM-induced PCD, we treated *e2fa* and *e2fb* with BLM and cell death was evaluated using propidium iodide staining of the root tips. No significant differences were revealed in the mutants suggesting that single E2Fa or E2Fb does not affect BLM-induced cell death (Figure 3.44A). In parallel, we used NtE2F and E2Fb overexpressing plants and similar results were obtained, suggesting that more sophisticated regulation exists in the root meristem compared to BY-2 cell suspension (Figure 3.44B).

Interestingly, it appears that the E2Fb-GFP signal is increased using 10^{-4} M BLM after 24 hours (Figure 3.44B). These results likely suggest stabilization of E2Fb during the DNA damage response.

3.3.8.3. Does NtE2F overexpression affect endoreduplication in response to BLM?

Several reports have shown that E2F upregulation induces entry into the S phase and thus could promote several rounds of endoreduplication (De Veylder et al. 2002). The change in root endoreduplication level was measured using a 3 days BLM or HU treatment (Figure 3.45). As a significant result, we considered a difference higher than 0,1 for the relative endoreduplication level. In control plants, NtE2F overexpressing plants had a higher relative endoreduplication (1,4 fold) compared to WT. However in response to BLM, WT plants increased their relative endoreduplication to two-fold but NtE2F plants harbored less variation compared to WT (1,2 fold). We also tested the effect of HU. In WT and NtE2F plants the relative endoreduplication level did not significantly changed. These data indicate that NtE2F overexpression increases the endoreduplication level in Arabidopsis roots that is in agreement with previous results (de Veylder et al. 2002), however in response to BLM the level of endoreduplication is reduced when NtE2F is overexpressed.

3.3.9. Cell cycle response linked to PCD induction in Arabidopsis

Our results have shown that BLM induces cell death in a small cell population in the root meristematic zone. Linked to the induction of cell death in WT plants we have previously shown an

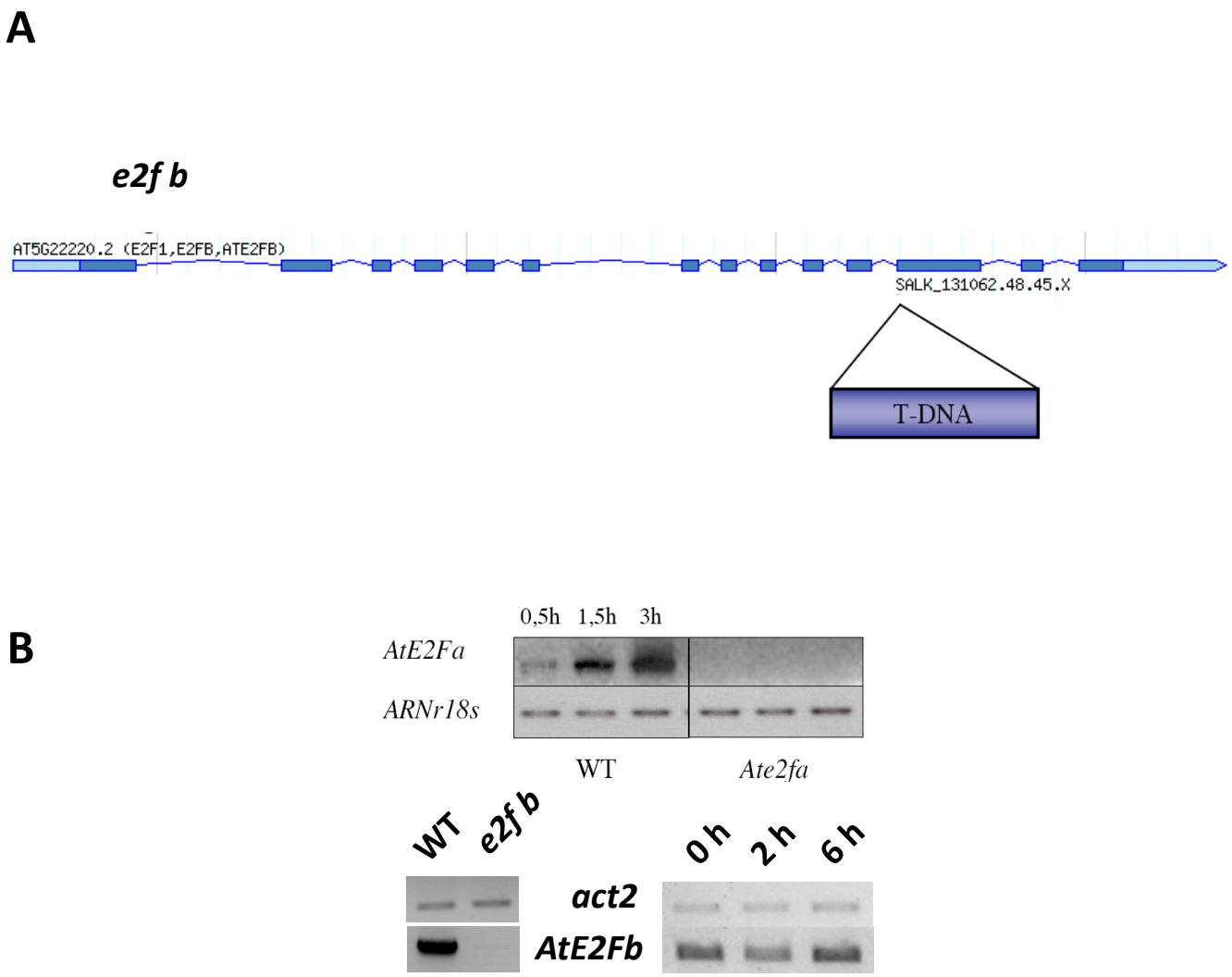


Figure 3.43.: Characterization of *e2fa* and *e2fb* mutants; **A:** Scheme of the localization of T-DNA position in *e2fb* mutant; **B:** Expression of *E2Fa* and *E2Fb* genes preformed by RT PCR analysis in *e2fa* (Roa et al. 2009) and *e2fb* mutants upon normal condition and BLM treatment (10^{-5} M).

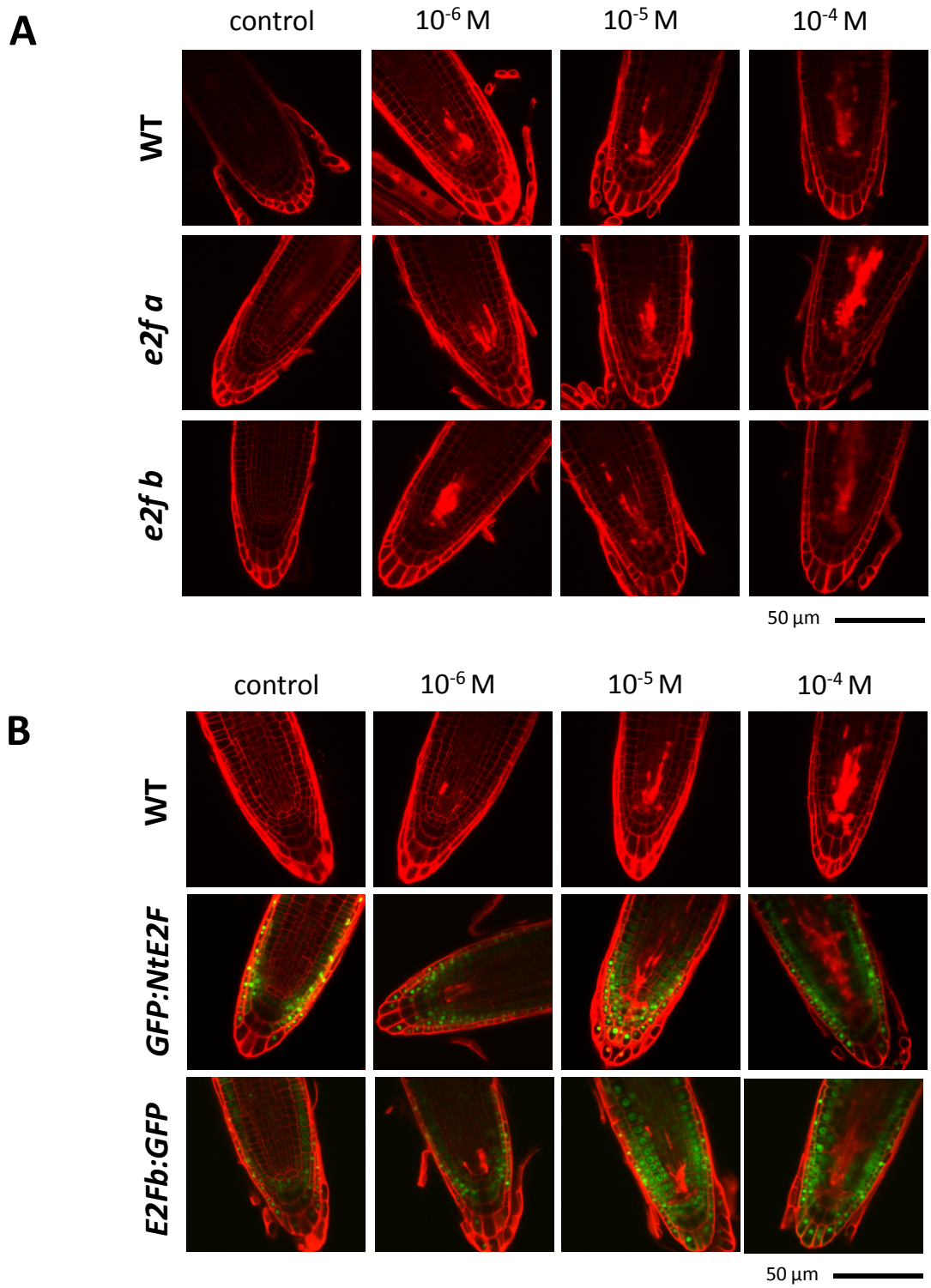


Figure 3.44: PI cell death detection in Arabidopsis root meristem after BLM treatment (10^{-6} M, 10^{-5} M, 10^{-4} M) in different genetic background during 24 hours. **A:** *e2fa* and *e2fb* mutants; **B:** Overexpression of E2Fb and NtE2F GFP fusion.

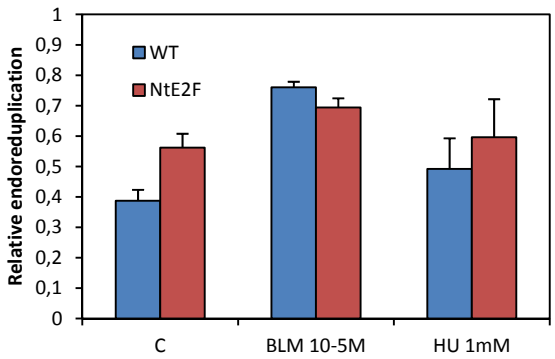


Figure 3.45: Relative endoreduplication levels in WT and NtE2F plants after 3 days of BLM (10^{-5} M) and HU (1 mM) treatment.

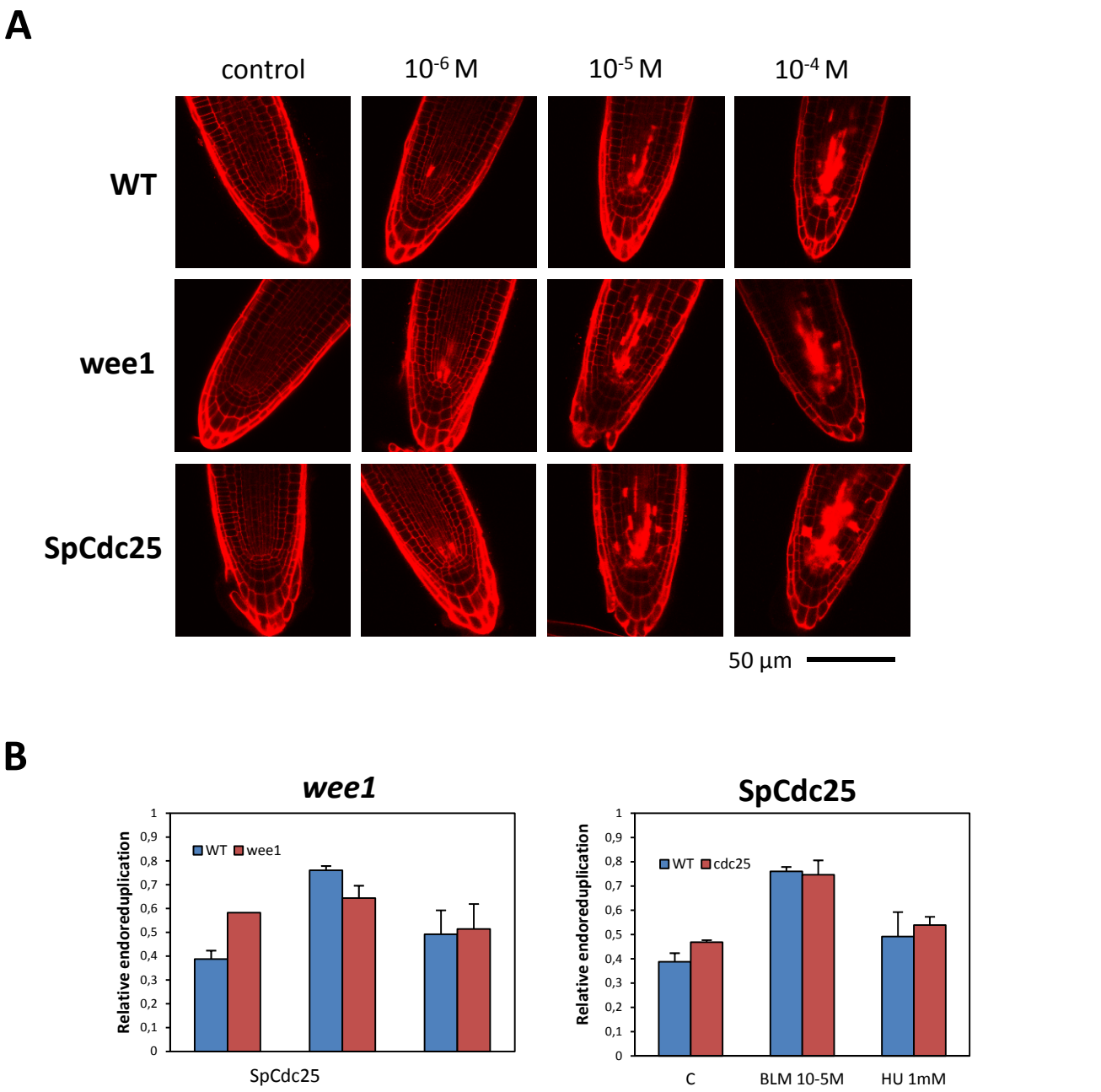


Figure 3.46: Arabidopsis *wee1* mutant and SpCdc25 OE line. **A:** Cell death detection after BLM treatment (10^{-6} M, 10^{-5} M, 10^{-4} M) in *wee1* and Spcdc25; **B:** Relative endoreduplication levels after BLM (10^{-5} M) and HU (1 mM) treatment in *wee1* and Spcdc25.

arrest of cell divisions, cell differentiation and switch to endocycle resulting in increased ploidy of the nuclei. Our data with BY-2 cells also suggested that during G2/M checkpoint the cells are the most sensitive to cell death induction and thus it seems that the decision to divide or undergo cell death takes place at this time. Taken these facts together we focused on two aspects – first on the role of cell cycle regulation and second on the role of endoreduplication as an alternative to the normal cell cycle progression during genotoxics-induced PCD.

3.3.9.1. Model of Wee1/Cdc25 regulation of the cell cycle

In mammals, cell cycle is regulated through sequential activations of different Cyclin/CDK complexes. Upon DNA damage, CDK activity is needed to be blocked by inhibitory phosphorylation of specific amino acid residues that leads to cell cycle arrest. An antagonist of WEE1, is CDC25 phosphatase returning the Cyclin/CDK complex to the active status. Final cellular response is given by interplay of WEE1 and CDC25 phosphatase (reviewed in Cools et de Veylder. 2009). In plants, the functional homologue of CDC25 phosphatase is still missing however its antagonist WEE1 was well documented as a downstream effector of ATM and ATR (de Schutter et al. 2007). Previously we have also shown an upregulation of *WEE1* upon BLM treatment in BY-2.

To analyze the effect of WEE1 in BLM-induced cell death, we analyzed the BLM response in the *wee1* T-DNA insertion mutant. An overexpression approach was excluded, because it seems that regeneration of *WEE1* overexpressing plants under the control of 35S promoter is not possible (de Schutter et al. 2007). As already mentioned that plants lack functional *Cdc25* homologue, we used Arabidopsis plants overexpressing *Cdc25* from *Schyzosacharomyces pombe*. *SpCdc25* when overexpressed is able to increase NtCDKB1 activity and thus overcome activated G2/M checkpoint in tobacco (Orchard et al. 2005). We used Arabidopsis plants overexpressing *SpCdc25* under the control of the constitutive 35S promoter. In spite of the heterologous expression of *SpCdc25* which was used only as a tool to modify cell cycle regulation, our approaches could help us to elucidate the crosstalk between cell cycle regulation and cell death induction.

Five DAG plants were exposed to BLM (10^{-6} M, 10^{-5} M, 10^{-4} M) for 24 hours and cell death induction in the root was analyzed using propidium iodide staining. No significant difference in cell death induction was observed in *wee1* and SpCdc25 plants compared to WT (Figure 3.46A).

3.3.9.2. Wee1 and SpCdc25 control of endocycle?

After longer genotoxics treatment we tested how the cell cycle regulation is affected by the lack of WEE1 kinase or gain of function of Cdc25 activity. As previously, we compared the levels of relative endoreduplication between different genotypes WT, *wee1* and SpCdc25 in roots after 3 days treatment with 10^{-5} M BLM or 1 mM HU (Figure 3.46B). The relative endoreduplication level was increased (1,5 fold) in *wee1* compared to WT in control plants. However in response to BLM, as for

NtE2F overexpressing plants the endoreduplication level was not significantly affected (1,1 fold) in *wee1* compared to WT. On the opposite, no significant differences were observed in response to HU between WT and *wee1*. Our data suggest that the lack of Wee1 increases number of endocycling cells in non treated cells, but reduces the endoreduplication level in response to BLM compared to WT. Alternatively, overexpression of SpCdc25 did not change the response in absence or presence of BLM or HU.

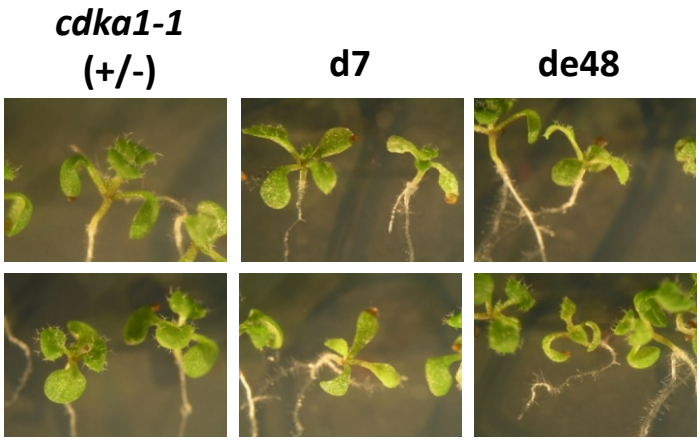
3.3.9. Does the inhibition of endoreduplication affects cell death?

For this study we used dominant-negative version of CDKA1;1 phospho-mimicry mutant named d7 and de48 showing both highly decreased CDKA1 activity at least in leaves. d7 plants are cdka;1 homozygous mutants expressing CDKA1;1^{T161D}, a phospho-mimicry at activatory residue 161, under the control of natural CDKA1;1 promoter. d7 plants are a "weak allele" partially restoring CDKA1;1 activity. Second, de48 plants are also cdka;1 mutants expressing CDKA;1^{T14DY15E}, a phospho-mimicry allele of inhibitory residues 14 and 15, under the control of natural cdka;1 promoter rendering kinase constitutively repressed. These plants have developmental and reproductive defects such as later germination, lanceolated cotyledons and leaves resulting from CDKA;1 deregulation. d7 and de48 plants have also glossy or shiny leaves due to the highly enlarged cells (Dissmeyer et al. 2007, personal communication, Figure 3.47A). We used these plants mainly because of reported strong inhibition of endoreduplication measured in leaves 1+2 and 3+4 (Dissmeyer et al. 2007).

Because of the sterility of d7 and de48 plants we used seed stock from heterozygous mother plants segregating homozygous plants. Ten days after the selection, we verified the endoreduplication levels in both dephosphomutants. Our results are shown in Figure 3.47B and represent the profile of DNA content of the whole 10 DAG old plants. As a control we used segregated plants which were not homozygous in *cdka;1* mutation, denoted as WT. In WT plants the number of C8 and C16 cells was 15 % and 6 % respectively whereas in d7 and de48 plants these numbers were decreased to 8 % for C8 and to 2% for C16. Previously, it was shown that endocycle is almost completely inhibited in d7 and de48 plants measured on 1st and 2nd pair of true leaves however our data suggest that the endoreduplication is still apparent at the level of whole plant (Dissmeyer et al. 2007 and 2009).

To address the question in which organs the endoreduplication is not blocked we dissected 21 DAG plants with the following parts to quantify DNA contents: roots, cotyledons, 1st + 2nd leaves, 3rd + 4th leaves and the rest of the plant involving hypocotyls, shoot apex and 5th and 6th leaves. The flow cytometry analyses showed that WT plants had a lowest number of endoreduplicated cells in apex fraction which progressively increased in the older parts of the plant (leaves 3+4) and (leaves 1+2) to a maximum of 16C in the cotyledons (Figure 3.48). In roots, the number of endoreduplicating cells was low and showed a little 16C fraction but the majority of the cells had 2C DNA content. When we analyzed the endoreduplication in the aerial parts of d7 and de48 plants compared to WT plants, we

A



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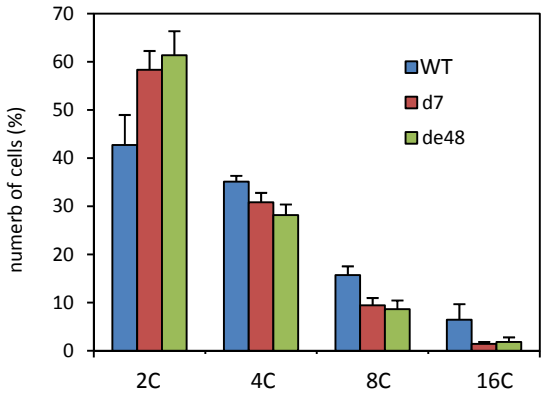

Figure 3.47: CDKA1-1 phospho-mimicry mutants d7 and de48. **A:** Phenotype of 10 DAG plantlets. **B:** Flow cytometry analysis of different part of d7 and de48 mutant plants.

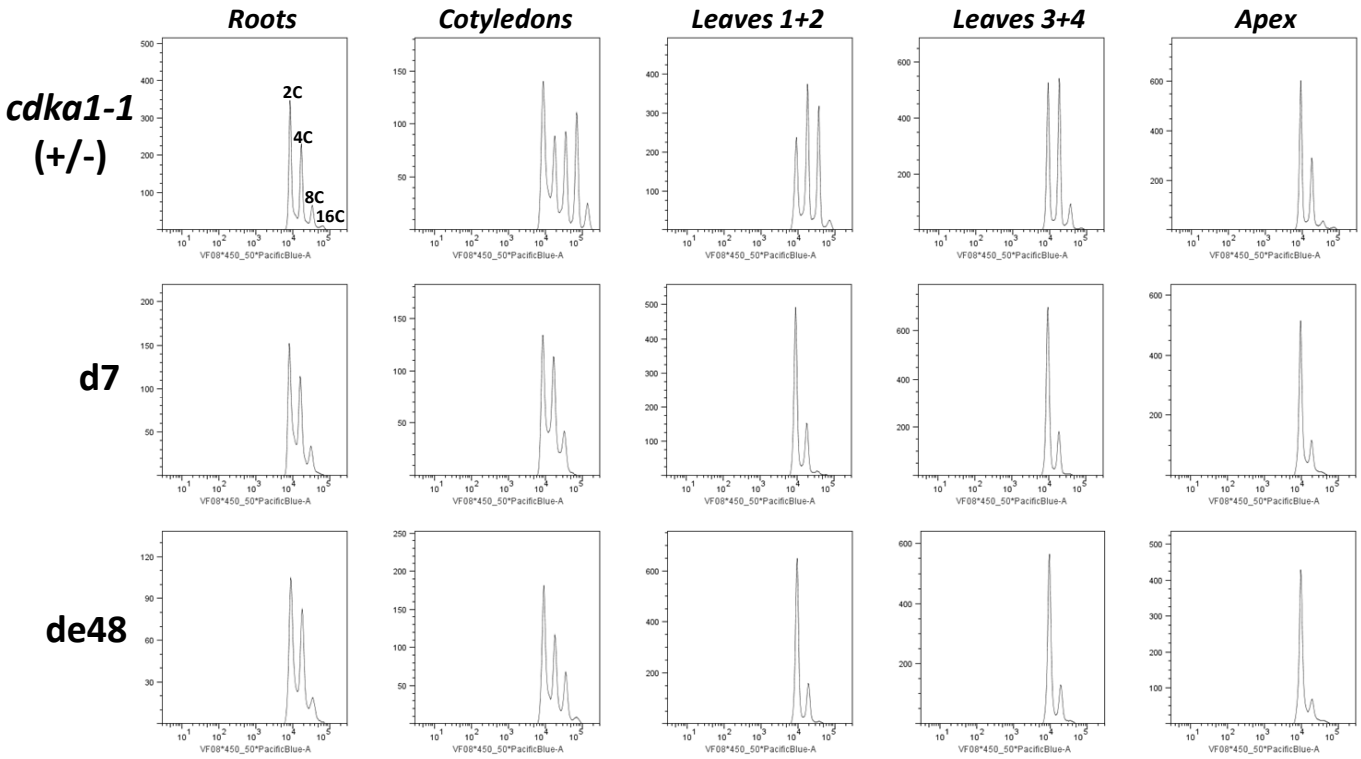

Figure 3.48: CDKA1-1 phospho-mimicry mutants d7 and de48. **A:** Phenotype of 10 DAG plants. **B:** Flow cytometry analyses of different part of d7 and de48 plants.

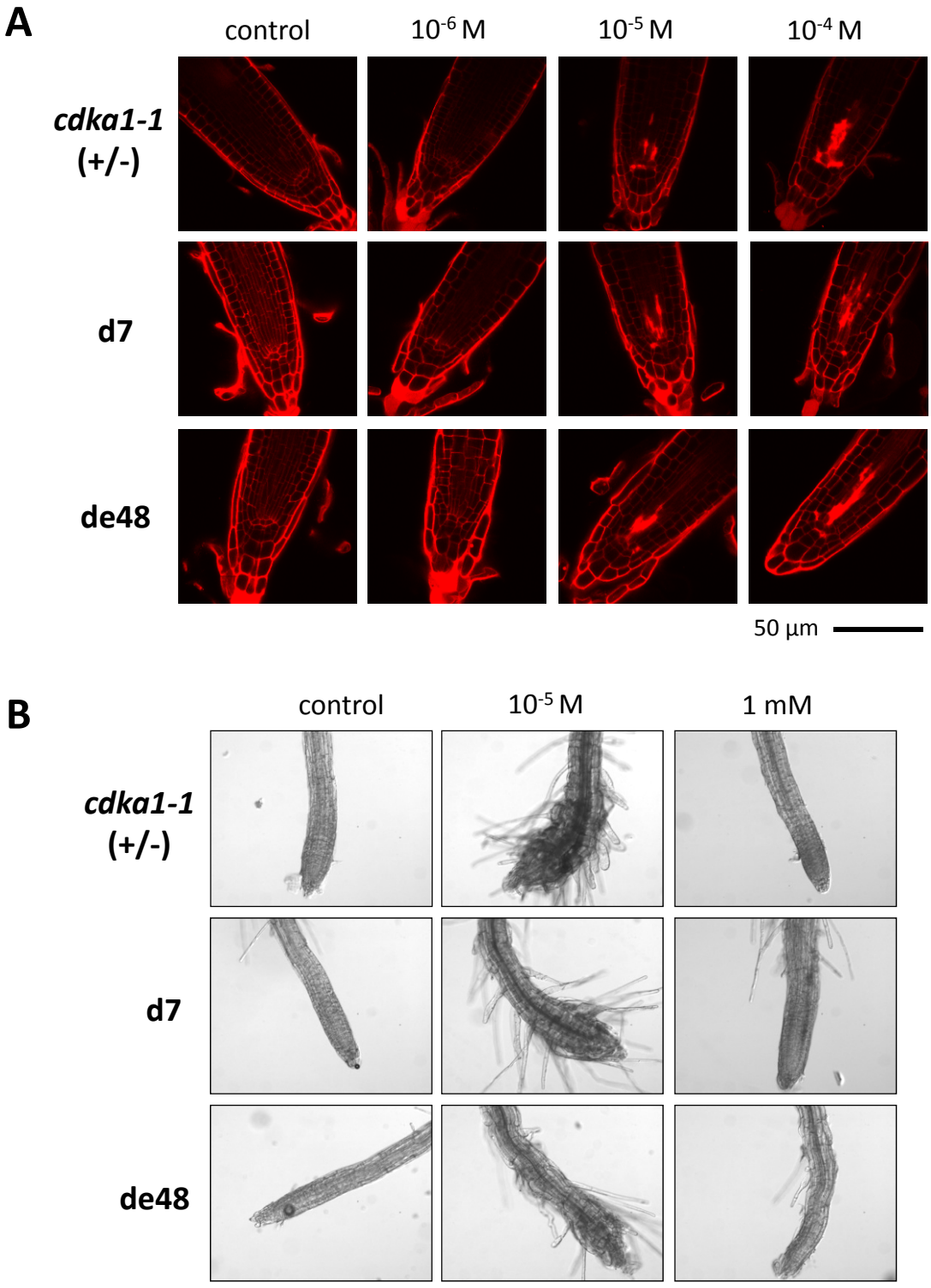

Figure 3.49: CDKA1-1 phospho-mimicry plants d7 and de48 in response to genotoxics. **A:** BLM treatment (10^{-6} M, 10^{-5} M, 10^{-4} M); **B:** Changes in root morphology after BLM (10^{-5} M) or HU treatment (1 mM) during 3 days.

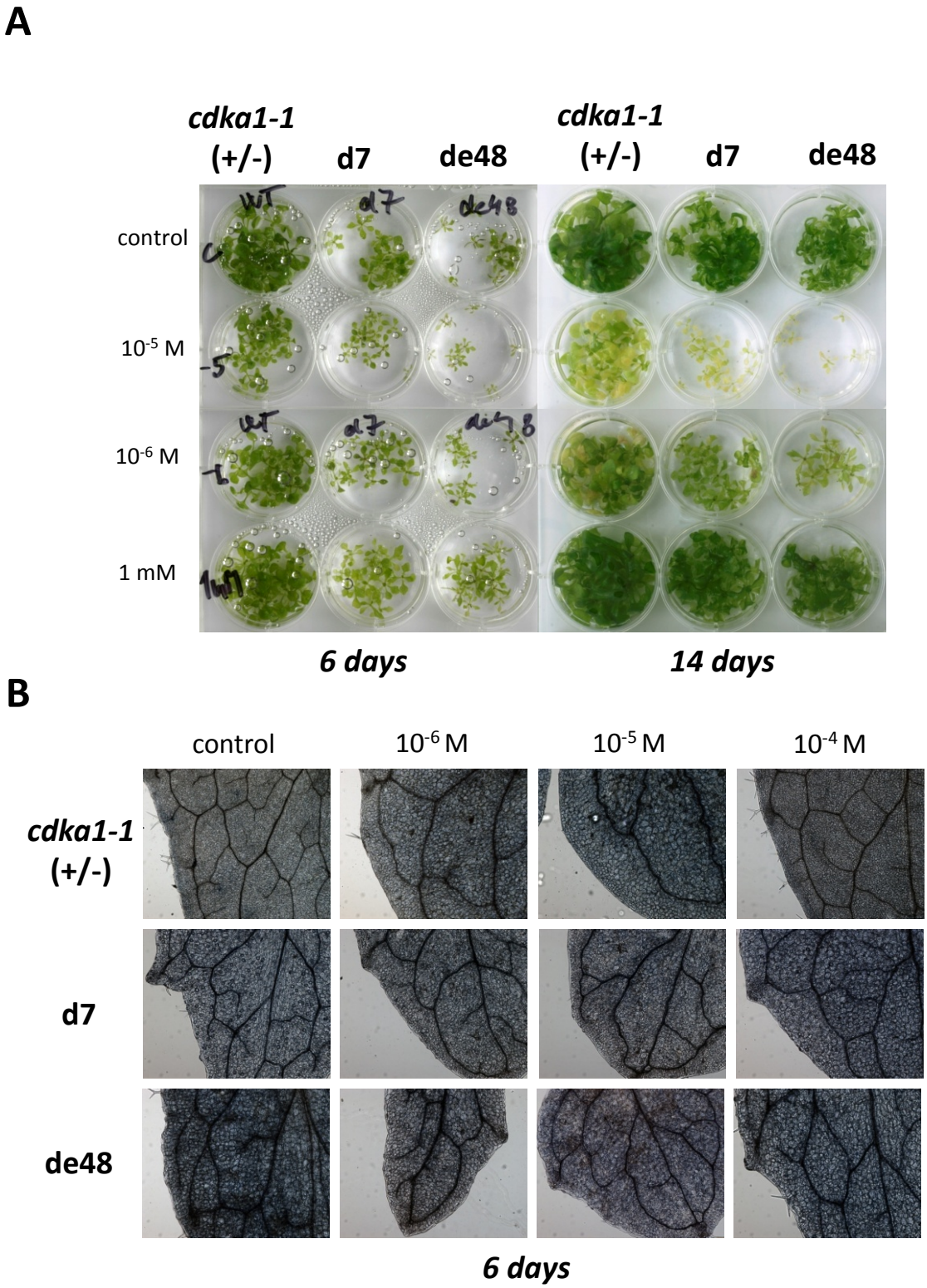

Figure 3.50: Long-term BLM treatment of CDKA1-1 phospho-mimicry mutants d7 and de48 with genotoxics. **A:** BLM (10^{-6} M, 10^{-5} M) and HU (1 mM) treatment in liquid medium; **B:** Detection of cell death with Evans blue staining after 6 days of BLM treatment (10^{-6} M, 10^{-5} M, 10^{-4} M).

observed no endoreduplicated cells in the mutants except for cotyledons where a little fraction of the cells had 8C DNA content. The roots of WT, d7 and de48 plants exhibit the same endoreduplication level present with a small fraction of 8C cells and a majority of 2C and 4C cells. To summarize we show that endoreduplication is strongly inhibited in aerial parts of d7 and de48 plants except for cotyledons, but was not affected in roots, suggesting that the inhibition of endoreduplication and the role of CDKA;1 activity in the control of endocycle is organ specific.

3.3.9.1. Cell death assay of CDKA;1 phospho-mimicry mutants

Despite of negative results suggesting that d7 and de48 plants do not have exhibit changes in root endoreduplication, we wanted to check if CDKA1;1 activity linked to endoreduplication process is modified in the mutants in response to BLM-induced cell death.

Ten DAG plants were transferred to medium containing BLM (10^{-5} M, 10^{-4} M) and after 24 hours the cell death was detected using propidium iodide (Figure 3.49A). A decreased CDKA;1 activity did not change the number of cells undergoing cell death upon BLM treatment suggesting that this activity is not necessary for cell death. As previously shown, the endoreduplication levels are decreased only in aerial parts of dephosphomutants but not in the roots. However, propidium iodide staining revealed that the reduction of CDKA;1 activity influences the overall architecture of root apical meristem. The cells in the root meristem were increased in volume and the regular organization of the cells around the QC was disrupted. As the total number of the cells forming root apical meristem decreased in dephosphomutants this was compensated by a cell size increased. According to the fact that area were dead cells are localized were comparable between WT and mutated plants, we hypothesized that for cell death induction the cell positioning within the meristem could be important for susceptibility of the cells to cell death.

We also analyzed the changes in root morphology of dephosphomutants exposed to 10^{-5} M BLM or 1 mM HU (Figure 3.49B). The results between all treatments were similar to those of WT suggesting that CDKA1;1 activity is not involved in such process.

In parallel, we checked the sensitivity of the mutants treated during 6 and 14 days with 10^{-6} M and 10^{-5} M BLM or 1 mM HU in liquid medium to see if some cell death is not induced in the leaves of d7 and de48 plants with suppressed endoreduplication. Plants treated with BLM were only growth retarded and there was no other sensitivity of the mutants to genotoxics. Only after 14 days of 10^{-5} M BLM treatment some chlorosis was apparent (Figure 3.50A). Simultaneously we used Evans blue staining to detect cell death in the leaves of dephosphomutants and WT plants which were transferred to liquid MS medium with BLM (10^{-6} M, 10^{-5} M, 10^{-4} M) for 6 days (Figure 3.50B). Observation under the microscope did not show any cell death induction. Taken these data together we

conclude that the inhibition of endoreduplication does not increase the sensitivity of leaf somatic cells to cell death induction and the cell death observed in the root tip does not require CDKA1 activity.

Discussion

Bleomycin induces PCD in plants

Using a tobacco BY-2 cells, we show that the DNA DSB inducer bleomycin is able to induce cell death in a time- and dose-dependent manner. This cell death was characterized by vacuolar disintegration, but was not accompanied with specific DNA fragmentation, typical for apoptotic-like PCD in plants (Jones 2000). BLM-induced cell death was accompanied by increased expression of genes encoding the negative regulator of cell death BI-1 and the stress response protein PR1b. *PR1b* genes are controlled by ERF (ethylene responsive factor) transcription factors via a GCC-box in their promoters and in tobacco ectopic expression of ERF leads to altered transcriptional regulation of *PR1b* enhancing plant tolerance to pathogen attack (Qin et al. 2006). Moreover, GGC-box containing promoters are required for systemic acquired resistance, one of the modes of plant defense mechanism against pathogens (Ward et al. 1991). In response to BLM, *PR1a* was not induced but we cannot exclude an early or late transient expression which we did not detect in our experimental conditions. BI-1 is a suppressor of cell death and its expression was observed during senescence or during different biotic and abiotic stresses (Yamada et al. 2009; Kawai-Yamada et al. 2004). Under stress conditions this gene is transiently upregulated suggesting its role during inhibition of precocious cell death. In Arabidopsis, BLM induced concomitant expression of PCD-marker genes implicated in ROS metabolism (*PRXcb*, *GST11*) or plant-pathogen interaction (*EDS1*, *PR1*) suggesting that in addition of DSB response, BLM may also induce a ROS response. This could not exclude that ROS can also induce DNA damage.

Taken these data together we suggest that in both BY-2 cells and Arabidopsis a general stress response is induced and the cell death observed in some fraction of the cells can be regarded as PCD. In Arabidopsis roots, BLM-induced PCD was restricted only to the root meristem where the cells maintain their division potential. Similar observations were recently published by two other research groups which used as a DSB inducers zeocin or γ irradiation (Fulcher et Sablowski 2009; Furukawa et al. 2010). It is still a question if PCD-marker genes whose expression we detected in Arabidopsis are specific to cell death induction or cell survival because in this plant material the ratio between the cell numbers undergoing cell death to the number of other cells which survive is relatively small. PCD-marker genes we used were previously described to be induced in *tso2* Arabidopsis mutants who exhibit elevated levels of cell death in parenchyma tissue (Wang et al. 2006) but plants are still alive, suggesting again that the expression of PCD-related genes might not be specific exclusively for dying cells. This hypothesis would be logic notably for genes implicated in ROS signaling since BLM through its complex mode of action is able to induce ROS in mammals (Chen et al. 2005). ROS cooperates with ethylene during various type of cell death including senescence or PCD after pathogen-attack (Asai et al. 2000) which shows an existing interconnection between ethylene-

dependent *PR1b* and ROS metabolism-related genes expression. We conclude that BLM induces stress response accompanied by ROS and probably also by ethylene accumulation. The implication of ROS and ethylene in response to chemical DSB inducers is supported by previous experiments with tomato suspension showing implication of these signaling molecules during CPT-induced cell death (de Jong et al. 2002).

We also tested replicative stress inducers HU and APC for their ability to induce cell death and our results demonstrate that in Arabidopsis root meristem the cell cycle arrest is activated but without subsequent cell death. We suggest that replicative stress induced by HU and APC does not promote cell death in the root meristem although UV-B induced replicative stress does (Furukawa et al. 2010). This difference might be caused by insufficient DNA damage induced by APC and HU after 1 day of treatment compared to UV-B which is in addition a potent inducer of ROS (discussed below).

BLM induces autophagic cell death

In BY-2 cells, BLM-induced PCD was accompanied by the formation of one big central vacuole which fulfilled a major part of the cell. Using BCECF-AM and Evans blue double staining we demonstrated that the vacuolar rupture occurs during BLM-induced PCD. The number of cells undergoing vacuole-mediated cell death was represented by living cells having disintegrated tonoplast (type II). The number of type II cells was proportional to BLM concentration and time of the treatment, suggesting the involvement of vacuole during BLM-induced cell death. In Arabidopsis we did not perform any cytological studies to describe the PCD character but previous studies revealed that zeocin, a chemical DSB inducer, is able to produce autophagic PCD suggested from the formation of autophagic structures in the cytoplasm. This cell death also lacked typical apoptotic characteristics as cell shrinkage, chromatin condensation and nuclear fragmentation (Fulcher et Sablowski. 2009). Therefore, we conclude that the treatment with chemical DSB inducers might lead to autophagic PCD in Dicots.

Vacuolar collapse was described as a key step of autophagic cell death resulting in a leakage of vacuolar hydrolases into the cytosol (Hatsugai et al. 2004, Kuroyanagi et al. 2005, van Doorn and Woltering, 2005). Using RT PCR analyses we have evaluated the transcriptional level of genes encoding several caspase-like proteases including VPEs and MCAs. VPEs represent a group of plant-specific cysteine proteases that exhibit caspase-1 activity and that are considered as main contributors of cell death execution during autophagic hypersensitive response (Hatsugai et al. 2004). Among the 4 *VPE* tobacco genes all of them were upregulated using high BLM concentration with the strongest response for *VPE1a*/*VPE1b*. Because of the close sequence homology between these two genes, we could not discriminate between the expressions of these genes. Nevertheless, *VPE1* induction was twice higher than the expression of *VPE2* and *VPE3* that could argue that Hatsugai et al. (2006) consider VPE1a/VPE1b as the most abundant VPEs during biotic stress induced by the tobacco

mosaic virus in tobacco plants. We propose that VPE1s are also implicated in abiotic stress response in tobacco. In Arabidopsis, *VPE α* was the only expressed *VPE* upon BLM treatment that was confirmed by kinetics study of the expression. *VPEα* and *VPE1a/b* are the genes which are highly up-regulated upon BLM treatment but the phylogenetic analyses of their encoded proteins did not show any important homology between the sequences (Figure 4.1.). Considering that upregulation of *VPE* under BLM treatment did not necessarily mean increased proteolytic activity, the transcriptional data indicated their presumable involvement in BLM-induced PCD. However we demonstrated that the *vpeα* mutant did not exhibit any difference in cell death induction under BLM exposure. We suggested that some functional redundancy may exist with some other VPE, especially with VPE γ with the function during stress response, could compensate the lack of *VPE α* gene (Knoshitia et al. 1999; Kuoryanagi et al. 2006). Therefore we performed direct detection of caspase activity using caspase-specific inhibitors in Arabidopsis and BY-2 cells.

In tomato, BY-2 and Arabidopsis animal caspase-specific inhibitors inhibit cell death cells under various biotic and abiotic stress conditions (Mlejnek et Procházka. 2004; Danon et al. 2004; de Jong et al. 2000) what is in contrast with our results. We did not observe any effect of caspase-1 and caspase-3 inhibitors on cell death induction triggered by BLM. This could be explained by methodological problem which is unlikely because caspase inhibitors we used were able to partially suppress cell death induced with 6-BAP, a cytokinin able to induce PCD involving caspase-like activities (Mlejnek et Procházka 2004). Other possibility is that BLM-induced cell death is independent of caspase-3 and caspase-1-like activities that would be in contrast to UV-C induced cell death observed by Danon et al. (2004) suggesting that cell death executioners may be involved. BLM and UV-C are also sensed by different DNA damage pathways, ATM and ATR that could explain such difference in cell death character (Garcia et al. 2003; Culligan et al. 2004).

Metacaspases are other caspase-like enzymes having a role during different types of plant PCD (He et al. 2008; Bozhkov et al. 2004; Suarez et al. 2004). We detected transient expression of *MCA8* and later expression of *MCA2*. Recently, *MCA2* was described as a negative regulator of pathogen-induced cell death and antagonist of MCA1 (Sanchez Coll, personal communication) suggesting a role in inhibition of precocious initiation of cell death execution similarly to BI-1. Arabidopsis *mca8* null mutants are less sensitive to cell death upon UV-C or H_2O_2 treatment in leaf parenchyma-derived protoplasts (He et al. 2008) but not upon zeocin treatment (Fulcher et Sablowski 2009) nor after a BLM treatment in the root meristem according to our results. This difference could be due to the induction of cell death by different cellular context or by different mode of action of used genotoxics which could be linked to ROS signaling (discussed below). In addition, the expression analyzes of caspase-like genes were performed on the whole Arabidopsis seedling. Therefore specific *MCA* or *VPE* expression could be linked to autophagy which does not lead immediately to cell death but only serves for the reconstitution of cell nutrients and structural components during stress

conditions. Longer exposure to stress could lead to the depletion of cellular reserves by autophagy and finally it could result in the chlorosis and senescence as we observed at the whole plant level after 2 weeks of BLM treatment.

Reports showing PCD induction after different genotoxics as UV-C or $CdSO_4$ exhibit more likely apoptotic-like PCD with typical DNA fragmentation (Danon et al. 1998; Fojtová et al. 2002; Kuthanova et al. 2008a). Though the DNA fragmentation was described even after non-specific cell disintegration (Kuthanova et al. 2008b), it is clear that different DNA-damage inducers cause PCD with different morphological features but it is not obvious why. Some comparative analysis of PCD morphotypes induced by different genotoxics is very difficult because the authors use different techniques for cell death characterization and there is not a clear methodological set of procedures for classification of plant PCD. In general, cell death with DNA fragmentation and the presence of caspase-like activities is automatically considered as apoptotic-like whereas (Danon et al. 1998, 2002) cell death characterized by vacuolization and formation of autophagic vesicles in the cytoplasm is considered as autophagic PCD (Fulcher et Sablowski 2009; Duan et al. 2010). It is important to point that in these experiments nobody can exclude an involvement of caspase-like activities, cell shrinkage or DNA fragmentation which might reimpose the classification of these cell death examples.

Signaling pathway leading to cell death initiation

The response to genotoxic treatments can be mediated by two closely related kinases ATM and ATR that are activated during the initial step of DNA damage recognition and are conserved among Eukaryotes (reviewed in Cools and de Veylder, 2009). ATM is primarily activated during DSB response, whereas ATR is activated after replicative stress such as hydroxyurea treatment or UV exposure (Garcia et al. 2003; Culligan et at. 2004). Recently, it was published that ATM is responsible for cell death in Arabidopsis upon zeocin treatment and ATM, ATR and SOG1 are needed for cell death induction upon UV-B and γ irradiation (Fulcher et Sablowski 2009; Furukawa et al. 2010). Our Arabidopsis data are in accordance with these results. Fulcher et Sablowski showed that zeocin and γ irradiation induces cell death which is blocked in *atm* mutant that was confirmed by the data of Furukawa et al. (2010) who has shown that late cell death response is ATR-dependent.

SOG1 is an integrator of cell death pathways from ATM and ATR and is capable to inhibit cell death upon UV-B and γ irradiation (Furukawa et al. 2010). Our results have shown that in *sog1-1* mutant as well as with caffeine treatment the BLM-induced cell death is strongly suppressed that complete previous results obtained with γ irradiation (Furukawa et al. 2010). SOG1 was also shown to be necessary for the expression of a variety of genes induced by γ irradiation (Yoshiyama et al. 2009). Our results have shown that *VPEα* expression is SOG1 and ATM-dependent. This result demonstrates that VPEα together with SOG1 and ATM are in one signaling pathway and reinforce our suggestion that VPEα could play a role during PCD execution although the caspase-1 inhibitors acting on VPEs

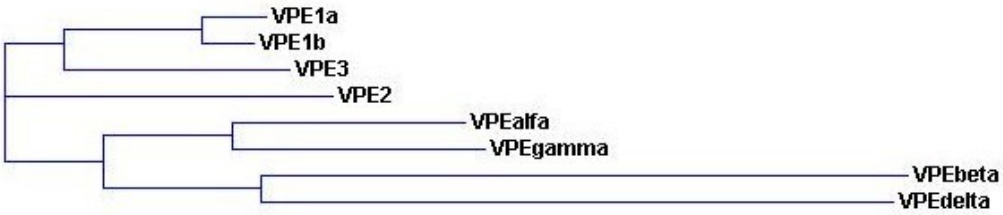

Figure 4.1: A phylogram of VPEs from *Nicotiana tabaccum* (VPE1a/b - VPE3) and *Arabidopsis thaliana* (VPEα – δ) protein sequences.

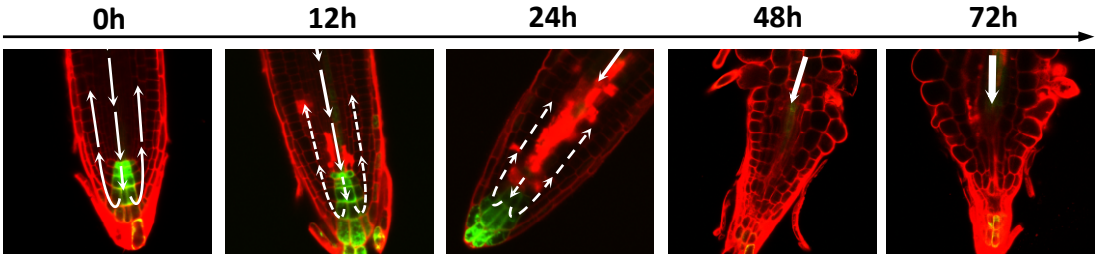

Figure 4.2: Auxin transport after 10^{-4} M BLM treatment in pro_{DR5}:GFP line. Cell death in the stele block the retrograde auxin transport leading to decrease of auxin concentration in cortex and endodermis and subsequent differentiation.

did not inhibited BLM-induced cell death in Arabidopsis, nor in BY-2 cells. It would be interesting to make a link between the execution phase of genotoxic-induced cell death and DNA damage pathways because it could give us valuable insight into plant PCD signaling. For example to directly describe a dependence of protease activity need for PCD execution on ATM, ATR or SOG1.

ATM mutation is available only in Arabidopsis, thus we inhibited DNA damage response by caffeine in tobacco cells. Caffeine, a specific inhibitor of ATM, is able to inhibit cell cycle arrest, G2/M or replication checkpoints induced by DNA damage alone or when mitotic cyclin B2 is upregulated, respectively in BY-2 cells (Weingartner et al. 2002, Carballo et al. 2006). But in the root of *Allium cepa* the caffeine fail to inhibit replicative-tress checkpoint mediated by ATR (Pelayo et al. 2001). In our BY-2 experiments caffeine inhibited BLM-induced cell death as well as the expression of PCD-related genes notably when high BLM concentration was used (10^{-4} M, but not after 10^{-5} M). Therefore we conclude that high concentration of BLM may induce cell death in an ATM-dependent manner that shows the link between DNA damage response and PCD signaling in BY-2 cells. In Arabidopsis, the extent of cell death was reduced by caffeine in the same extent as in *sog1-1* mutant which is downstream target of both ATM and ATR (Yoshiama et al. 2009) so we conclude that in Arabidopsis caffeine likely inhibit not only ATM but also ATR.

As already mentioned, a direct role of ATM kinase, but not ATR, was also demonstrated during cell death induction in Arabidopsis treated with zeocin (Fulcher et al. 2009). Also the UV-B irradiated roots the cell death was enhanced in *atm* mutant, Furukawa et al. (2010) proposed that in *atr* the SSB lesions are transformed to DSB and then sensed via ATM possibly leading to enhanced cell death. These findings indicate a possible conservation of ATM role in activating PCD pathways upon DNA damage in higher plants compared to ATR.

Interestingly, lower BLM concentration induced only minor decrease of cell viability, which was not affected by caffeine treatment in BY-2 cells. It indicates that in the exponentially growing culture a small fraction of cells probably dies independently of the ATM-signaling, similarly as observed in mammal cells after γ irradiation (Hirao et al. 2002). But what could be the other mechanism and the biological relevance of ATM-independent cell death under low DNA damage? We propose that there are several possibilities – first the implication of another signaling pathway mediated by other still unknown protein; second hypothesis comprises indirect effect of BLM via generation of ROS (Mahmutoglu et al. 1987). We suggest that production of hydroxyl radicals, superoxide and hydrogen peroxide which are positive regulators of plant cell death could mediate ATM-independent cell death during BLM response. The hypothesis of PCD triggered independently on DNA damage pathways through ROS accumulation is in accordance with the observations of Furukawa et al. (2010) who observed that γ irradiated roots of *atm/atr* double mutant still present some dead cells in the meristem.

Interesting is also to compare the cell death-inducing potential of different exogenous stressors leading to stalled replicative fork in plants. Why cell death is observed in root tips upon UV-B irradiation roots but not in response to HU or APC? This might be also explained by the possible implication of ROS which is directly and strongly generated by UV irradiation and not to such extent by HU or APC. ROS involvement in cell death induction could be investigated in WT and plants harboring modified sensitivity to ROS using ROS generating (UV-B) or non-generating (HU) genotoxics.

Is Wee1/Cdc25 mode of cell cycle regulation involved in BLM-induced cell death?

BLM treatment induced in WT BY-2 cells an increase of the cell fraction with duplicated DNA content (4C) suggesting the activation of the G2/M checkpoint. Similar results were observed in Arabidopsis cells which in addition underwent several rounds of endocycle. G2/M checkpoint was shown to be activated in an ATM-dependent manner and characterized by increased *Wee1* expression after γ irradiation or the treatment with BLM-type antibiotics zeocin (Carballo et al. 2006, De Schutter et al. 2007).

In Arabidopsis we tested a well known downstream effector of AMT/ATR pathway, the kinase WEE1 but the *wee1* mutant plants leading to checkpoint deregulation (De Schutter et al. 2007) did not affect BLM-induced cell death in the root meristem. This result is consistent with previous observations using zeocin (Fulcher et Sablowski 2009). *Wee1* mutants are hypersensitive to HU shown as a retarded root growth under replicative stress conditions (De Shutter et al. 2007) but the analyses of cell death in the root meristem after replicative stress treatment was still not tested. These results could give us an idea whether WEE1 kinase participate during cell death induction triggered after replicative stress checkpoint controlled by ATR kinase. Indeed, our results showed already that HU induced PCD in the meristematic cells in *atr* concluding that ATR is more important for DNA repair and cell survival. *WEE1* expression is induced by γ irradiation, zeocin in Arabidopsis (De Schutter et al. 2007) and we showed also that a tobacco *WEE1* homologue is induced upon BLM treatment in BY-2 cells. It was proposed that WEE1 do not have an essential role during normal cell cycle regulation but only during stress response when it targets downstream effector proteins such as CDKBs (not CDKA), plant specific G2/M cell cycle regulators (Dissmeyer et al. 2009). However the role of CDKB in DNA damage response remains to be elucidated.

WEE1 antagonists a kinase CDC25 that was identified in Arabidopsis but without regulatory N-terminal domain; however plants lacking or overexpressing *CDC25* gene have a normal phenotype suggesting that this gene does not play a critical role during plant cell cycle progression under non-stressed conditions (Dissmeyer et al. 2009). However, tobacco cells transformed with CDC25 homologue from Schizosacharomyces pombe have a shortened G2 phase and Arabidopsis plant overexpressing SpCDC25 can bypass checkpoint G2/M by positive regulation of CDKB1 activity

(Orchard et al. 2005). Our results demonstrate that SpCDC25 plants have PCD response to BLM comparable to WT, suggesting that SpCDC25 and hypothetically its plant targets (such as CDKB1) do not modulate cell death response.

How E2F can inhibit BLM-induced cell death?

In mammals, transcription factors E2F are implicated in cell proliferation, DNA repair and cell death by direct gene regulation or by protein-protein interactions (Figure 1.10) (reviewed in Iaquinta et Lees, 2007). The involvement of E2F factors in the DNA-damage response was recently also shown in plants (Roa et al. 2009). We demonstrated the effect of tobacco NtE2F overexpression on BLM induced cell death but also the ability of NtE2F to modulate cell cycle checkpoint regulation that was not shown till now. During our studies we used BY-2 cells in which NtE2F was overexpressed alone without its co-activator DP as with Arabidopsis E2Fs in other studies (de Veylder et al. 2002; Magyar et al. 2005). We suppose that the NtE2F was effective without DP, as it was already shown by Rossini et al. (2002) and Mariconti et al. (2002). We suggest that the levels of internal NtDP partner can be spontaneously adjusted by increased E2F protein as demonstrated by Magyar et al. (2005).

In synchronized BY-2 cells, at low BLM concentration (10^{-5}M) WT cells were the most susceptible for cell death when the BLM was added just before G2/M checkpoint but NtE2F overexpression bypassed the G2/M checkpoint that led to reduced mortality. This data indicates that G2/M checkpoint is crucial for cell death induction when low DNA damage occurs. On the other hand, G2/M checkpoint deregulation by NtE2F resulted in broken chromosomes and during cytokinesis formation of micronuclei, similarly as observed in Hesperidin-treated BY-2 cells with aberrant chromosome segregation (Kurihara et al. 2006). Interestingly, in synchronized culture we saw a high difference in cell death induction between WT and NtE2F cells but not when non synchronized cells were treated with low BLM concentration. This could be explained by the low cell number in G2 in non-synchronized culture compared to synchronize one.

Arabidopsis plants when exposed to low doses of BLM exhibited slower inhibition of cell division arrest. These data indicates that as in BY-2 cells, NtE2F ectopic expression in Arabidopsis modulates the activation of G2/M checkpoint however some more detailed analysis are needed to confirm this hypotheses. NtE2F plants also exhibit increased level of endoreduplication in roots that is in agreement with previous observations that E2Fa/Dap overexpression leads to cell division and enhanced endoreduplication (de Veylder et al. 2002). Similarly to BY-2, we detected deregulation of cell cycle checkpoint control in Arabidopsis plants because in BLM-treated inflorescence, we observed anaphase bridges in the cells of highly dividing tissue of pistils. On the other hand, NtE2F overexpression did not inhibit cell death in Arabidopsis root meristem as observed in BY-2 cells and similar results were obtained for E2Fb. One reason could be the heterologous expression of NtE2F although our previous results show that NtE2F could complement *e2fa* mutant during DNA damage

response (Julien Lang, personal communication). Another possibility how to explain why NtE2F or E2Fb did not have an inhibitory effect on BLM-induced cell death is the more complex response in multicellular Arabidopsis roots which could be influenced by developmental context. This discrepancy could be explained by using of E2F overexpressing Arabidopsis cell culture.

In response to high BLM concentration (10^{-4} M), entry into the mitosis was totally blocked in both WT and NtE2F BY-2 cells that indicates that DNA damage induced by BLM activated G2/M checkpoint and stopped cell cycle progression, probably due to too high DNA damage, as documented by accumulation of 4C nuclei (G2 phase) when NtE2F cells were treated during G2 phase. Moreover, the activation of G2/M checkpoint even in NtE2F cells is preferred because of the absence of micronuclei formation in BLM-treated non-synchronized culture. However, even under this higher BLM concentration, NtE2F overexpression significantly reduced mortality. It means that under higher BLM concentration, the cell death pathways should to be inhibited via NtE2F by another mechanism independent on G2/M cell cycle checkpoint. The expression analysis suggested that it might be mediated through downregulation of *VPEs* or other PCD-related genes, possibly causing the inhibition of the vacuolar rupture.

The considerable decrease in genome integrity in NtE2F cells under lower BLM concentration did not result in increased but decreased cell death. This may be connected with different living strategies of animals and plants and different needs to cope with cell cycle deregulation after DNA damage that could lead to cancer development in animals but not in plants (Hefner et al. 2006, Inzé et de Veylder 2006). Tobacco plants could also tolerate much higher level of DNA damage because Nicotiana tabacum species is a natural allopolyploid where genome duplication probably occurred during evolution (Mured et al. 2002) and this could buffer DNA mutation accumulated in the genome during the lifetime.

Sedentary plants, with the inherently flexible development, can tolerate much higher doses of DNA damage in general and therefore they may not need such strict regulation of DNA repair/cell death decision through E2F as known in animals. Microarray data searching for E2Fa target genes in Arabidopsis also did not show the direct control of E2Fa on known cell death regulators (Naouar et al. 2009), although PCD genes could be under the control of E2Fb for which the wide gene target analyses was not performed. Furthermore, the plant homologues of E2F-targeted pro-apoptotic genes such as p53, BH3-only Bcl-2 proteins or several caspases were not identified in plants. We propose that E2F-mediated control of cell death induction was suppressed in plants throughout the evolution.

Endoreduplication is a consequence of cell death in the root meristem

In Arabidopsis we analyzed PCD induction in the root tips similarly as shown in Fulcher et Sablowski (2009) and recently Furukawa et al. (2010) upon zeocin treatment, UVB and γ irradiation,

respectively. In a short term treatment we observed time- and dose- dependent induction of cell death in the meristem, mainly in the initial cells and in the stele. Second important feature of the plant exposure to BLM was increased endoreduplication levels after longer treatment (3d). Previous observation showed that the endocycle as well as G2 checkpoint genes are induced in response to zeocin in Arabidopsis (Ramirez-Parra et al. 2007).

When the roots were exposed to BLM for longer period, the cells in the cortex and epidermis started to increase in volume and the epidermal cells differentiated that is in complete opposite response of the cells localized in the central root zone (in the stele). These cells underwent rapid cell death and at higher concentrations of BLM the cells of this region were all almost death. Thus we can distinguish between two different cellular responses within one root which was delimited by endodermis with a barrier of Casparian strip.

We compared the effect of cell death induction by different genotoxics in wide range of genetic background such as plants lacking the genes implicated in DNA damage response, cell cycle regulation or execution of cell death. As mentioned, long genotoxics treatment led to the arrest of root growth as well as differentiation coupled with endoreduplication. We showed that similarly to BLM this phenotype is induced by CPT, another DSB inducer. When the roots were treated only with HU we observed just arrest of growth which was not coupled with increased differentiation and endoreduplication. Similar phenotype was already observed in WT plants after γ irradiation (Ricaud et al. 2007; Culligan et al. 2004), in *atr* mutant after replicative stress induced by HU treatment but not in WT (Culligan et al. 2004). Thus this differentiation and endoreduplication response is specific to WT plants where cell death was induced in the root meristem by DSB damage and not by HU-mediated replicative stress.

Depending on genetic background the endoreduplication and differentiation of the root cells was observed even during replicative stress suggesting that under certain conditions the cell death could occur after replicative stress. In our experiments HU induced PCD in *atr* mutant plants which exhibited the same response as WT or *atr* roots after DSB induction. Also the results of others showed that replicative stress inducers such as APC, HU induced differentiation of the root till the root tip (Culligan et al. 2004).

According to Fulcher and Sablowski (2009) zeocin-induced cell death is completely inhibited in plants lacking functional ATM kinase. During our long-term BLM treatment experiments we remarked that *atm* mutant exhibits the same modification in root morphology and increased endoreduplication as WT. We suggested that in *atm* cell death is not completely blocked but only retarded. If not, upon BLM treatment the *atm* plants would exhibit the same root morphology as WT roots exposed to HU. Our supposition was recently confirmed by Furukawa et al. (2010) who showed that the cell death in γ irradiated *atm* roots is only delayed compared to the WT. These data confirms

that cell death differentiation and endoreduplication is just a consequence of PCD in the root meristem and it is not an active and direct response to genotoxics mediated via ATM pathway.

The mechanism of cell death-induced root endoreduplication

Our analysis with pro^{WOX5}:GFP and pro^{DR5}:GFP lines showed a complete reorganization of the meristem after long exposure of the roots to BLM. Auxin concentration rose in the stele whereas the auxin maxima in the QC and columella disappeared at high BLM concentration. Similar changes in auxin redistribution were observed Arabidopsis roots after γ irradiation but not in such high extent probably due to the low level of DNA damage or too short period of observation after the irradiation (Ricaud et al. 2007). Auxin transport is mediated by PIN proteins which are auxin efflux carriers and by their specific localization in the plasma membrane they affect the directionality of auxin fluxes within the plant tissue (Wiśniewska et al. 2006). In roots, the auxin is transported acropetally through the stellar tissue to the root cap columella where the direction of the phytohormone fluxes is reversed to basipetal and diverted to the cortical and epidermal cells (Marchant et al. 1999). Thus we suggest that the auxin accumulation in the distal stellar tissue is a consequence of cell death in the stele close to the QC which enables directed auxin transport in the cortex and epidermis resulting in decreased auxin concentration in these tissues (Figure 4.2).

WOX5 is a gene specifically expressed in the QC. Previous results indicate that QC is more resistant to cell death upon zeocin treatment (Fulcher et Sablowski 2009). Longer genotoxic exposure induced disappearance of *WOX5* expression in the QC. When massive cell death occurred in the stellar tissue, W*OX5* expression was apparent in the endodermis, cortex and finally was specific for differentiated and swelled epidermal cells that could signify the potency to induce cell division. Maintenance or differentiation of Arabidopsis root meristem is controlled by local auxin levels which are determined by auxin biosynthesis and intercellular transport. Auxin response factors ARF10 and ARF16 repress the *WOX5* transcription and restrict it to the quiescent center (Ding and Friml 2010).The disturbance of auxin fluxes caused by cell death in the meristem could abolish the repression of WOX5 in the surface root layers. But supplementary experiments with other markers involved in the maintenance of the root apical meristem are required to propose some significance of WOX5 redistribution and a model which could explain why WOX5 is not repressed in surface root layers.

Stem cells in the apex of the meristem generate daughter cells which undergo several additional divisions and the proximal meristem and differentiate at the distal transition zone (TZ) which encompasses the boundary between dividing and differentiating cells. For meristem maintenance and root growth the rate of cell division must be equal to differentiation. This balance is controlled by the crosstalk of auxin and cytokinin signaling (Dello Ioio et al. 2008a). A simplified model is that auxins and cytokinins are antagonists – auxin promotes cell division whereas cytokinins

promote cell differentiation (Dello Ioio, Scaglia Linhares et al. 2008b). Recently the regulation of the critical role in the regulation of balance between cell division and differentiation or endoreduplication was demonstrated by Ishida et al. (2010). Taking into account this model we conclude that in Arabidopsis root, cell differentiation after longer BLM treatment occurred due to the lowered concentration of auxin in the epidermal and cortex cells, thus as a consequence of PCD which blocks the basipetal auxin transport. Enhanced root differentiation was observed also after disruption of TRH1 carrier which makes an important part in auxin transport through the root cap and thus mimics the effect of cell death in the root meristem on auxin transport in genotoxics-independent way (Vincente-Agullo et al. 2004).

This model of control of endoreduplication by auxin in Arabidopsis root could explain the behavior of Arabidopsis and BY-2 cell suspension cultures which never exhibited increased endoreduplication levels. Both cell cultures are auxin-dependent and this phytohormone was added into the standard cultivation medium. We suppose that the induced endoreduplication by BLM in Arabidopsis is in cell suspensions blocked by auxin which is always present in culture medium. In agreement with this hypothesis is the endoreduplication induction by auxin withdrawal from the culture medium known as auxin starvation (Quélo et al. 2002).

Are B-type CDKs cell death regulators?

In conclusion, what could be the pathway on which we should focus to reveal the control of cell death upon DNA damage in plants? In tobacco BY-2 cells, it was shown that G2/M transition is linked to increased CDKB1 protein levels and activity (Sorrel et al. 2001). The overexpression of a dominant-negative mutated form of CDKB1 increased the 4C fraction, supposing the positive regulation of G2/M checkpoint by CDKB1 (Porceddu et al. 2001). Also, the overexpression of a core cell cycle regulator CDC25 from Schyzosacharomyces pombe increased the level of CDKB1 and thus bypassed the G2/M checkpoint activated by inhibition of the cytokinin signaling (Orchard et al. 2005). B-type CDKs help to protect CDKA/CYC complexes from the inhibition through phosphorylation of CDK inhibitors ICK/KRP members, triggering their destruction (reviewed in Inzé et de Veylder, 2006). As the expression level and activity of the mitotic CDKB1 is induced by both E2Fa and E2Fb in tobacco (Boudolf et al. 2004, Magyar et al. 2005; Sozzani et al. 2006), we suggest that at low BLM concentration (10^{-5} M) overexpression of NtE2F could suppress cell death by inhibited the G2/M checkpoint probably via increased CDKB activity. It is further supported by the fact, that E2F most efficiently reduced cell death induced by BLM treatment in G2 phase in BY-2 cells (before G2/M checkpoint).

Also other observations prompt that CDKBs could have a specific role during plant cell cycle checkpoint and PCD response control. It was demonstrated that in Arabidopsis roots the exposure to zeocin induced decreased *CDKB2;1* expression (Adashi and Umeda, personal communication).

Dissmeyer et al. (2009) demonstrated that WEE1 kinase does not target CDKA kinase but probably other proteins and one of the hot candidates are plant-specific CDKBs. We showed that CDKA1;1 controls the endoreduplication only in the green parts of Arabidopsis suggested from flow cytometry analyses. Thus in the roots it could be interesting to analyze if CDKB do not play a role during the regulation of this process. Moreover, suppressed CDK1;1 activity does not modify cell death induction in root meristem that also prompt us to test that it could be CDKBs. Taken together, propose that this plant specific CDK could be a unique plant-specific regulator of the response leading to cell division, differentiation or cell death.

General conclusion

The main objective of this work was to characterize the DNA damage response leading to cell death in two models; BY-2 cells as unicellular model and Arabidopsis as a plant model. We have particularly investigated the role of E2F transcriptional factor during BLM-induced cell death. We suggest that the final cellular response to BLM treatment is the interplay between two effects of this drug which depends on used BLM concentration and time of the treatment. As a general conclusion we present two simplified models, one for BY-2 (Figure 4.3) and a second for Arabidopsis (Figure 4.4). However, lot of supplementary experiments is needed to be done to confirm our hypothetical regulation based on our experiments as well of the experiments of others which were published in recent years.

When BY-2 cells are exposed to BLM, there are two effects of this genotoxic. First, when low DNA damage is sensed by ATM kinase activating a kinase WEE1 which could target and inhibit G2/M plant specific B-type CDKs leading to the arrest of the cell cycle at G2/M boundary that allows DNA repair (Figure 4.3). ATM-independent PCD induction might be mediated by ROS which are induced during cell stress response. When BY-2 cells are exposed to high BLM concentration, PCD induction occurs according to increased DSBs generated directly by BLM or indirectly by ROS where induced SSBs are converted to DSBs at the stalled replication forks. Such damage is mainly processed through ATM pathway which might involve VPEs, however we did not proved direct implication of VPEs during the execution of BLM-induced cell death. However, we have no answer concerning the involvement of the kinase ATR in BY-2 system because the loss of ATM function is not available in BY-2.

E2F transcriptional factors may control the activity of CDKBs and thus mediate the crosstalk between G1/S and G2/M checkpoints. In BY-2, we suggest that NtE2F can inhibit BLM-induced cell death by several pathways: i) by downregulation of VPEs by unknown mechanism, ii) by controlling B-type CDKs which could in this case negatively control PCD via inhibitory phosphorylation of positive regulators or executors of PCD at G2/M transition and iii) by positive regulation of DNA repair pathway at G1/S (Lang et al., in preparation). We note that pathways i) and ii) could partially overlap.

In Arabidopsis, the model of regulation proposed for BY-2 may be partly redundant but in addition ATR kinase and SOG1 factor are integrated in the response. We suggest that similarly to BY-2 BLM induces DSB and ROS. The cell cycle is arrested via ATM, ATR and WEE1 pathways which might target B-type CDKs. ATM-dependent cell death control pro death VPEs which are likely

positive regulators of cell death. ATM-independent pathway is governed by ROS, positive regulator of cell death which is still detected in the *atm/atr* double mutants after γ irradiation (Furukawa et al. 2010). ROS could also generate DNA SSB leading to replicative stress which activates ATR kinase and concomitant arrest of cell cycle mediated by WEE1. Accumulation of unrepaired SSBs induced by ROS, will be again converted into DSBs at the stalled replication forks which can induce a secondary activation of ATM that could amplify the cell death induction. It was shown that WEE1 does not phosphorylate CDKA1 (Dissmeyer et al. 2009), so a possible role of CDKBs is proposed.

We demonstrated that the root endoreduplication is not under the direct control of DNA damage pathway but we hypothesize that it could be regulated by B-type CDKs and auxin and cytokinin crosstalk. We suggest that this cell cycle regulator could play an important role in the control of G2/M checkpoint where decision to cell cycle progression, endoreduplication or cell death activation takes place. Thus it would be interesting to test the role B-type CDKs in DNA damage response.

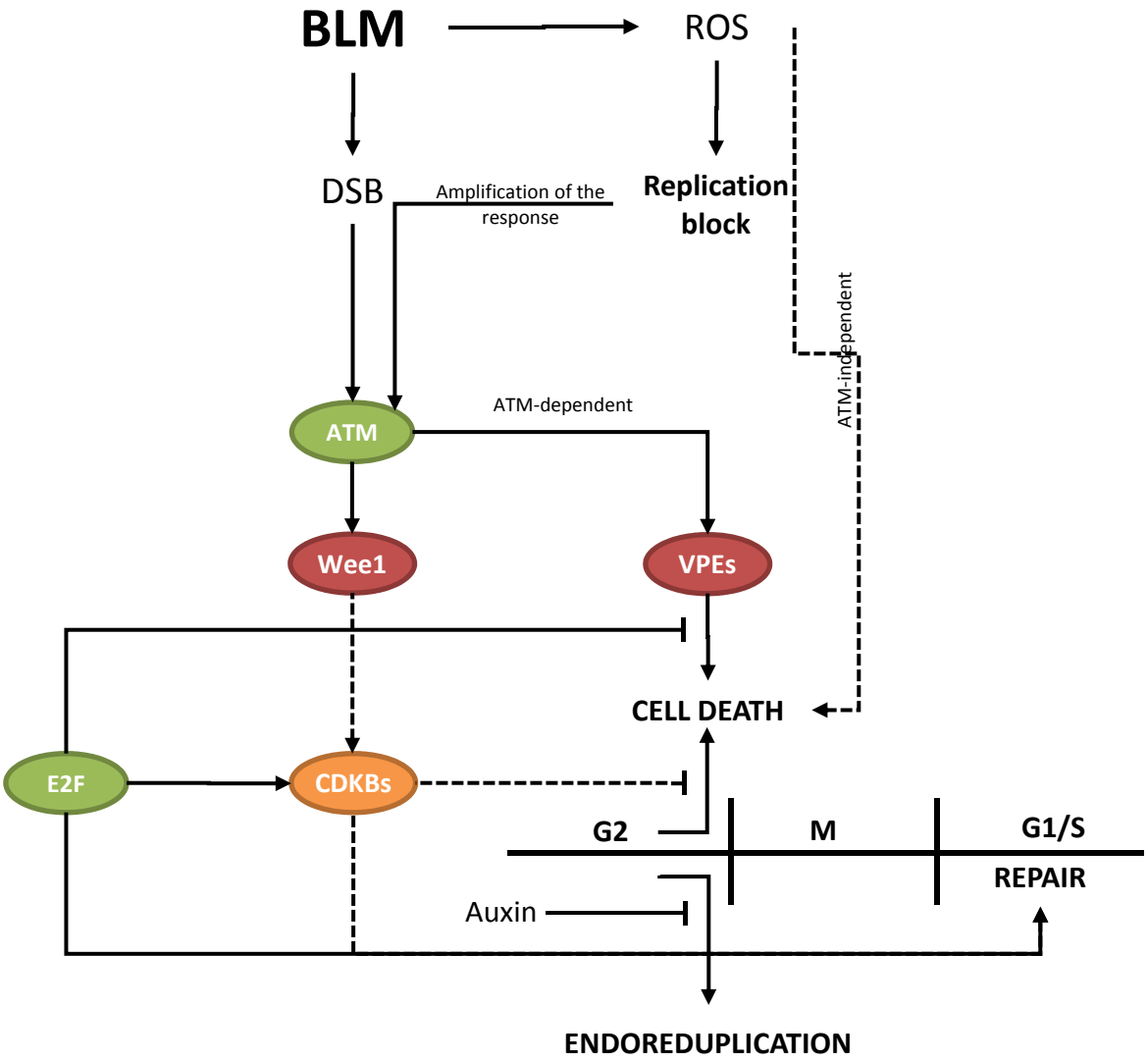

Figure 4.3: Hypothetical model of BLM-induced cell death regulation by two independent pathways ATM-dependent and ATM-independent. in BY-2. Full lines represent experimentally proven interactions; dashed lines denote hypothetical situations.

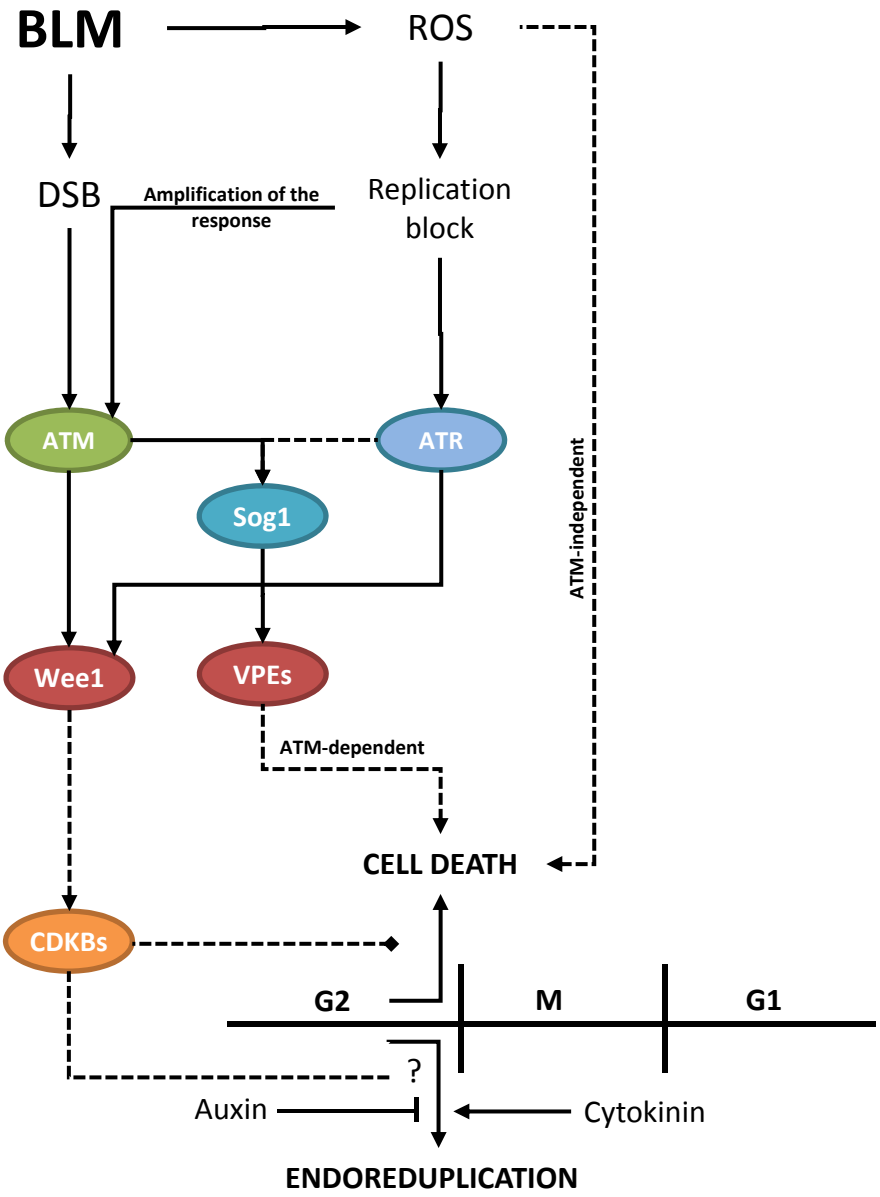

Figure 4.4: Hypothetical model of BLM-induced cell death regulation by two independent pathways ATM-dependent and ATM-independent in Arabidopsis. Full lines represent experimentally proven interactions; dashed lines denote hypothetical situations.

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Abstract

In multicellular organisms, programmed cell death (PCD) is an essential mechanism during development, morphogenesis and during the interaction with the environment. The regulation of PCD is highly regulated and conserved throughout the evolution. Animal apoptosis is the best described PCD type which is characterized by the condensation of cytoplasm, specific DNA fragmentation and the formation of apoptotic bodies which are finally engulfed by neighboring cells. Due to the structural specificities of plant cells, plant PCD exhibits rather autophagic character.

In mammals, DSBs-induced PCD is mainly governed by ATM kinase. This response also involves downstream ATM effectors like p53 and E2F factors. P53 stabilization leads to the cell cycle arrest in G1/S whereas E2F can promote apoptosis by activating PCD-related genes including caspases. PCD could be induced also by E2F ectopic expression. In spite of a conservation of PCD signaling among Eukaryotes, number of animal PCD regulators was not identified in plants (caspases, p53), thus in plants the PCD response induced by DSBs is still poorly understood. On the contrary, E2F transcriptional factor is conserved between animals and plants but its role during plant PCD was not evaluated till now.

The main goal of my thesis was to characterize the PCD in response to DNA damage induced by DBSs in two models: unicellular non differentiated system of tobacco BY-2 cell suspension and Arabidopsis roots, a well defined multicellular structure. BY-2 cell treatment with a DSB inducer bleomycin led to rapid cell cycle arrest in G2/M, transcriptional activation of ROS- and PCD-related genes, disintegration of vacuole and subsequent autophagic PCD. We have demonstrated that the pro-death E2F function observed in animals is lost in plants. In addition E2F deregulation led to PCD inhibition or to increased genomic instability through a bypass of the G2/M checkpoint depending on dose of BLM. In Arabidopsis, DBSs induced not only PCD but also endoreduplication specific for surviving tissues. According to our results, we propose a model of DSB response which strongly depends on genotoxic concentration. We suggest a distinct role of vacuolar processing enzymes (VPEs) and the factor SOG1 which are plant specific. Whereas PCD seems to be a direct effect of DSB sensed through ATM signaling, endoreduplication is only indirect developmental consequence of cell death in Arabidopsis meristem. Taking our results together we demonstrate plant specificities of DNA damage induced PCD probably coming from the different living strategies between animals and plants.

Abstrakt

Programovaná buněčná smrt (PCD) je důležitý mechanizmus, se kterým se u mnohobuněčných živočichů setkáváme nejen během vývoje a morfogeneze, ale i během jejich interakce s prostředím. PCD je přesně regulovaný proces, který je navíc evolučně velmi konzervovaný. Jedním z nejlépe popsaných typů PCD je apoptóza u živočichů, která se vyznačuje smrštěním cytoplasmy, specifickým štěpením DNA a tvorbou apoptotických tělísek, které jsou nakonec pohlceny okolními buňkami. U rostlin se však díky specifické stavbě rostlinné buňky setkáváme s PBS autofagyckého typu.

PCD může být spuštěna během nepříznivých podmínek okolního prostředí, zejména během přetrvávajícího poškození DNA v podobě dvouřetězcových zlomů (DSBs) molekuly DNA. U savců spuštění PCD navozené DSBs molekuly DNA vyžaduje aktivitu kinázy ATM a dalších proteinů jako například E2F a p53. Stabilizace p53 má za následek zastavení buněčného cyklu v kontrolním bodu G1/S zatímco E2F transkripční faktor spustí apoptózu aktivací PCD specifických genů, například kaspáz. Bylo popsáno, že E2F může navodit buněčnou smrt i během pouhé overexprese. Navzdory vysoké konzervovanosti signálních drah PCD u eukaryot, některé regulátory PCD (např. kaspázy nebo p53) nebyly dosud u rostlin identifikovány, proto toho o signalizaci rostlinné PCD moc nevíme. Vyjímkou je E2F transkripční faktor, který byl nalezen jak u rostlin tak živočichů ale jeho role během rostlinné PCD zatím nebyla popsána.

Hlavním cílem mé dizertační práce bylo vysvětlit, jakým způsobem je kontrolována PCD navozena DSB poškozením DNA ve dvou různých modelových organismech. Prvním modelem byla suspenzní kultura nediferencovaných buněk tabáku BY-2 a druhým kořen Arabidopsis, který je naopak dobře prostudovaným souborem diferencovaných buněk. Navození DSB v buňkách BY-2 pomocí genotoxika bleomycinu (BLM) vedlo k zastavení buněčného dělení, aktivaci kontrolního bodu G2/M, aktivaci genů spojených s ROS i PCD a nakonec k prasknutí vakuoly, což je typický znak autofagické PCD. Dále jsme ukázali, že pozitivní regulace PCD pomocí E2F pozorovaná u živočichů u rostlin s vysokou pravděpodobností neexistuje. Naopak, v závislosti na použité koncentraci BLM a tudíž rozsahu poškození DNA overexprese E2F buď inhibovala buněčnou smrt anebo zvyšovala tzv. nestabilitu genomu díky narušení kontrolního bodu G2/M. Po ovlivnění rostlin Arabidopsis BLM jsme kromě indukce PCD detekovali značný nárůst buněk s endoreduplikovanou DNA. Na základě našich výsledků jsme navrhli model buněčné odpovědi na DSB, která značně závisí na koncentraci aplikovaných genotoxik. Předpokládáme důležitou úlohu pro rostliny specifických proteinů - tzv. Vacuolar processing enzymes a také transkripčního faktoru SOG1. Dále jsme ukázali, že zatímco PCD je přímým důsledkem poškození DNA, endoreduplikace je pouze důsledkem buněčné smrti v kořenovém meristému Arabidopsis. Poškození DNA vede k rozdílným reakcím rostlinných a živočišných buněk což je zřejmě způsobeno jejich rozdílnými životními strategiemi.

Résumé

Chez les organismes multicellulaires, la mort cellulaire programmée (MCP) est un mécanisme essentiel au cours du développement, la morphogenèse ou lors de la réponse aux stress. C'est un processus hautement régulé et conservé au cours de l'évolution. Chez les animaux, l'apoptose représente le type de MCP la mieux décrite, et se caractérise par une condensation du cytoplasme, une fragmentation spécifique de l´ADN génomique et la formation de corps apoptotiques qui sont finalement phagocytés par les cellules avoisinantes. En raison des spécificités structurelles de cellules végétales, la MCP chez les plantes est de type plutôt autophagique.

La MCP est induite dans diverses conditions environnementales défavorables notamment en réponse aux dommages de l´ADN telle que les cassures de l´ADN Double Brin (CDB). Chez les mammifères, la MCP induite par les CDBs est essentiellement sous le contrôle de la kinase ATM. Cette réponse met en jeu des effecteurs en aval d´ATM comme les facteurs E2F ou p53. La stabilisation du facteur p53 conduit à l´arrêt du cycle cellulaire en G1/S alors qu' E2F peut favoriser l´apoptose en activant des gènes régulateurs de la MCP, dont les caspases. L´apoptose peut être également induite par l´expression ectopique du facteur E2F.

Chez les plantes, la réponse aux CDBs conduisant à la mort cellulaire est encore mal comprise. Malgré une conservation de la régulation de la MCP chez les Eukaryotes, certains régulateurs de la MCP animale n'ont pas été identifiés chez les plantes (les caspases, p53). Au contraire, le facteur de transcription E2F est conservé entre les animaux et les plantes mais son rôle dans la régulation de la MCP n´est pas encore établie.

L´objectif principal de mon projet de thèse était de caractériser la MCP en réponse aux dommages de l´ADN induit par CDBs à travers 2 modèles différents : un système unicellulaire non différencié avec la suspension BY-2 du tabac et les racines d´Arabidopsis, une structure multicellulaire bien définie. Un traitement des cellules BY-2 avec un inducteur des CDBs la bléomycine conduit à l´arrêt du cycle cellulaire en G2/M, l´activation transcriptionnelle de gènes liés aux ROS et la MCP, la désintégration de la vacuoles et enfin la MCP autophagique. Nous avons démontré que la fonction pro-apoptotique d´E2F observée chez les animaux a été perdue chez les végétaux au cours de l´évolution. En outre, en fonction de la dose de la BLM, une dérégulation d´E2F conduit à l´inhibition de la MCP ou à l´augmentation de l´instabilité génomique liée à un défaut de contrôle du point de contrôle G2/M. Chez Arabidopsis, les CDBs n'induisent pas seulement de la MCP mais aussi de l´endoréduplication spécifique dans les cellules qui ont survécues. Selon nos résultats, nous proposons un modèle de la réponse aux CDBs qui dépend fortement de la concentration des génotoxiques. Nous proposons également un rôle particulière pour les Vacuolar processing enzymes (VPEs) et le facteur SOG1 qui sont spécifiques aux végétaux. La MCP induite par les génotoxiques semble être un effet directe des CDBs à travers la signalisation ATM. Par contre l´endoréduplication observée n´est qu´une conséquence indirecte de la MCP dans le méristème liée au développement. Nous avons mis en évidence des spécificités végétales de la MCP liée aux dommage à l´ADN qui pourraient être la conséquence des différentes stratégies de vie entre les plantes et les animaux.

Appendix

Primers used for sqPCR

gene	Accession N°	Forward 5´	Reverse 3´
Actin2	At3g18780	AGACATCATGGTGTCATGGTTG	TCAAGACGGAGGATGGCATGAG
PRXcb	At3g49120	AGTTAAGGTCGGACCCTCGT	CTCTCCTTCCCAAAGGAACC
GST11	At1g02920	CTTGTTTGGGAGCAAGTC	GAAGGCCTAGAAGTGATG
PR1	At2g14610	CTTGTAGGTGCTCTTGTTCTTCCC	TGCCTCTTAGTTGTTCTGCGTAGC
EDS1	At3g48090	CGCTATCACAAGGAAGAAGCAGG	CACACAACGAGGCTCAAGGTAAAC
MCA1	At1g02170	CGTATCCCGACCAAGCAAAAC	CATAGCCATCAACTTCATCACCG
MCA2	At4g25110	TGTTGTTGCTGGTGGACTGCTC	TTGGGGAAAGGAAAAGGGC
MCA3	At5g64240	TACTTAGACAACACCAACCGCAAC	AACTGCTCTCTTCTTCCCAAACG
MCA4	At1g79340	CGAAAAAGGCGGTGCTTATTG	TGAGTAGAGGATTCATCGGTGTCG
MCA5	At1g79330	CGGAGACTGGAGAAGACGATGATAC	GATGAGACCACCACTGTGACAAGAG
MCA6	At1g79320	CGGTAAGAACATCAGACAGGCG	CGGAAGGAACAATACACTCATCG
MCA7	At1g79310	GGCAAAGAGAGCGTTGTTGATAGG	GGTTGAGTGTAAGATTCGTCGGTG
MCA8	At1g16420	GGAATCAACTACCCAGGAACCG	CCACCACTATGACAAGAGTCCGAG
MCA9	At5g04200	GCAACTACCCTAACACAAGGAACG	TTGGCACCTGTCGGCTTTAC
VPEα	At2G25940	CGAAGAACGAGGAGAATCCAAGAC	GACCGCTATCTACAACTTTCCCAC
VPEβ	At1G62710	CGGAAGATGGGTCAAGGAAGAAG	ATCGTCAACCAAAGGCAAACC
VPEγ	At4G32940	GGAACAAACCCTGCCAATGAC	GAACCTTCTGGTGCTTTTCGG
VPEδ	At3G20210	TTCTGGCTTGGTCAATCCGC	GCTGTCCTGTTGTTCTTGTGGAAG
WEE1	AJ715532	AGAGCCAGCCGATTGAAGAGGGT	GGCCCTGATTCTGGCAGCGG

Primers used for qPCR

gene	Accession N°	Forward 5´	Reverse 3´
VPE 1a/b	AB075947/8	AGTGTCCGCCCTGCTGGTCA	ATGCCTCCACCATCTGCGCC
VPE 2	AB075949	CTGTCAGTTGTCGCCGCCGT	TCCAGCAACCAACACAGCCCA
VPE 3	AB075950	GCCGCCGACGGACGTAATGT	CGGCAAGAAGGACGGCCCAT
PR1a	X06361	TCCCACTCTTGCCGTGCCCA	AGGCTGCTACCTGGTCGTCCC
PR1b	X66942	GCAGCATTCCGGTGGCCCTT	CTTTACTGCTCCCGCCGCGT
BI-1	AF390556	TCGGCGTCGTCTCGCAATCG	AGCTCCAGCAGCCGAAGCAAC
MCAII	EU869285	GCTGTATCAACGATGTTAAGC	GTCCCATGTCCACTGTAATG
18S	NC006581	GGTCTTCAACGAGGAATTCC	CGACTTCTCCTTCCTCTCAA
Genotyping		LP	RP
mca2	SALK_084393	TCCAAACTTCTGCAATGAAGG	ACGGTACCACTATGACAAGCG
mca8	RATM13-5036	ACCGTTTACCATCATCACTCG	AGGTGGCGAAGAAAGCTTTAG
e2fb	SALK_131062	TCCAAGATTTTGTTGTCGCTC	TCTTCTCACTGGGGAAACATG
LBb1.3	T-DNA specific	ATTTTGCCGATTTCGGAAC	
Amplification of the full CDS of mutated gene		Forward 5´	Reverse 3´
mca2	At4g25110	GTTGTTGC TGGTGGACTG CTC	GAGAAGGGCTTCTCATATACAGC
mca8	At1g16420	GTTACGTGGCTGTGTCAACGAC	GTAGCATATAAATGGTTTATCAAC
e2fb	At5g22220	TGTCTGAAGAAGTACCTCAAC	AGCTACCTGTAGGTGATCTC

NtE2F amplification with etag	
Forward 5´	ATGGGCGCTCCAGTGCCTTATCCAGATCCACTTGAACCTCGCATGCGCAGCCACATCATCA
Reverse 3´	TCAGCTCCCAGTTGTATTTGCAGAAGGCAGTTCAGTGGTAC
cloning of etag-NtE2F to pENTR201	
Forward 5´	GGGGACAAGTTTGTACAAAAAAGCAGGCTCTATGGGCGCTCCAGTGCC
Reverse 3´	GGGGACCACTTTGTACAAGAAAGCTGGGTGTTCAGCTCCCAGTTGTATTTGC
E2Fb amplification with etag	
Forward 5´	ATGGGCGCTCCAGTGCCTTATCCAGATCCACTTGAACCTCGCATGTCTGAAGAAGTACCTCAAC
Reverse 3´	TCAGCTACCTGTAGGTGATCTCGTAGCAGTGGATTCTTCTGGTGTTGGGC
cloning of etag-E2Fb to pENTR201	
Forward 5´	GGGGACAAGTTTGTACAAAAAAGCAGGCTCTATGGGCGCTCCAGTGCC
Reverse 3´ for C-term GFP fusion	GGGGACCACTTTGTACAAGAAAGCTGGGTGTGCTACCTGTAGGTGATC
Reverse 3´ for N-term GFP fusion	GGGGACCACTTTGTACAAGAAAGCTGGGTGTCAGCTACCTGTAGGTGATC

General abbreviations

6-BAP	**6-b**enzyl **a**mino**p**urin
APC	**ap**hidi**c**olin
APC	**A**naphase **p**romoting **c**omplex
BLM	**bl**eo**m**ycin
BY-2	Nicotiana tabacum L. cv **B**right **Y**ellow **2**
CDS	**cod**ing **s**equence
Col-0	*Arabidopsis thaliana*, ecotype Columbia-0
CPD	**c**yclobutane **p**yrimidine **d**imers
CPT	**cam**p**t**othecin
DAG	**d**ay **a**ter **g**ermination
DBS	**d**ouble **s**trand **b**reak
DEVD	caspase-3 specific motif
dsDNA	**d**ouble **s**tranded DNA
FDA	**f**luorescein **dia**cetate
FISH	**f**luorescence ***in situ*** **h**ybridization
GFP	**g**reen **f**luorescent **p**rotein
HU	**h**ydroxy**u**rea
MI	**m**itotic **i**ndex
MMC	**m**ito**m**ycin **C**
MMS	**m**ethyl**m**ethan**s**uphonate
MS	**M**urashige **S**koog
NLS	**n**uclear **l**ocalisation **s**ignal
PCD	**p**rogrammed **c**ell **d**eath
PCR	**p**olymerase **c**hain **r**eaction
PI	**p**ropidium **i**odide
QC	**q**uiescent **c**enter
qPCR	**q**uantitative **PCR**
ROS	**r**eactive **o**xygen **s**pecies
sqPCR	**s**emi**q**uantitative **PCR**
SSB	**s**ingle **s**trand **b**reak
ssDNA	**s**ingle **s**tranded DNA
TUNEL	**t**erminal transferase d**U**TP **n**ick **e**nd **l**abeling
UV	**u**ltra**v**iolet
WT	**w**ild **t**ype
YVAD	caspase 1-specific motif

Abbreviations of the most cited genes

ATM	**A**taxia **t**elangiectasia **m**utated
ATR	**A**TM and **R**ad3 **r**elated
BARD1	**B**RCA1-**a**ssociated **R**ING **d**omain protein **1**
BCL-2	**B-c**ell **l**ymphoma **2**
BI-1	**B**ax-**i**nhibitor **1**
BRCA1	**Br**east **ca**ncer **1**
CAK	**C**DK-**a**ctivating **k**inase
CDC	**C**ell **d**ivision **c**ycle
CDK	**C**yclin-**d**ependent **k**inase
CYC	**Cyc**lin
EDS1	**E**nhanced **d**isease **s**usceptibility **1**
GST11	**G**lutathion **S-t**ransferase **11**
ICK	**I**nhibitor of **c**yclin-dependent **k**inase
KRP	**K**IP-**r**elated **p**rotein
MCA	**M**eta**ca**spase
PR1	**P**athogenesis-**r**elated **1**
PRXcb	**Pe**ro**x**idase **cb**
RBR	**R**etino**b**lastoma-**r**elated
SOG1	**S**uppressor **o**f **g**amma response **1**
VPE	**V**acuolar **p**rocessing **e**nzyme
WOX5	**W**uscher-related homeob**ox 5**

Author´s publications

Book chapter

Lincker F., Roa H., Lang J., Sanchez-Calderon L., **Smetana O**., Cognat V., Keller M., Mediouni C., Houlné G. and Chabouté M.E. (2008) Plant E2F factors in cell cycle development an DNA damage response "Control of cellular physiology by transcription factors E2F", Yoshida K., ed (Kerala India: Research Signpost) chap 2:17-31.

Submitted publications

Smetana O., Široký J., Houlné G., Opatrný Z., Chabouté ME.: Induction of autophagic programmed cell death by the genotoxic treatment with bleomycin and analysis of the effect of *E2F* overexpression in BY-2 cells. Author´s contribution: design of experiments, performance of all experiments except FISH analysis, writing of the manuscript.

Lang J., **Smetana O.**, Sanchez-Calderon L., Lincker F., Genestier J., Houlné G., Chabouté ME: Novel aspects of the DSB cellular response in plants. Author´s contribution: part of experiments (cloning of constructs, RT sqPCR analysis, microscopy), and contribution to manuscript preparation.