universität wien

DIPLOMARBEIT

Titel der Diplomarbeit

Cell cycle and DNA damage dependent control of transcription of the DNA repair gene *AtCOM1*

Verfasser

Stefan Bailey

angestrebter akademischer Grad

Magister der Naturwissenschaften (Mag. rer. nat.)

Wien, 2012

Studienkennzahl lt. Studienblatt:	A 490
Studienrichtung lt. Studienblatt:	Diplomstudium Molekulare Biologie
Betreuerin / Betreuer:	Dr. Peter Schlögelhofer

Table of contents

1 Abstract

The maintenance of genomic integrity is of crucial importance for all living organisms. The genome is however constantly threatened by a multitude of different DNA lesions, that can be caused by internal stresses, or exogenous genotoxic agents. Therefore, the ability to sense and repair DNA damage in a timely manner is essential. A large variety of proteins are involved in this DNA damage response. COM1, which is highly conserved from yeast (COM1/SAE2) to humans (CtIP), is one of these proteins. The plant homologue AtCOM1 is essential for homologous recombination (HR) and meiotic progression. It is expressed at basal levels in mitotic cells and at strongly enhanced levels in cells undergoing the endocycle. Its transcription is strongly enhanced in response to ionizing radiation depending on the checkpoint kinase ATM (Ataxia telangiectasia mutated). It is essential for HR and acts in cooperation with the MRN/X complex (Mre11, Rad50, Nbs1/Xrs1), in response to DNA double strand breaks (DSBs). *Atcom1* mutants are sterile due to defects in meiotic double strand break (DSB) processing and subsequent DNA repair and are sensitive to the interstrand crosslinking agent Mitomycin C.

This study presents data about the cell cycle and tissue specific, regulatory dynamics of *AtCOM1* expression. Evidence is provided, that an E2F transcription factor (TF) binding site in the promoter is essential for the transcription of *AtCOM1* and that, in the absence of genotoxic stress, the gene is expressed in mitotic cells, independent of ATM at a basal level. Ionizing radiation (IR) leads to a strongly enhanced expression of *AtCOM1*, that depends on ATM. The *AtCOM1* promoter activity is however restricted to the meristems. At least one of the six plant E2Fs, E2Fa is shown to be enriched at the E2F transcription factor binding site of the *AtCOM1* promoter. Alterations of the expression levels of different E2F TFs lead to altered expression levels of *AtCOM1*.

The results presented in this thesis lead to the hypothesis, that the transcription of the DNA repair gene *AtCOM1* depends on E2F and ATM dependent cell cycle control. *AtCOM1* promoter activity seems to be restricted to the meristems and is strongly upregulated in response to IR. This upregulation of *AtCOM1* is dependent of ATM. These results suggest, that IR-induced DSBs lead to an ATM dependent switch

from proliferation to endocycle in meristematic cells. In endocycling cells E2Fa activity is strongly enhanced, leading to the upregulation of *AtCOM1.*

2 Zusammenfassung

Die Aufrechterhaltung der genomischen Unversehrtheit ist von entscheidender Bedeutung für alle lebenden Organismen. Das Genom wird jedoch permanent von einer Vielzahl verschiedener DNA Schäden bedroht, die interne Ursachen haben, oder von exogenen, genotoxischen Stoffen verursacht werden können. Daher ist die Fähigkeit DNA Schäden schnellstmöglich zu erkennen und zu beseitigen entscheidend. Eine Vielzahl an Proteinen sind an dieser Antwort auf DNA Schädigungen beteiligt. COM1, das von der Hefe (COM1/SAE2) bis zum Menschen konserviert ist, ist eines dieser Proteine. Das homologe Pflanzenprotein AtCOM1 ist essentiell für homologe Rekombination und den Ablauf der Meiose. Eine basale Expression von *AtCOM1* findet in mitotischen Zellen statt und eine stark erhöhte *AtCOM1* Expression findet in Zellen statt, die eine besondere Form des Zellzyklus durchlaufen, das Endocycle. *AtCOM1* wird, als Reaktion auf DNS-Doppelstrangbrüche, abhängig von der Checkpoint Kinase ATM (Ataxia telangiectasia mutated) aktiviert und treibt im Zusammenspiel mit dem MRN/X Komplex (Mre11, Rad50, Nbs1/Xrs1) die homologe Rekombination voran. *Atcom1* Mutanten sind steril, aufgrund von Störungen bei der Prozessierung von DNS-Doppelstrangbrüchen und der darauffolgenden DNS Reparatur, außerdem reagieren sie sensitiv auf die Substanz Mitomycin C, die einander gegenüberliegende DNA Stränge quervernetzt.

Diese Arbeit präsentiert Daten über die Zellzyklus – und gewebsspezifische, regulatorische Dynamik der *AtCOM1* Expression. Es werden Hinweise dafür gegeben, daß eine E2F Transkriptionsfaktorbindestelle im *AtCOM1* Promotor entscheidend für die Transkription des Gens ist. Außerdem wird gezeigt, daß *AtCOM1*, in Abwesenheit von genotoxischem Stress, in mitotischen Zellen, unabhängig von ATM auf einem basalen Niveau exprimiert wird. Ionisierende Strahlung führt zu einer stark erhöhten, ATM abhängigen Expression von AtCOM1. Die Aktivität des *AtCOM1* Promotors ist jedoch auf die Meristeme beschränkt. Es wird nachgewiesen, daß zumindest einer, der sechs E2F Faktoren, nämlich E2Fa an

der E2F Bindestelle des *AtCOM1* Promotor angereichert ist. Durch die Veränderung der Expressionsniveaus, unterschiedlicher E2F Faktoren, wird die Expression von *AtCOM1* differentiell modifiziert.

Die Ergebnisse, die in dieser Arbeit präsentiert werden, führen zu der Hypothese, daß die Transkription des DNS-Reparatur-Gens *AtCOM1* durch die E2F- und ATM-abhängige Kontrolle des Zellzyklus reguliert wird. Die Aktivität des *AtCOM1* Promotor scheint auf die Meristeme beschränkt zu sein und wird als Antwort auf ionisierende Strahlung, in Abhängigkeit von ATM stark hochreguliert. Diese Ergebnisse führen zu der Annahme, daß durch ionisierende Strahlung verursachte DNS-Doppelstrangbrüche zu einem ATM-abhängigen Einsetzen des Endocycle in Zellen der Meristeme führen. In diesen Zellen ist die E2Fa Aktivität stark erhöht, was der Grund für die vermehrte AtCOM1 Expression sein könnte.

3 Introduction

In contrast to animals, plant development is largely post-embryonic, as plants have to be able to flexibly adapt to environmental conditions, given their sessile lifestyle. New organs, such as roots, stems, leaves, and flowers, originate from life-long iterative cell divisions followed by cell growth and differentiation (Inze and De Veylder 2006). These cell divisions take place at specialized zones, called meristems. Flowers and leaves are produced at the floral and shoot meristems, respectively. Root meristems continuously add new cells to the growing root. The cells at the meristems are pluripotent, so their progeny can become committed to a variety of developmental fates (Inze and De Veylder 2006). Many differentiated plant cells have the ability to de-differentiate, thereby requiring pluripotentiality (Steward 1970; Grafi and Avivi 2004). Quiescent root pericycle cells, for example, can be stimulated to undergo cell divisions and to form lateral roots de novo (Casimiro, Beeckman et al. 2003; Inze and De Veylder 2006).

Another aspect, making plant development unique, is the fact, that plant cells are surrounded by rigid cell walls, preventing any cell migration. The number of cells produced at the meristems and the cell division plane is thus important for determining the organization of plant tissues (Di Laurenzio, Wysocka-Diller et al. 1996; Inze and De Veylder 2006). Another interesting aspect of plants, is that they do not develop tumors, except as specific responses to certain pathogens (Doonan and Hunt 1996).

To understand the role of cell division in plant development and growth, it is required to understand the cell cycle and the basic machinery controlling it. Given its importance for growth and development, the cell cycle is one of the most comprehensively studied biological processes (Inze and De Veylder 2006).

In response to DNA damage the cell cycle is stopped, giving a complex DNA repair machinery the time to repair the DNA damage, before replication and cell division continue. This study is about the cell cycle dependent regulation of the expression of a specific DNA repair factor.

3.1 The Cell Cycle

The eukaryotic cell cycle is divided into four distinct phases. In the G1 (gap1) phase cells increase in size and prepare for DNA replication, which occurs during S (synthesis) phase. After DNA replication, the cell enters G2 (gap2) phase, in which cell growth continues and preparations for cell division are made. Cell division is taking place during M (mitosis) phase. (Figure 1).

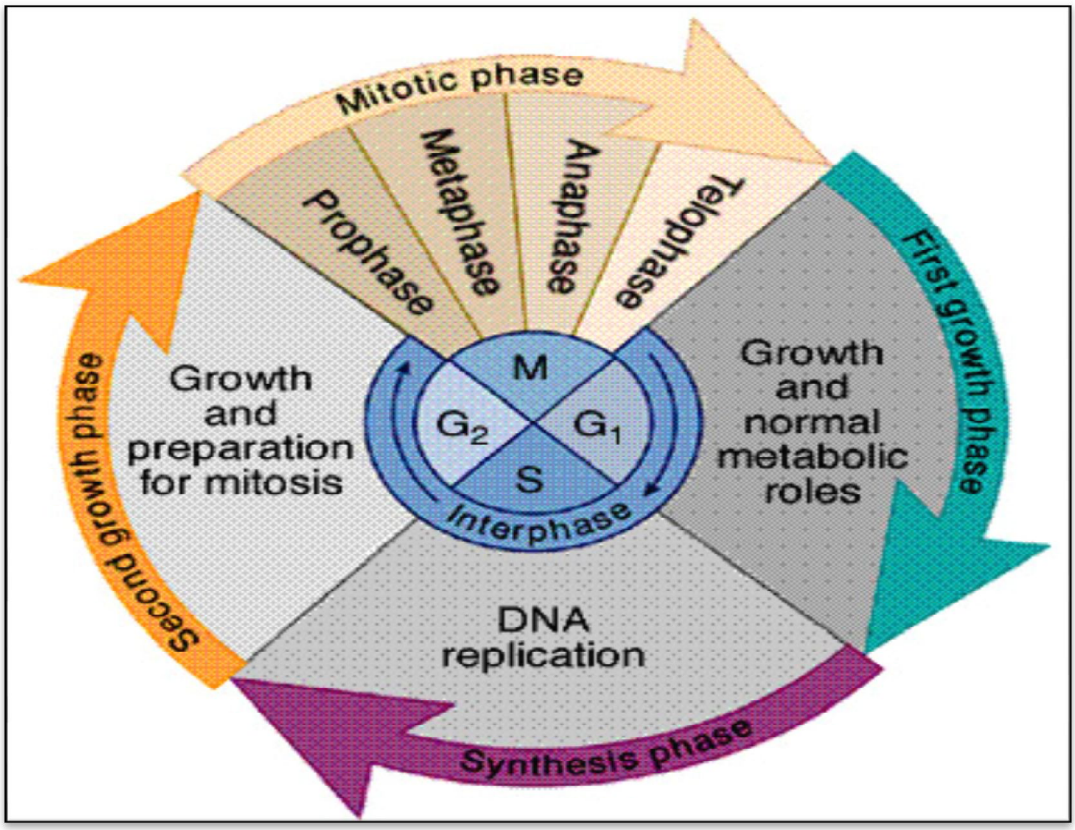


Figure 1 – Schematic overview of the cell cycle and its four distinct phases (Furler 2012)

The cell cycle is tightly regulated, as alterations of the cell cycle machinery can cause severe developmental defects via unrecognized replication errors, or uncontrolled cell division, for instance. In all eukaryotes, cyclins and cyclin dependant kinases (CDKs) are two major classes of regulatory molecules, that determine a cell´s progression through the cell cycle. In plants there are two different classes of CDKs: CDKAs play a pivotal role in both the G1/S and G2/M transitions, whereas CDKBs accumulate at the G2- and M-phase and are essential for regulating the G2/M transition (Porceddu, Stals et al. 2001). These CDKs require binding of cyclins for their activity. Plants contain many more of these regulatory proteins, than previously described in other organisms - *Arabidopsis thaliana* contains at least 32

cyclins with a putative role in cell cycle progression(Inze and De Veylder 2006). D-type cyclins (CYCD) are conserved between plants and animals and are responsible for triggering the G1/S transition through their association with CDKAs (Dewitte, Scofield et al. 2007). One of the targets of CDK-CYCD complexes is the retinoblastoma related protein (RBR). Upon phosphorylation through CDKA, RBR dissociates from E2F transcription factors, which, in their RBR-free form, activate genes required for S-phase entry. B-type cyclins on the other hand play an important role in the G2/M transition and intra M-phase control, while A-type cyclins are reported to regulate the S-to-M phase control.

The activity of CDK/cyclin complexes is regulated by phosphorylation and dephosphorylation, interaction with regulatory proteins and protein degradation (Pines 1995; Zhao, Harashima et al. 2012). Yeast CDK/cyclin complexes are inhibited by phosphorylation of an N-terminal Tyr residue in the CDK partner. In vertebrates this CDK phosphorylation occurs on an N- terminal Tyr and a Thr residue. Tyr phosphorylation is catalyzed by the WEE1 kinase and is counteracted by the phosphatase CDC25 (Inze and De Veylder 2006). Plants also possess a WEE1 kinase, that is putatively involved in the inhibitory phosphorylation of CDKs (Sorrell, Marchbank et al. 2002; Vandepoele, Raes et al. 2002). However in *Arabidopsis* and other plants, whose entire genome sequences are available, no *CDC25* gene, encoding a CDK-phosphatase could be identified (Vandepoele, Raes et al. 2002; Bisova, Krylov et al. 2005). Cell cycle control is a very complex network of many proteins working in concert, the exact molecular events controlling the cell cycle still need to be subject of further studies.

The cell cycle is an ordered and unidirectional process, that cannot be reversed. At certain positions, checkpoints prevent the cycle from further progression, to ensure that all requirements for the next phase are met. Several checkpoints were designed to make sure, that damaged, or incomplete DNA is not passed on to daughter cells. The two main checkpoints are the G1/S checkpoint and the G2/M checkpoint, while the former is the rate-limiting step of the cell cycle, also known as restriction point. Cyclin-dependant kinase inhibitors (CKIs) can inhibit progression of the cell cycle, by binding and inhibiting CDKs. Plant CKIs all share a C-terminally located 31-amino-acid domain, involved in the binding of CDKs and cyclins, that is essential for the

inhibitory activity of the proteins (Wang, Fowke et al. 1997; De Veylder, Beeckman et al. 2001; Jasinski, Perennes et al. 2002; Coelho, Dante et al. 2005).

Cell cycle regulation is of pivotal importance for plant growth and development. Plants contain more genes encoding core cell cycle regulators than other organisms (Dewitte, Scofield et al. 2007). The large number of regulatory genes might reflect the high developmental plasticity of sessile plants to respond to both intrinsic developmental signals and extrinsic environmental cues. The different cyclins might posses a wide range of expression patterns and confer different substrate specificities (Inze and De Veylder 2006).

3.2 Endoreduplication

The normal cell cycle is characterized by a round of DNA replication (S-phase) followed by mitosis and cytokinesis (M-phase). The two phases are separated by the two gap phases (G1 and G2). Many plant cells however undergo endoreduplication, a cell cycle mode, where cells undergo iterative DNA replications without subsequent mitosis (Sugimoto-Shirasu and Roberts 2003). The ploidy level varies between tissue and species. In *Arabidopsis thaliana*, nuclei with up to 32C are detected (Galbraith, Harkins et al. 1991; Inze and De Veylder 2006). It is believed, that endoreduplication plays an important role in the differentiation process of postmitotic cells, since the onset of endoreduplication often characterizes the switch from proliferation to differentiation (Kondorosi, Roudier et al. 2000). It is suggested that endoreduplication requires nothing more elaborate than a loss of M-phase cyclin-dependent kinase activity (CDKB) and oscillations in the activity of S-phase cyclin dependant kinase (CDKA) (Larkins, Dilkes et al. 2001). The mitotic cycle and the endocycle have DNA replication in common and probably use the same machinery. In fact overexpression studies of DNA replication stimulating genes have shown that both cycles were enhanced (Schnittger, Schobinger et al. 2002; Kosugi and Ohashi 2003).

Another hypothesis is that mutations are buffered by the endocycle. To assure the availability of functional copies of the genome, several copies are produced. A recent study has correlated DNA double strand breaks (DSBs) with the onset of endoreduplication. It is proposed, that DSB signals affect the expression of cell cycle regulators, such as CDK suppressors, thereby switching the mitotic cycle to the endocycle. A possible benefit could be, that DNA damaged cells are prevented from

proliferation and also from dying. Plant cells, unlike animal cells, cannot migrate within tissues, cell death usually leaves behind an opening in the local tissue structure (e.g., xylem in vascular tissue). *Arabidopsis* may therefore have acquired the strategy of actively inducing endoreduplication to prevent such gaps from arising in damaged tissue and thus to guarantee uninterrupted development during the life cycle (Adachi, Minamisawa et al. 2011).

The cell cycle, including the endocycle, is a complex machinery, that can only function accurately, if specific proteins are only active at specific timepoints. One way to control abundance and activity of proteins is to regulate the expression of the genes coding for these proteins.

3.3 Promoters and Transcriptional Regulation

The regulation of gene expression is one of the most intensively studied areas of biology. Only some eukaryotic genes are expressed constitutively, while expression of most genes is regulated in response to environmental and physiological conditions. The regulation of transcription, the first committed step in gene expression, is achieved via the interaction of transcription factors (TFs) with *cis* acting regulatory elements (CAREs) (Holt, Millar et al. 2006). These regulatory elements are part of the eukaryotic gene promoters, which are in general composed of three functional regions. The first element is the core promoter, a region flanking the transcription start site (TSS), upon which the enzymatic transcription machinery assembles. The second region is the proximal promoter, which is located upstream of the core promoter and contains the cis regulatory elements (Wray, Hahn et al. 2003; Zhang 2007; Uanschou 2009). The third part is the distal promoter, which is situated further upstream or may be also present in downstream regions and introns (Zhang 2007). The distal promoter contains additional regulatory elements that often possess a weaker influence on gene expression regulation (Zhang 2007; Uanschou 2009). In eukaryotes, a vast number of highly diverse proteins is involved in the regulation of gene expression. In contrast to prokaryotes, where the basal state of transcription is non-limited, meaning that RNA polymerase has unlimited access to DNA promoters, it is very well restrictive in eukaryotes (Struhl 1999). Eukaryotic DNA is associated with histones, proteins, that package the DNA in the nucleus. The basic unit of eukaryotic DNA packaging is the nucleosome. It consists of a segment of DNA

wound in sequence around a histone octamer, consisting of two copies each of the core histones H2A, H2B, H3 and H4. Approximately 147 bp of DNA are wrapped around this histone core and the nucleosomes are connected by stretches of linker DNA. This DNA/protein complex is called chromatin. Tightly packing chromatin can prevent binding of transcription factors and association of the transcription machinery with specific genes. A loose and thus active chromatin structure is referred to as euchromatin, while the most tightly packed nucleosome arrays are called heterochromatin. Chromatin structure alternates between these two conformations, in response to a variety of cellular signals.

RNA-polymerases are enzymes, transcribing DNA to RNA. In eukaryotic organisms, there are three different RNA polymerases, which are responsible for the synthesis of rRNA (Pol I), mRNA (Pol II), and tRNA, 5S rRNA, and other small RNA molecules (Pol III), respectively (Riechmann 2002). The transcription of protein encoding genes, to yield mRNA is exclusively carried out by Pol II. This is a multi-subunit complex, that requires general transcription factors (GTFs) to recognize promoter sequences and initiate transcription (Cramer, Bushnell et al. 2001).

Sequence specific DNA binding transcription factors are responsible for the selectivity in the regulation of gene expression. They bind to specific sequence motifs and can act as transcriptional activators or repressors and are often themselves expressed in a tissue, cell-type, temporal, or stimulus-dependent specific manner (Riechmann 2002). They can act as landing platforms for different components of the transcription machinery, or act in concert with chromatin remodelling complexes, which are responsible for reorganizing chromatin structure, making the DNA more, or less accessible for proteins like RNA polymerases and their cofactors. The correct regulation of gene transcription is essential for the maintenance of normal cellular homeostasis. In order to regulate gene expression, cells maintain tight control over transcription factors (Tansey 2001). There are several means to accomplish a tight control over transcription factors. One of them is, that kinases, which are part of the transcriptional machinery, phosphorylate TFs, thus marking them for ubiquitin-dependant degradation, or inducing conformational changes, thereby inactivating the proteins. Transcription factor activity can be inhibited by binding of interacting proteins. The expression of genes encoding TFs can be repressed and TF binding

sites can be blocked by transcriptional repressors (Berckmans and De Veylder 2009; Borghi, Gutzat et al. 2010; Magyar, Horvath et al. 2012).

Another way to control gene expression is by modification of epigenetic marks. Epigenetic processes are heritable and reversible alterations in the expression patterns of genes, without changing the underlying DNA sequences. Examples for epigenetic modifications are histone acetylation, or methylation, the former usually leading to the formation of a looser chromatin structure (euchromatin), making genes more accessible and thus more likely to be activated, while the latter usually leads to a compaction of the chromatin structure (heterochromatin), leading to gene inactivation (Holliday 1990; Nottke, Colaiacovo et al. 2009). Other histone modifications altering chromatin structure are phosphorylation, sumoylation and ubiquitylation. Other epigenetic changes are mediated by the production of different splice forms of RNA, or by the formation of double-stranded RNA (RNAi) (Bird 2007; Reik 2007).

It is of critical importance for an organism to tightly regulate gene expression in order to maintain normal development and an intact cell cycle. The E2F (E2 promoter binding factor) transcription factor family is a group of transcription factors, playing an important role in proliferation and development of higher eukaryotes.

3.4 E2F transcription factors

E2F transcription factors were first described in 1986, when a HeLa cell factor was detected, that appeared to mediate transcriptional stimulation of the adenoviral E2 promoter (Kovesdi, Reichel et al. 1986). This factor was termed E2 promoter binding factor (E2F).

E2F transcription factors are present in all higher eukaryotes and regulate genes, that are involved in proliferation, differentiation, development and apoptosis. They play a critical role in cell cycle progression, as they stimulate the expression of genes, required for the onset of S-phase and DNA replication (Rossignol, Stevens et al. 2002). In mammals eight *E2F* genes have been characterized, while there appear to be only six *E2F* genes in *Arabidopsis* (E2Fa-f). They all bind to a highly conserved consensus sequence (Figure 2).

Figure 2 - Sequence logo of the overrepresented motif found in the set of 181 putative E2F target genes (Vandepoele, Vlieghe et al. 2005).

The first three *Arabidopsis* E2Fs (E2Fa-c) share a common domain organization with their mammalian counterparts. They all contain a domain for the binding of cylinA, a DNA binding domain, a transcriptional activation domain, which harbours a binding site for the retinoblastoma related protein (RBR), as well as a binding site for one of the two possible dimerization partners DPa and DPb. Binding of a dimerization partner (DP) is required for successful DNA target sequence binding. E2Fa and b preferentially bind to DPa, while E2Fc prefers DPb (del Pozo, Diaz-Trivino et al. 2006; Sozzani, Maggio et al. 2006). If RBR is bound by these transcription factors, the ability to activate gene expression is suppressed. The other three E2Fs, E2Fd, e and f, also known as DEL2, 1 and 3 possess two DNA binding sites, enabling them to bind promoter regions independent of DPs, but contain none of the other conserved domains, which is why they are considered to be transcriptional repressors (Figure 3). As mentioned before, the RBR protein controls the transcription factor activity of the E2Fa-c/DP dimers. E2Fa and E2Fb are known to activate genes, that are required for G1 to S phase transition, in complex with DPa, while E2Fc has been shown to delay cell division and repress the expression of S phase genes in overexpression studies (del Pozo, Boniotti et al. 2002)

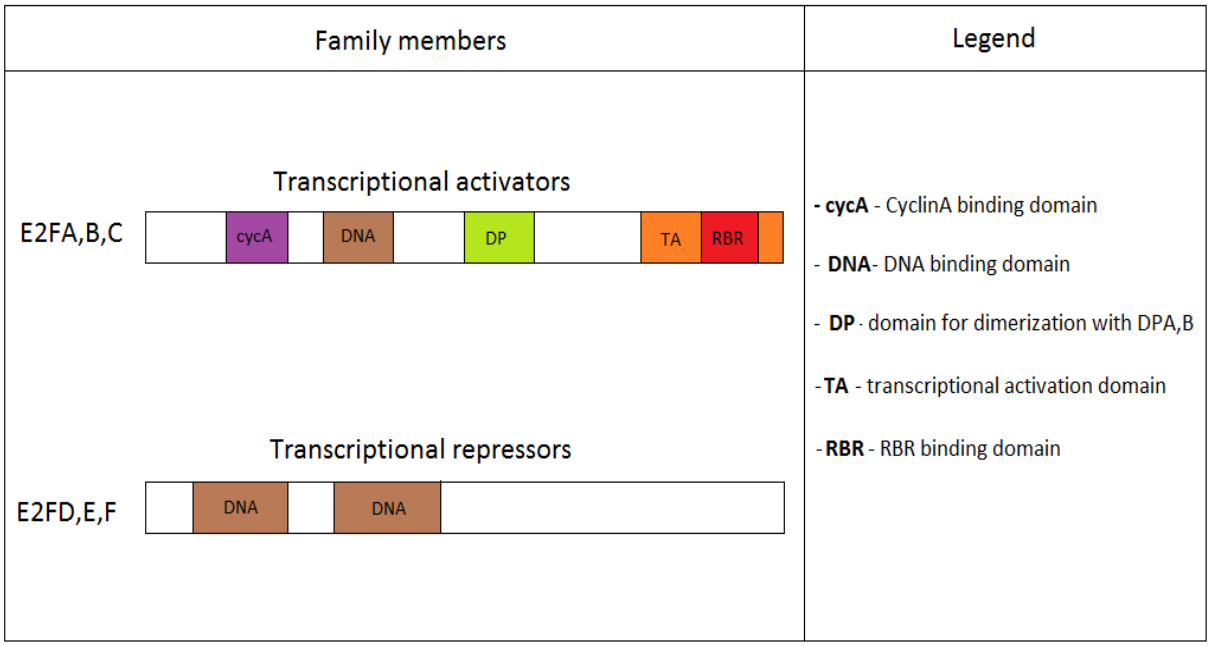


Figure 3 - The six *Arabidopsis* E2F transcription factors and their conserved functional domains

According to current models, RBR is bound to the E2F/DP dimer in its hypophosphorylated form. Upon phosphorylation of RBR by CDKA, in complex with CYCD3, E2Fs are released, thereby regaining their transcriptional activity and thus triggering G1 to S phase transition (Figure 4) (Nakagami, Sekine et al. 1999; Berckmans and De Veylder 2009).

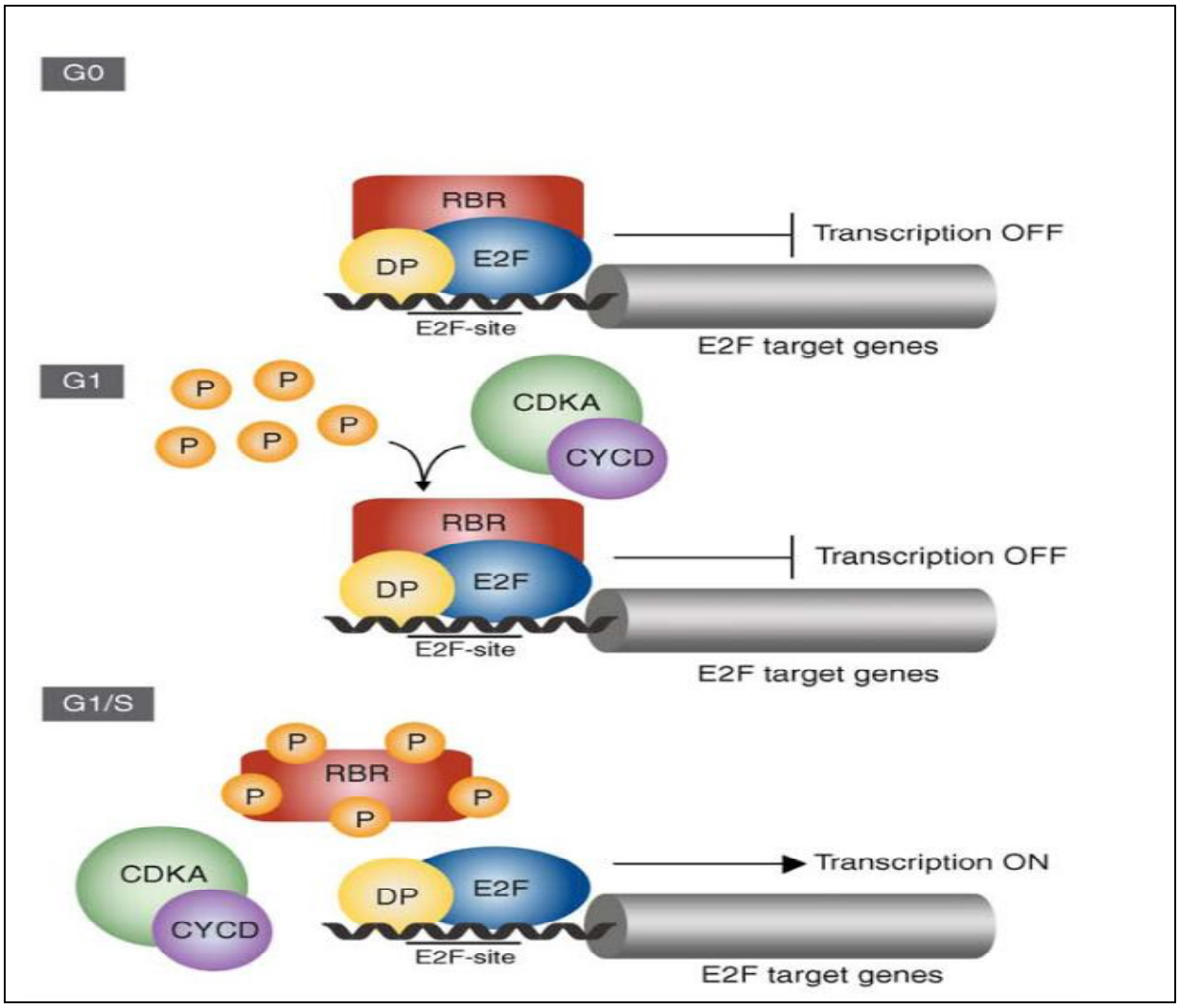


Figure 4 – InG_0, when the E2F/DP heterodimer is bound by RBR, the transcriptional activity of the complex is repressed. When cells are committed to divide CDKA is bound by CYCD, thus hyperphosphorylating the RBR protein. The E2F/DP complex is released and activates genes necessary for DNA replication (Berckmans and De Veylder 2009).

The RBR/E2F/DP pathway also plays an important role for the transition from proliferation to the endocycle. Plant development comprises an extensive cell proliferation phase, followed by a period of differentiation, often linked with the start of endoreduplication, an alternative cell cycle, during which the cell increases its DNA content, without cell division (Berckmans and De Veylder 2009). It was shown, that E2Fe/DEL1 represses the expression of genes, involved in the switch from the cell cycle to the endocycle in proliferating cells, thereby restraining their expression to endoreduplicating cells and inhibiting endocycle onset (Lammens, Boudolf et al. 2008). All of the six plant E2F transcription factor genes, except E2Fa, contain an

E2F binding site in their promoter regions, regulating their expression levels. It was shown, that *E2Fe/DEL1* expression is antagonistically regulated by E2Fb and E2Fc. E2Fb induces *DEL1* expression, while E2Fc is a repressor of *DEL1* (Figure 5) (Berckmans, Lammens et al. 2011).

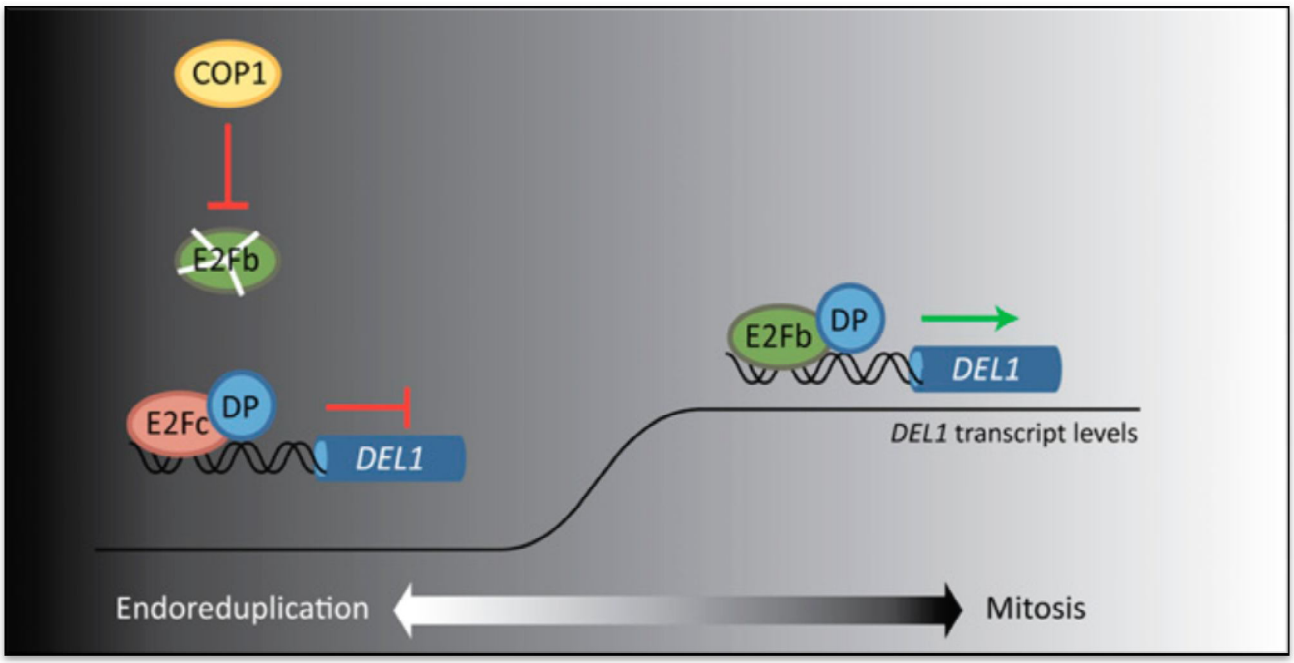


Figure 5 – Under light conditions E2Fb activates DEL1 expression, which inhibits endocycle entry. Under dark conditions, COP1 marks E2Fb for degradation, leaving E2Fc as the most abundant E2F. E2Fc inhibits DEL1 expression, allowing the cell to enter the endocycle.

E2Fc was also shown to repress genes in G2 control, thereby promoting endocycle (del Pozo, Diaz-Trivino et al. 2006). While E2Fb stimulates S phase and M phase genes, making it a driver of the cell cycle, E2Fa only stimulates S phase genes and is necessary and sufficient for both endoreduplication and proliferation. It regulates growth by maintaining proliferation indirectly through its RBR bound form within the meristems and promoting endocycle by activating S phase genes outside the meristems. The RBR/E2F/DP pathway comprises an interconnected gene regulatory network, regulating the balance between proliferation and endoreduplication (Figure 6) (Magyar, Horvath et al. 2012).

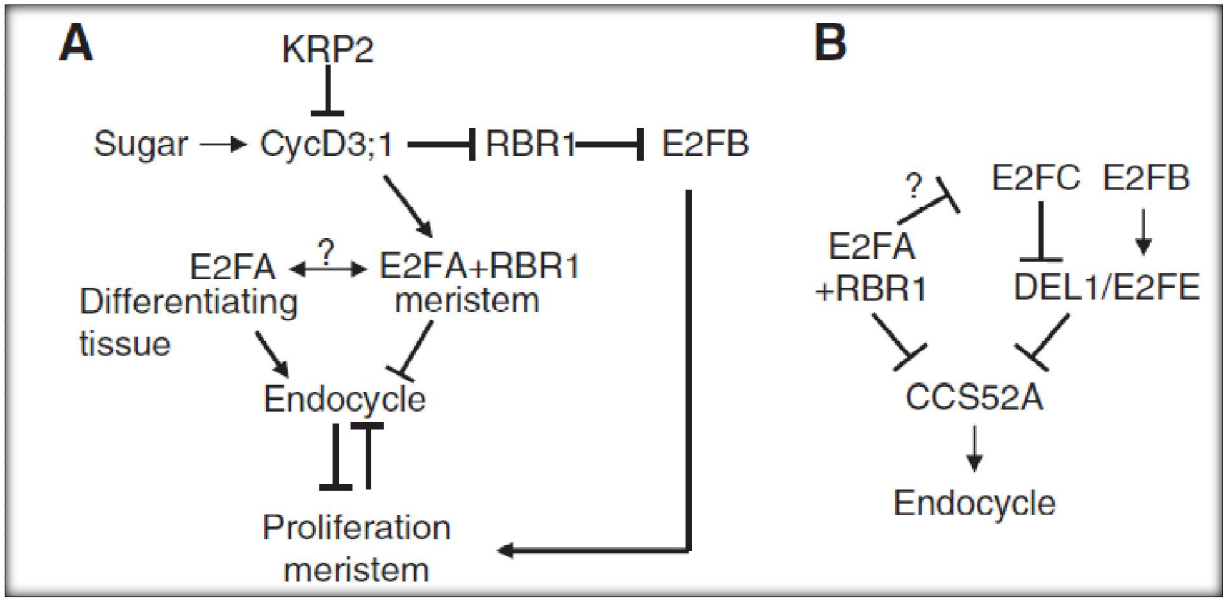


Figure 6 – **(A)** RBR represses E2Fb and is released by CYCD3. RBR-free E2Fb induces cell proliferation, by activating genes involved in cell cycle progression. The RBR-E2Fa complex is more stable in proliferating cells within the meristem and inhibits activation of genes involved in the switch from proliferation to endreduplication. E2Fa is released by an unknown mechanism and stimulates endocycle in cells committed to differentiation.
(B) E2Fa inhibits endocycle in its RBR bound form by repressing the activity of E2Fc and endocycle activating genes. DEL1 represses endocycle activating genes and is antagonistically and competitively regulated by E2Fb and E2Fc (Magyar, Horvath et al. 2012).

Complex interplay between E2Fs, DPs and RBR is crucial for a cell to properly regulate its cell cycle and to adapt to environmental stresses. As mentioned above, DNA damage can lead to endocycle onset (Adachi, Minamisawa et al. 2011). This is however only one aspect of a broad variety of cellular responses to DNA damage.

3.5 DNA damage signalling

It is of critical importance for all cellular organisms to maintain their genomic integrity. For this purpose, the ability to repair DNA in a timely manner is essential. Genome integrity is continuously threatened by exogenous agents, such as radiation and chemical mutagens, causing a variety of DNA lesions, as well as by internal stresses, resulting from cellular metabolism, such as reactive oxygen species, or stalled replication forks (Amiard, Charbonnel et al. 2010). Unrepaired DNA damage can lead to a variety of mutations, cell death, senescence and tumorigenesis. There is abroad

variety of DNA alterations caused by several internal and external stresses, like chemical modification of bases (alkylation of bases, depurination, deamination etc.), DNA crosslinks (e.g. thymidine dimers), DNA single strand breaks (SSBs) and DNA double strand breaks (DSBs). DSBs are the most toxic lesions, as a single unrepaired double strand break can cause cell death (Bennett, Lewis et al. 1993). DNA damage responsive pathways are activated to sense and repair the lesions. These DNA damage responses (DDRs) can lead to cell cycle arrest,- to provide time for repair -, apoptosis, or endocycle entry. The major regulators of the DDR are the protein kinases Ataxia telangiectasia mutated (ATM) and ATM and Rad3 related (ATR), which belong to the phosphatidyl inositol 3-kinase family (PIKKs). ATR responds primarily to stalled replication forks and single stranded DNA (ssDNA), with replication protein A coated ssDNA activating its kinase activity (Amiard, Charbonnel et al. 2010). ATM however is recruited and activated in response to DSBs by the highly conserved MRN complex, which recognises DSBs by its ability to bind DNA ends (Harper and Elledge 2007) ; (Paull and Gellert 1999). The MRN complex consists of three proteins, Meiotic recombination 11 (MRE11), Radiation sensitive 50 (RAD50), and Nijmegen Breakage Syndrome 1 (NBS1) and is involved in DNA damage repair, DNA replication, meiosis, and telomere maintenance (Czornak, Chughtai et al. 2008; Mimitou and Symington 2009; Lamarche, Orazio et al. 2010). After ATM is recruited to sites of double strand breaks, it is activated by its interaction with MRN and thereupon phosphorylates a large number of downstream targets, including the histone variant H2AX, which in its phosphorylated form (γ-H2AX) is a well established cellular marker of DNA double strand breaks, that plays a key role in the recruitment and accumulation of DNA repair proteins at sites of DSBs (Paull, Rogakou et al. 2000; Kinner, Wu et al. 2008). Other targets of ATM are proteins involved in cell cycle checkpoint control, DNA repair and apoptosis, making it a major regulator of the cell cycle in response to DSBs.

The protein kinase ATR, which is known to respond primarily to ssDNA and stalled replication forks, does not require the MRN complex for its activity in response to replicative stress. ATR is recruited to RPA (replication protein A)-coated ssDNA by ATRIP (ATR interacting protein). ATR-ATRIP is then loaded close to DNA lesions by RAD17 onto the 9-1-1 complex (RAD9-RAD1-HUS1)(Ricaud, Proux et al. 2007). The 9-1-1 checkpoint clamp is another sensor complex that functions in DNA damage

checkpoint signal transduction, being recruited to the stalled forks (Kanoh, Tamai et al. 2006). ATR, which is activated by the 9-1-1 complex, phosphorylates downstream targets, leading to cell cycle arrest. ATR is however also considered as part of the long-term response to DNA DSBs, hereby depending on the MRN complex (Cimprich and Cortez 2008; Adachi, Minamisawa et al. 2011). After a DSB has been detected and bound by the MRN complex, the nuclease activity of MRE11 leads to resection of the DNA ends, resulting in single stranded DNA (ssDNA), required for homologous recombination. These ssDNA overhangs lead to the activation of ATR in response to DSBs, which is needed to maintain the cell cycle arrest and keep the cell from entering M phase (Mimitou and Symington 2009; Lamarche, Orazio et al. 2010). ATM and ATR are sensor kinases that relay the damage signal to transducer kinases Chk1 and Chk2 and to downstream cell-cycle regulators. Plants also possess ATM and ATR orthologs but lack obvious counterparts of downstream regulators (Melo and Toczyski 2002; Culligan, Tissier et al. 2004; Adachi, Minamisawa et al. 2011).

Arabidopsis thaliana plants mutated for either ATM or ATR are viable. *atm* mutant plants grow only small siliques, bearing very few seeds and are thus partially sterile (Garcia, Bruchet et al. 2003). Plants mutated for ATM are also hypersensitive to gamma-radiation and methylmethane sulfonate (Garcia, Bruchet et al. 2003). *atr* mutant plants, by contrast, are fully fertile, but *atm atr* double mutants are completely sterile, because of severe chromatin fragmentation. *atr* mutant plants show delayed cell cycle arrest in response to DNA damage. ATR is involved in the correct loading of recombinases to sites of meiotic DSBs and is thus an integral part of the regular meiotic program (Culligan, Tissier et al. 2004; Culligan, Robertson et al. 2006; Culligan and Britt 2008; Kurzbauer, Uanschou et al. 2012). Most of the factors involved in the DNA damage response are conserved between vertebrates and plants. However the fact, that mammalian *atr* knockout mutants are not viable, suggests, that the regulatory mechanisms, underlying the DDR are diverged between plants and animals (Britt and May 2003; Culligan, Tissier et al. 2004; Adachi, Minamisawa et al. 2011). In fact animals posses a kind of master regulator, that governs most aspects of a cell´s response to DNA damage, the transcription factor p53 (Yoshiyama, Conklin et al. 2009). It can integrate a variety of environmental signals, to produce a response appropriate for the cell´s developmental stage (Rozan and El-Deiry 2007). Homologs of p53 have not been identified in yeasts and plants.

Model yeasts (*Schizosaccharomyces pombe and Saccharomyces cerevisiae)* seem to lack such a master regulator, a transcription factor, that governs the majority of the transcriptional response to DNA damage. The sensors and many of the transducers and effectors involved in the DDR are however conserved between fungi and animals (Gasch, Huang et al. 2001). It has been hypothesized, that yeasts lack this additional layer of integration and regulation, because they are single celled organisms and thus lack an apoptotic DNA damage response, requiring this additional factor. Also the response of a unicellular organism to DNA damage is sophisticated, while a multicellular species, with various tissue types may require another level of organisation. In plants, like in yeasts most of the DDR genes are conserved. Plants also possess a single transcription factor, central transcription factor processing most DNA damage signals and participating in pathways governed by both ATM and ATR. This transcription factor, called SOG1 (suppressor of gamma response 1) is unrelated to p53, unique to plants and seems to have appeared only after the origin of multicellularity in plants (Yoshiyama, Conklin et al. 2009). It was shown recently, that DNA damage caused by gamma radiation (DSBs) and UVB irradiation (blocked replication) both lead to apoptosis in root and shoot stem cells of *Arabidopsis*, and that this DNA damage dependant, programmed cell death in the stem cell niche is activated by SOG1 and by either ATM, or ATR (Fulcher and Sablowski 2009; Furukawa, Curtis et al. 2010). However, the exact mechanism, by which programmed cell death (PCD) is induced in *Arabidopsis* stem cells, in response to DNA damage remain unclear (Figure 7).

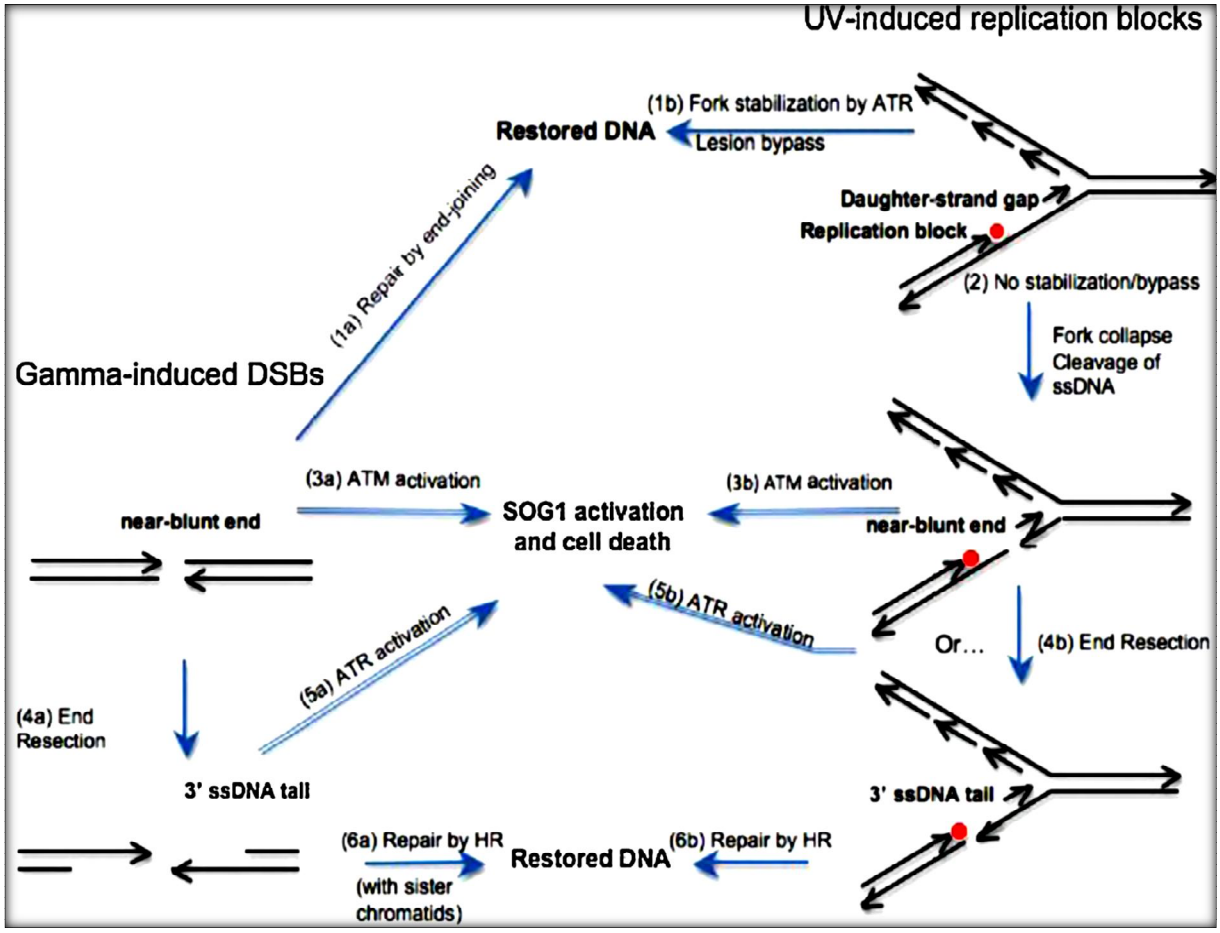


Figure 7 – Overview of common model pathways for ATM/ATR/SOG1 responses to gamma radiation-induced DSBs and UV-induced replication blocks in *Arabidopsis* stem cells. (1a) Gamma induced DSBs are repaired by NHEJ(non-homologous end joining). (3a) ATM is activated in response to DSBs, which leads to SOG1 activation and cell death. (4a) End resection of DSBs leads to 3´ssDNA overhangs. (5a) These overhangs lead to activation of ATR, which results in cell death, via SOG1 activation. (6a) 3´ssDNA overhangs are repaired by HR (homologous recombination. (1a) Stalled replication forks are stabilized by ATR and DNA is restored. (2) Stalled replication forks, that are not stabilized, collapse. The ssDNA is cleaved. (3b) The resulting DSBs from replication fork collapse lead to ATM activation. SOG1 activation by ATM leads to cell death. (5b) When DSBs caused by fork collapse are resected, the ssDNA overhangs cause activation of ATR. SOG1, which is activated by ATR, causes cell death. (6b) DSBs are repaired by HR. (Furukawa, Curtis et al. 2010).

In the stem cell niche PCD seems to be a viable way to remove DNA-damaged cells. DNA double strand breaks are, as mentioned before, the most lethal of all DNA lesions and can also be induced via the processing of stalled replication forks. There are at least two mechanisms, by which cells cope with DSBs. One is to delay cell division in order to repair the damage, the other is to induce apoptosis. A recent study suggested, that there is a third mechanism, namely endoreduplication. It was

proposed, that DSB signals affect the expression of cell cycle regulators, such as CDK suppressors and CDKB in a way, that the mitotic cell cycle is switched to the endocycle. They found, that both, the ATM-SOG1 and the ATR-SOG1 pathway can induce endocycle entry and that either one suffices for endocycle induction to assure the availability of functional copies of the genome (Figure 8). Another possible benefit could be, that DNA damaged cells are prevented from proliferation and also from dying. Plant cells, unlike animal cells, cannot migrate within tissues, cell death usually leaves behind an opening in the local tissue structure (e.g., xylem in vascular tissue). *Arabidopsis* may therefore have acquired the strategy of actively inducing endoreduplication to prevent such gaps from arising in damaged tissue and thus to guarantee uninterrupted development during the life cycle (Adachi, Minamisawa et al. 2011).

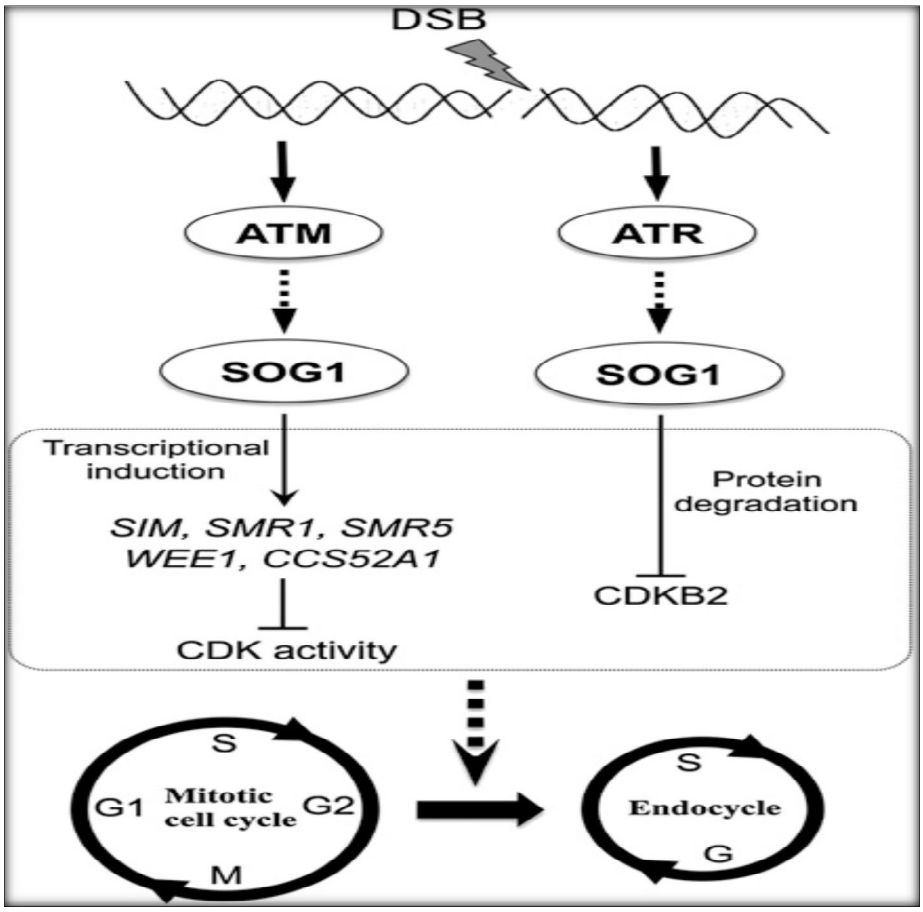


Figure 8 – Model of endocycle induction via the ATM/ATR-SOG1 pathways. ATM-SOG1 induces the expression of CDK suppressors, such as WEE1 and CCS52A1, and of the CDK inhibitors SIM, SMR1, and SMR5. ATR-SOG1 reduces the level of CDKB via proteasome-mediated degradation. Either pathway suffices for endocycle onset (Adachi, Minamisawa et al. 2011).

3.6 Somatic DNA double strand break repair

As described in the previous paragraph, there is a broad variety of DNA lesions, but DSBs are the most toxic. They are potentially lethal to the cell as they lead to mitotically unstable acentric chromosome fragments and the consequent loss of essential genes (Lieber 2010; Lloyd, Wang et al. 2012). DSBs can also lead to a variety of chromosomal rearrangements, such as duplications, translocations and deletions, all of which are precursors to genome instability and tumorigenesis (Bernstein and Rothstein 2009). In eukaryotes, two major pathways have evolved for the repair of these DNA double strand breaks, namely homologous recombination (HR) and non-homologous end joining (NHEJ). They are both conserved from yeast to humans (Hays 2002). During HR, the damaged chromosome enters into synapsis with, and retrieves genetic information from, an undamaged DNA molecule, with which it shares extensive sequence homology. NHEJ brings about the ligation of two DNA double stranded ends without the requirement for extensive sequence homology between the DNA ends and does not require synapsis of the broken DNA with an undamaged partner molecule (Jackson 2002). Simple eukaryotes like yeasts mainly use HR to repair DSBs, while in higher eukaryotes NHEJ is predominant in G1 and G0 phases of the cycle and HR during S and G2 phases.(Johnson and Jasin 2000). In general, NHEJ is preferred by organisms that contain large amounts of non-coding repetitive DNA, where randomly inserted breaks are less likely to fall within an open reading frame (Kurzbauer 2008). Central to NHEJ in all eukaryotes is the Ku protein, a heterodimer of the two subunits Ku70 and Ku80, that recognizes and binds to DNA double strand breaks. In vertebrates, Ku serves as the DNA targeting subunit of the DNA-dependant protein kinase catalytic subunit (DNA-PKcs), which together with Ku forms the DNA-PK holoenzyme (Critchlow and Jackson 1998; Smith and Jackson 1999; Jackson 2002). So far no plant homolog of the DNA-PKcs has been identified. The C-terminus of DNA-PK shares homology with the catalytic domains of the phosphatidyl inositol 3 kinase like (PIKK) family, which includes ATM and ATR (Hartley, Gell et al. 1995; Smith and Jackson 1999; Abraham 2001). Once it has bound a DSB, DNA-PK displays protein Ser/Thr kinase activity and phosphorylates proteins, involved in NHEJ, like Ku70, Ku80, Artemis, Xrcc4 and DNA-PKcs itself (Gottlieb and Jackson 1993; Jackson 2002). The Artemis protein, which is involved in the maturation of the DSB, and the Xrcc4/DNA ligase IV heterodimer, which

catalyzes the resealing of the DNA ends, are then recruited to the DSB site (Lees-Miller and Meek 2003; Meek, Gupta et al. 2004; Bleuyard, Gallego et al. 2006). Counterparts of most of these NHEJ proteins have been identified in *Arabidopsis* via sequence similarities with vertebrate proteins, supporting the notion that NHEJ in plants strongly resembles the process in mammals described above (Bleuyard, Gallego et al. 2006). Most DSBs cannot be directly religated and require limited processing, that sometimes includes the activity of DNA-polymerases, before NHEJ can take place. Therefore, NHEJ is rarely error-free and deletions of various lengths are usually introduced. The artemis protein possesses both exo- and endonucleolytic activities and performs ATM dependant processing of DSBs, cleaving hairpins and other DNA structures (Ma, Schwarz et al. 2005). The MRN complex, which also possesses exonucleolytic, endonucleolytic and DNA unwinding activities is also involved in the processing stages of NHEJ and stimulates the ligase activity of the Xrcc4/DNA ligase IV heterodimer (Huang and Dynan 2002; Jackson 2002). Canonical NHEJ in plants involves the Ku70/Ku80 heterodimer, which binds to DNA ends and recruits a number of other proteins including the DNA ligase IV/XRCC4 complex which repairs the break (Grawunder, Wilm et al. 1997; Downs and Jackson 2004; Lloyd, Wang et al. 2012). As mentioned earlier, no homolog of DNA-PKcs has been identified in plants so far. When the canonical NHEJ pathway becomes compromised, an alternative NHEJ pathway substitutes its function (Mladenov and Iliakis 2011), the B-NHEJ (backup-NHEJ) pathway. It is believed, that instead of the Ligase IV/XRCC4 complex, Ligase III functions in concert with XRCC1 and is regulated by PARP1 in the B-NHEJ pathway (Wang, Zeng et al. 2001; McKinnon and Caldecott 2007; Mladenov and Iliakis 2011). DNA ligase III is only present in vertebrates. There is evidence for histone H1 playing a role in B-NHEJ, probably by aligning DNA ends prior to ligation (Rosidi, Wang et al. 2008). Knowledge of these NHEJ pathways in plants remains less developed, than in other model organisms, but there are apparent differences in *Arabidopsis,* like the absence of known orthologs of proteins like DNA-PKcs and Ligase III (Charbonnel, Gallego et al. 2010; Charbonnel, Allain et al. 2011).

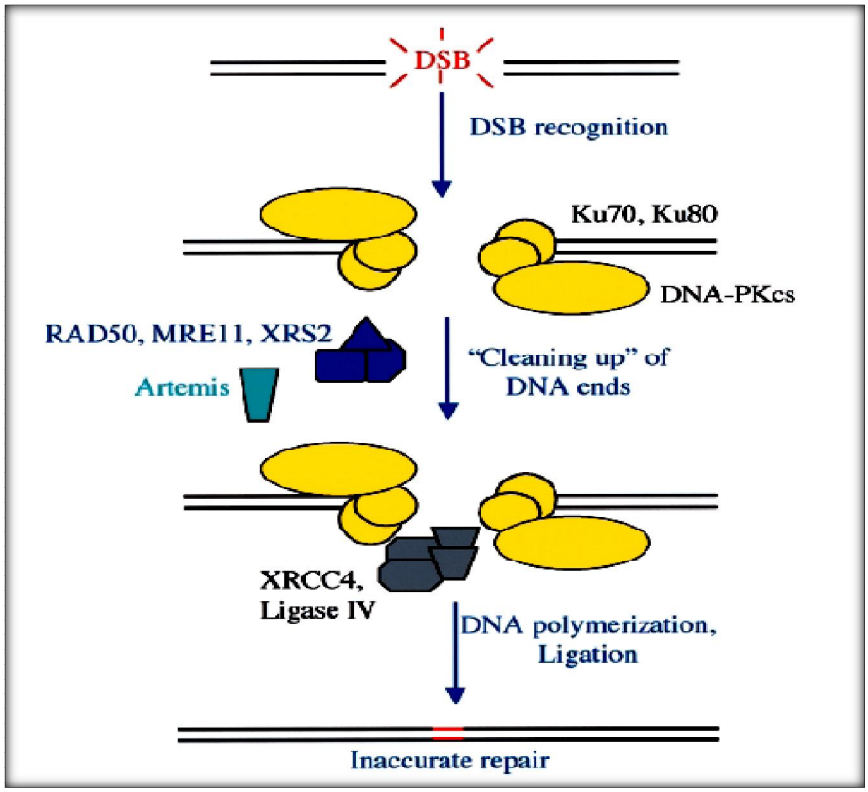


Figure 9 – Schematic representation of the NHEJ pathway. Ku binds to free DNA ends and recruits DNA-PKcs. Ku then recruits the XRCC4/Ligase IV complex, which is activated by DNA-PK mediated phosphorylation and religates DNA ends. The MRN complex and Artemis are required for DNA end processing, before ligation (Jackson 2002).

As mentioned before, in cells, that are in the S or G2 phase of the cell cycle, DSBs are predominantly repaired via HR, which preferentially uses a homologous template from either the sister chromatid or the homologous chromosome to repair the damage (Bernstein and Rothstein 2009). The cell cycle state however is not the only deciding factor, which repair pathway is used, also the type of lesion dictates the response. A single endonuclease induced DSB during G1 phase triggers NHEJ, while a DSB caused by ionizing radiation (IR) is preferentially targeted to the HR pathway (Barlow, Lisby et al. 2008). In the budding yeast *Saccharomyces cerevisiae,* DSBs, that are repaired by HR, are recognized and bound by the MRX/N (Mre11, Rad50, Xrs2 in yeast/NBS1 in higher eukaryotes) complex. Then the protein Sae2/COM1(CtIP in mammals; Ctp in fission yeast) is recruited to the site of DNA damage and bound to Rad50, after being phosphorylated by the kinase Cdc28, which is a homolog of the human CDK1 (Gravel, Chapman et al. 2008; Huertas, Cortes-Ledesma et al. 2008; Uanschou 2009). These findings have been made in

yeast and mammals (Huertas, Cortes-Ledesma et al. 2008). Although the *Arabidopsis* CDK1/CDC28 sequence orthologue CDKA;1 has been implicated in HR, there is currently no evidence, linking it to DSB processing (Dissmeyer, Nowack et al. 2007). This activates the nuclease activity of Mre11 and the initial resection of the DSB, resulting in 50–100 nucleotide ssDNA 3′ overhangs (Borde 2007; Bernstein and Rothstein 2009). In the second step, these overhangs are then further extended. This further processive reaction can occur by two independent mechanisms: one that utilizes Sgs1/BLM and Dna2 and the other using Exo1/hEXO1 (Gravel, Chapman et al. 2008; Nimonkar, Ozsoy et al. 2008; Bernstein and Rothstein 2009; Mimitou and Symington 2009). In *S.cerevisiae* the single stranded DNA is coated by replication protein A (RPA), which recruits the Rad52 epistasis group proteins (Rad51, Rad52, Rad54, Rdh54/Tid1, Rad55 and Rad57) that promote the invasion of the homologous DNA molecule (Krogh and Symington 2004). The strand-exchange protein Rad51 is essential for effective homology search. During synapsis, Rad51 facilitates the formation of a physical connection between the invading DNA substrate and the homologous duplex DNA template, leading to the generation of heteroduplex DNA (D-loop) (Klapstein, Chou et al. 2004; Chen, Yang et al. 2008; Krejci, Altmannova et al. 2012). A D-loop (displacement loop) is a DNA structure, where the two strands of a dsDNA molecule are separated by an invading DNA strand, which is complementary to one of the two main strands, thereby displacing the other one (Kasamatsu, Robberson et al. 1971). The Rad52 epistasis group seems to be well conserved among eukaryotes and orthologues have been found in vertebrates and plants (Bleuyard, Gallego et al. 2005). Like vertebrates *Arabidopsis* possesses six RAD51 paralogues (AtRAD51; AtRAD51B; AtRAD51C; AtRAD51D; AtXRCC2; AtXRCC3. However, only AtRAD51, AtRAD51C and AtXRCC3 are required for HR) (Osakabe, Yoshioka et al. 2002; Bleuyard, Gallego et al. 2005). The role of RAD51, as a recombinase is consistent. After Rad 51 mediated homology search and strand invasion and the formation of a D- loop, the 3´end serves as a starting site for new DNA synthesis, using the intact strand of the sister chromatid or homologous chromosome as a template (Krogh and Symington 2004; Bleuyard, Gallego et al. 2006; Krejci, Altmannova et al. 2012) This is succeeded by branch migration and resolution of the Holliday junctions that are formed during the process (Bleuyard, Gallego et al. 2006). The function of Rad51 is largely conserved among eukaryotes,

its regulators and their functions, however can vary among organisms. Human Rad52, for example, which shares structural and biochemical similarity with yeast Rad52 has not been shown to possess recombination mediator activity (Krejci, Altmannova et al. 2012). The central Rad51 mediator function is carried out by BRCA2 (breast cancer 2) in human cells (Thorslund and West 2007; San Filippo, Sung et al. 2008). RAD52 and BRCA2 homologs have been identified in plants. In contrast to yeast, where Rad52 is essential for HR, mutation of *RAD52* has only a moderate effect on homologous recombination in *Arabidopsis* (Samach, Melamed-Bessudo et al. 2011). AtBRCA2 has been shown to be required for proper meiotic synapsis and the recruitment of AtRAD51 (Seeliger, Dukowic-Schulze et al. 2012).

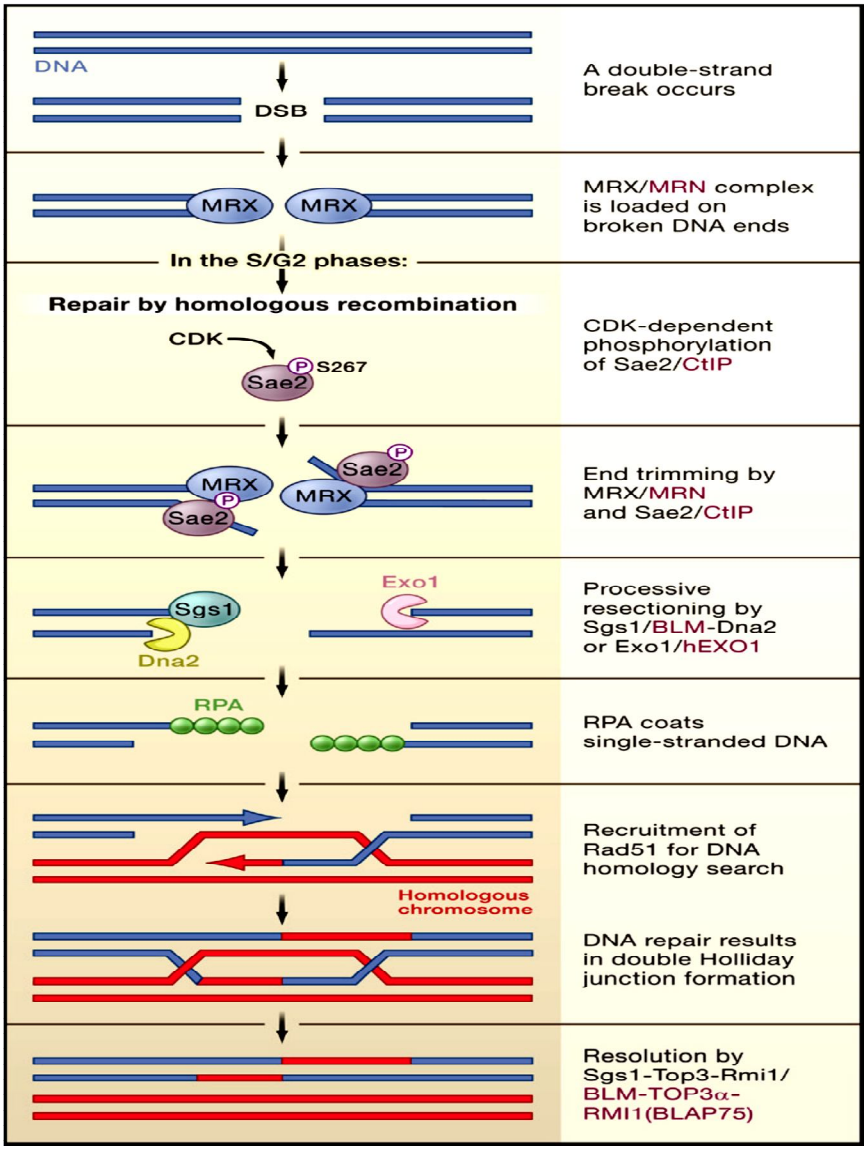


Figure 10 – DSB repair via the HR pathway. MRX is loaded onto DNA ends and recruits Sae2, which is phosphorylated by CDK. After resection of the DSB, the ssDNA overhangs are coated by RPA. A D-loop is formed, in this case with the homologous chromosome. The double Holliday junction is then resolved, which leads to a non-crossover product, in this example (Bernstein and Rothstein 2009).

DSBs are the most toxic of all DNA lesions and can be repaired by means of NHEJ or HR. There is however a biological process, that is essential for sexual reproduction, where DSBs are introduced intentionally to promote HR between maternal and paternal chromosomes, namely meiosis.

3.7 Meiosis

Meiosis is a specialized kind of cell division in sexually reproducing organisms, where a single round of DNA replication is followed by two rounds of nuclear division (meiosis I and II). The process of meiosis begins with a diploid cell, containing a maternal and a paternal copy of each chromosome and produces four haploid gametes. (Edlinger and Schlögelhofer 2011; Osman, Higgins et al. 2011). During meiosis, genetic information between maternal and paternal chromosomes is mutually exchanged, leading to novel combinations of genetic traits in the following generation. This is a significant source of the genetic diversity in sexually reproducing organisms. The first round of nuclear division, meiosis I is referred to, as reductional division, since homologous chromosomes are separated and thus ploidy is reduced. Genetic information between maternal and paternal chromosomes is mutually exchanged via homologous recombination during meiosis I, leading to novel combinations of genetic traits in the following generation, while meiosis II is mechanically similar to mitosis, where sister chromatids are separated (Edlinger and Schlögelhofer 2011). Meiosis I and II both comprise four stages: prophase, metaphase, anaphase and telophase. Prophase I is further divided into five sub-stages: leptotene, zygotene, pachytene, diplotene, and diakinesis (Ma 2006; Kurzbauer 2008). In the leptotene stage chromosomes condense and lateral elements of the synaptonemal complex assemble. The synaptonemal complex (SC) is a protein complex, that forms between homologous chromosomes during meiosis and facilitates pairing and HR (Zickler and Kleckner 1999). During zygotene, pairing of the homologous chromosomes takes place. In order to start homologous recombination, DSBs are formed and repaired, using the nonsister chromatids of the homologous chromosomes. These COs (crossovers) are matured in pachytene. Genetic material of the homologous chromosomes is recombined. The synaptonemal complex is degraded during diplotene, but homologous chromosomes remain tightly

bound at chiasmata (Hamant, Ma et al. 2006). The chromosomes are then further condensed in the diakinesis stage and aligned along an equatorial plane during metaphase I, before the homologous chromosomes are separated in anaphase I and may decondense in telophase I. After completion of the creation of two daughter cells with half the number of chromosomes, meiosis II can take place, which is comparable with mitosis, is also divided into the four stages: prophase II, metaphase II, anaphase II and telophase II and results in the production of four haploid gametes (Figure 11). Meiotic recombination is an important mechanism, that increases genetic diversity among individuals in a population and may have contributed to the success of eukaryotes (Ma 2006). In order for meiotic recombination to take place, a DNA double strand break has to be introduced to facilitate further strand invasion and exchange of genetic material. These DSBs are formed by a protein complex, whose catalytically active subunits are Spo11 proteins.

The Spo11 protein is virtually found in all eukaryotes and shares homology with the A-subunit of topoisomerase VI in the archaeon, *Sulfolobus shibatae.* The Arabidopsis genome encodes, unlike those of mammals and yeast, where only one Spo11 is present, three Spo11 homologues, AtSPO11-1, AtSPO11-2 and AtSPO11-3 (Hartung and Puchta 2000; Shingu, Mikawa et al. 2010; Edlinger and Schlögelhofer 2011). Only AtSPO11-1 and AtSPO11-2 are essential for meiosis, whereas AtSPO11-3 is needed for somatic endoreduplication (Grelon, Vezon et al. 2001; Stacey, Kuromori et al. 2006). As an intermediate of the DNA cleavage process, Spo11 proteins remain covalently linked to the 5´ termini of single-stranded DNA at the incision sites and have to be removed by a downstream process. (Bergerat, de Massy et al. 1997; Keeney, Giroux et al. 1997; Neale, Pan et al. 2005; Edlinger and Schlögelhofer 2011).

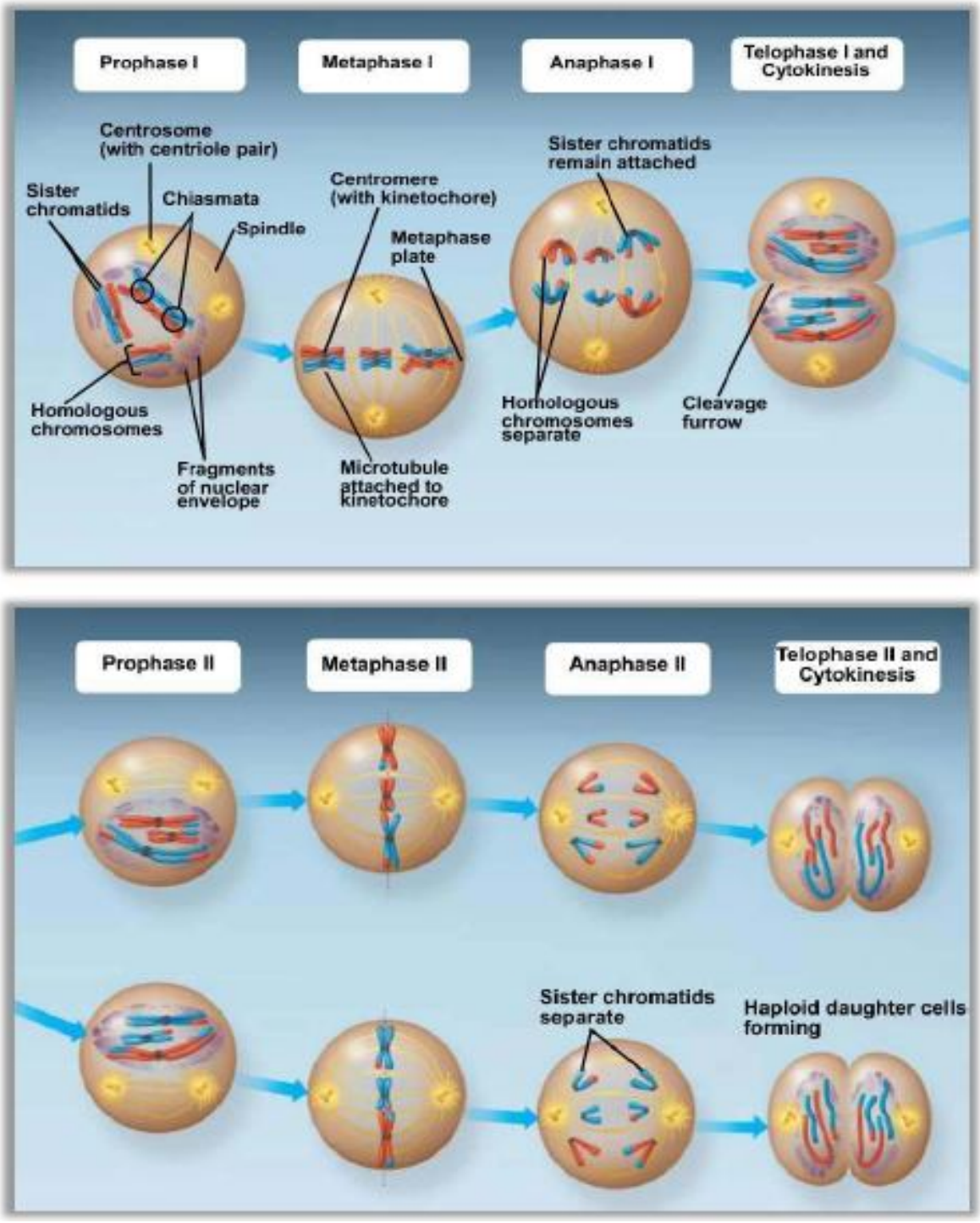


Figure 11 – Overview of meiosis. Four haploid daughter cells are formed, starting form one diploid cell. HR occurs during first meiotic division. See text for description (Campbell NA 2009).

In order to release Spo11 from the DSB site, the DNA is nicked at a distance from the incision site by the endonucleolytic activity of Mre11, in conjunction with Com1/Sae2, and resected towards Spo11 by the 3´-5´exonuclease activity of Mre11 (Longhese, Bonetti et al. 2009; Mimitou and Symington 2009). Spo11 is then released from the

cleavage site with a short DNA oligonucleotide remaining attached to the protein. Long stretches of ssDNA have to be created, that can probe for matching sequences on homologous chromosomes, or sister chromatids, respectively. This is done by 5´-3´ strand resection, starting at the Mre11 mediated ssDNA lesions. The precise mechanisms of DSB processing are not yet fully understood. In the yeasts *S. pombe* and *S. cerevisiae* DNA-end resection is accomplished by the exonucleases Exo1 and Dna2, together with the helicase Sgs1 (Figure 12) (Longhese, Bonetti et al. 2010; Manfrini, Guerini et al. 2010; Garcia, Phelps et al. 2011).

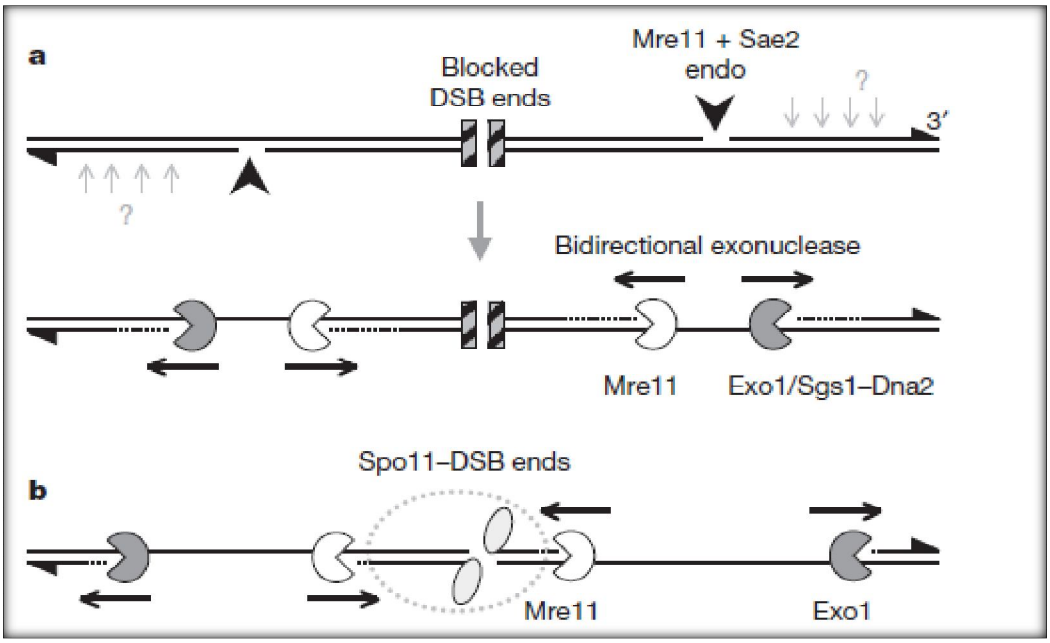


Figure 12 – Model for bidirectional resection of DSBs by Mre11 and Exo1. (a) DSBs with blocked ends cannot be processed by NHEJ, but are processed via HR. Mre11 nicks the single strand in concert with Sae2 and bidirectional resection of the strand commences. (b) In meiotic cells, this blocking of DSB ends is accomplished by covalently bound Spo11 (Garcia, Phelps et al. 2011).

The stretches of ssDNA are coated by the protein RPA, which recruits Rad 52 and accessory proteins, that facilitate the loading of the strand exchange proteins Rad51 and Dmc1 in yeast (Krogh and Symington 2004; Edlinger and Schlögelhofer 2011). In higher eukaryotes the BRCA2 protein has been found to be essential for this step (Longhese, Bonetti et al. 2010). Homologues of the two strand exchange proteins Rad51 and Dmc1have been found in *Arabidopsis.* After DNA loading, these two proteins start with the search for a stretch of DNA with significant homology, with a

strong preference for the homologous chromosome, rather than the sister chromatid (Schwacha and Kleckner 1997; Hamant, Ma et al. 2006; Neale and Keeney 2006). Rad 51 functions in mitosis, as well as in meiosis, while Dmc1 is meiosis specific. The strand invasion process, mediated by the two proteins, can follow one of two pathways: the double-strand break repair (DSBR) pathway or the synthesis-dependent strand-annealing (SDSA) pathway (Maloisel, Bhargava et al. 2004; Ma 2006; McMahill, Sham et al. 2007; Kurzbauer 2008) (Figure 13).

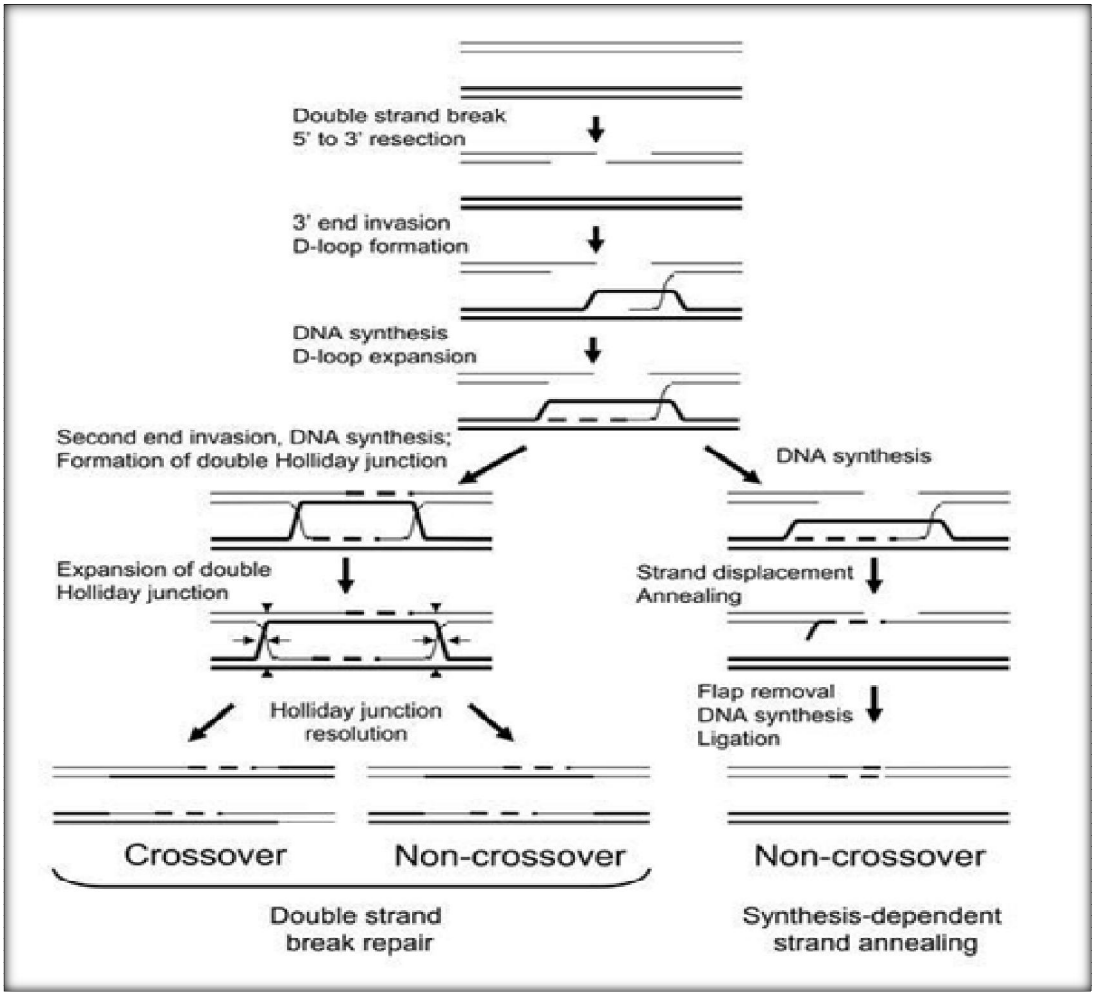


Figure 13 – Models for homologous recombination. The DSBR model (left) and the SDSA model (right). Both start with a DSB, 5′ to 3′ resection to 3′ single strands, the invasion of one end into the intact recombination partner to form a D-loop, and some DNA synthesis. In the canonical DSBR model, the second end invades, leading to the formation of the double Holliday junction, which expand and then can be resolved to either crossover or noncrossover. In the alternative model only noncrossovers can be generated. The D-loop is disrupted by displacement of the extended end. The displaced end is then annealed to its prior partner (Ma 2006).

Both pathways start with the invasion of one end into the intact recombination partner to form a D-loop, and some DNA synthesis using the intact strand as a template. In the canonical DSBR model, 3´-end extension enlarges the D-loop, making it possible for the second strand to pair, leading to the formation of a double Holliday junction (dHJ). This structure expands and can be resolved to either crossover (CO), or non-crossover (NCO). Usually dHJs resolve into crossovers. In the SDSA pathway the D-loop is disrupted by displacement of the extended 3`-end. The displaced end is then annealed to its prior partner and the result is a non-crossover event (Ma 2006; McMahill, Sham et al. 2007).

3.8 The DNA repair protein COM1/SAE2/CtIP

As extensively alluded above, one of the central DNA repair proteins in somatic and meiotic HR is COM1/SAE2/CtIP/Ctp1. It is a highly conserved gene from yeast to humans, that is known to be required for meiotic DSB ends resection by cooperating with the MRX complex and for specific mitotic DNA repair events (Ji, Tang et al. 2012). In 1997 it was simultaneously identified in the budding yeast *Saccharomyces cerevisiae* by two independent research groups and it was termed COM1 (Completion of Meiosis 1) (Prinz, Amon et al. 1997) and SAE2 (Sporulation in the Absence of Spo Eleven) (McKee and Kleckner 1997). In these mutants several events, important for meiosis, are blocked, like homologous pairing, SC formation and meiotic DSB resection, indicating the importance of COM1/SAE2 for meiosis (Ji, Tang et al. 2012). In SAE2 mutants, the normal DSB repair by HR is also blocked in mitotic cells and inverted chromosome duplications are produced (Lobachev, Gordenin et al. 2002). COM1/SAE2 exhibits endonuclease activity, is phosphorylated by the yeast holmologues of the checkpoint kinases ATM and ATR, Tel1 and Mec1, it is one of the first proteins detected at somatic DSB sites and interferes with DNA replication checkpoints (Lisby, Barlow et al. 2004; Clerici, Mantiero et al. 2006; Lengsfeld, Rattray et al. 2007). COM1/SAE2 homologs have been identified in mammals (CtIP) (Schaeper, Subramanian et al. 1998), fission yeast (Ctp1) (Limbo, Chahwan et al. 2007), *Caenorhabditis elegans* (COM-1) (Penkner, Portik-Dobos et al. 2007), *Arabidopsis* (AtCOM1) (Uanschou, Siwiec et al. 2007) and *Tetrahymena* (COM1) (Lukaszewicz, Howard-Till et al. 2010). In all organisms deficiency of the

COM1/SAE2 homologs leads to defects in DSB resection and the following processes. A recent study in rice (*Oryza sativa)* found, that loss of function of the rice homologue OsCOM1 leads to the absence of homologous pairing and recombination, but also entangled nonhomologous chromosomes and massive acentric fragments at anaphase I. These findings lead to the suggestion, that in addition to its established role in HR, OsCOM1 also plays a role in the inhibition of nonhomologous interaction during rice meiosis (Ji, Tang et al. 2012).

The mammalian Sae2 counterpart CtIP is recruited to sites of DSBs and complexes with BRCA1 to control the G2/M checkpoint (Yu and Chen 2004; Yu, Fu et al. 2006). It is phosphorylated by ATM and promotes HR in cooperation with MRN, in response to DNA DSBs (Greenberg, Sobhian et al. 2006; Sartori, Lukas et al. 2007). Mutations in CtIP lead to hypersensitivity against DSB inducing agents in mammalian cells and to early embryonic lethality of knockout mice (Chen, Liu et al. 2005; Sartori, Lukas et al. 2007).

The *Arabidopsis* homologue, AtCOM1 was first described as a new growth related *Arabidopsis* gene strongly induced by ionizing radiation, AtGR1 (*Arabidopsis thaliana* Gamma response 1) with an expression profile similar to that observed for several plant cell cycle related proteins (Deveaux, Alonso et al. 2000). AtGR1 expression was shown to be expressed at basal levels in mitotically dividing cells and at a strongly enhanced level in endoreduplicating cells. On the other hand ionizing radiation-induced DNA damage led to transcriptional activation and protein accumulation of AtGR1, especially in germ line tissue, that never undergoes endoreduplication (Deveaux, Alonso et al. 2000). AtCOM1 is essential for male and female meiosis, it acts downstream of AtSPO11-1 and upstream of AtDMC1 during meiosis. It is needed for regular turnover of SPO11-1 and processing of meiotic DSBs (Uanschou, Siwiec et al. 2007). Exposure to IR (ionizing radiation) leads to a strong accumulation of AtCOM1 transcript in somatic tissues (Deveaux, Alonso et al. 2000; Uanschou, Siwiec et al. 2007). This transcriptional increase was shown to be induced by the checkpoint kinase ATM (Garcia, Bruchet et al. 2003). Interestingly, only treatment with the interstrand crosslinker and alkylating agent Mitomycin C (MMC) leads to inhibition of growth of homozygous Atcom1-1 mutant seedlings, while all other treatments, causing different types of DNA damage, including IR, did not affect homozygous mutants, compared to heterozygous mutant and wildtype

seedlings. Thus, in somatic cells, AtCOM1 seems to be needed for the repair of interstrand crosslinks, but not for other types of DNA damage. These results led to the assumption, that upon DNA damage, ATM induces the transcription of a broad spectrum of DNA repair related genes, but the actual type of damage determines which gene products are effectively needed for repair (Uanschou, Siwiec et al. 2007). *Atcom1-1* mutants are sterile, due to severe DNA fragmentation during meiosis, but vegetative development is not effected under non-stress conditions. *Atspo11-1/Atcom1-1* double mutant plants do not show this DNA fragmentation phenotype, indicating, that the fragmentation is caused by the inability to repair meiotic DSBs without AtCOM1(Kurzbauer 2008).

3.9 Analysis of the AtCOM1/GR1 promoter

A promoter analysis is fundamental to asses in which tissue and developmental stage a specific gene is expressed and which environmental cues trigger activity of the promoter. *AtCOM1* is an example of a DNA repair gene, essential for homologous recombination. To learn more about the mechanisms of DNA damage repair, it was of great interest to study the cell cycle, DNA damage and tissue specific control of the *AtCOM1* promoter.

The dynamics of induction of the *AtCOM1* promoter were described in a previous diploma thesis (Kurzbauer 2008). It was known, that *AtCOM1* transcription is strongly induced after treatment with ionizing radiation (Deveaux, Alonso et al. 2000), as well as, that AtCOM1 mediates resistance against the intrastrand crosslinker Mitomycin C (Uanschou, Siwiec et al. 2007). It was also known, that other genotoxic treatments did not affect growth of *Atcom1-1* mutant seedlings, but there was no information about changes of *AtCOM1* transcription levels in response to other genotoxins, aside from MMC and IR. So, one of the objectives of the thesis was to analyze the effects of different DNA damaging substances on *AtCOM1* promoter activity, via qPCR analysis. It could be verified, that after treatment with IR, *AtCOM1* expression is strongly induced in two week old seedlings and that this induction depends on ATM, as it had been shown before (Deveaux, Alonso et al. 2000; Ricaud, Proux et al. 2007). Seedlings treated with the intrastrand crosslinker MMC only showed strong induction of *AtCOM1* expression after overnight incubation. An explanation for this

observation might be, that DSBs are not directly introduced, but occur later, during S-phase (Sognier and Hittelman 1986; Kurzbauer 2008). This effect could not be observed in *Atatm* mutant seedlings and was severely diminished in *Atatr* mutants. Treatment with the DNA scission agent Bleomycin, which introduces DSBs and SSBs lead to a strong induction of *AtCOM1* expression already after two hours. Again this effect was abolished by the mutation of *AtATM* and reduced by the mutation of *AtATR*. The other tested genotoxic agents, Cisplatin, Hydroxyurea (HU) and Methylmethane-sulfonate (MMS) had no effect on *AtCOM1* expression. Cisplatin forms Cisplatin-DNA adducts, that may block replication and transcription, and it also forms intrastrand crosslinks between adjacent purines and other adducts, that are mainly repaired via nucleotide excision (Zamble and Lippard 1995; Moggs, Szymkowski et al. 1997; Kurzbauer 2008). HU and MMS lead to slowing down or even blocking of replication, which might indirectly lead to the occurrence of DSBs, but no DSBs are directly introduced (Beranek 1990; Lundin, North et al. 2005; Kurzbauer 2008).
It could be shown, that the induction of AtCOM1 is dependent on the nature of the DNA damage. Directly introducing DSBs by IR and Bleomycin leads to a strong and fast induction of transcription, while MMC, which might lead to a delayed and indirect occurrence of DSBs, shows delayed transcriptional induction of AtCOM1. Agents, that introduce other lesions have no effect (Kurzbauer 2008).

The second objective of the thesis was, to conduct a promoter deletion analysis, combined with a GUS assay, in order to learn about the cis- and trans-regulatory factors, controlling the *AtCOM1* promoter. The analysis of the *AtCOM1* promoter started with an in silico promoter prediction, that involved the web-tools SCOPE and WEEDER (Pavesi, Mereghetti et al. 2004; Carlson, Chakravarty et al. 2007) to screen for consensus sequences of transcription factor binding sites (Uanschou 2009). The assumed promoter region has been set to about 1000 base pairs (bp) in length and reaches into an upstream gene of opposite orientation with unknown function. It contains two putative DOF (DNA-binding with One Finger) transcription factor binding sites (-644; -524), three putative bZIP (basic-leucine zipper) transcription factor binding sites (-587; -536; -517) and one putative E2F transcription factor binding site (-311). The 5´UTR (untranslated region) is 284 bp long and interrupted by an intron (Figure 14) (Kurzbauer 2008; Uanschou 2009).

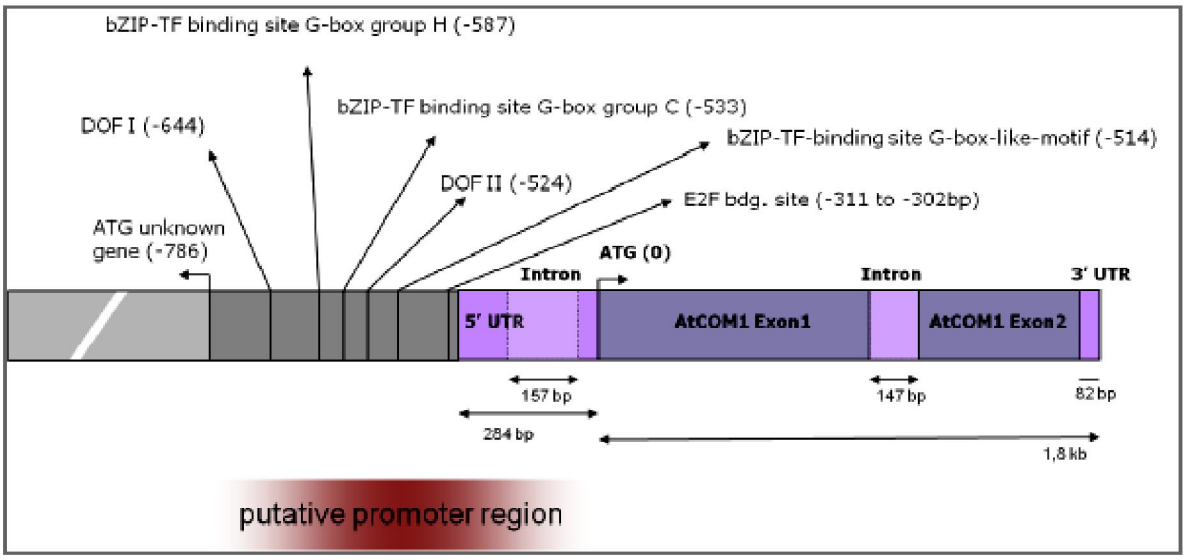


Figure 14 – The putative promoter region of *AtCOM1* contains two putative DOF, three bZIP and one E2F transcription factor binding sites. The gene is 1,8 kb in length and consists of two exons, that are separated by a 147 kb long intron (Kurzbauer 2008).

Several promoter fragments were created by PCR and ligated into the vector pCBK04, substituting a CaMV 35S promoter, in front of the GUS reporter gene. In a GUS assay, the gene product of the GUS gene - β-glucuronidase – hydrolyzes 5-bromo-4-chloro-3-indolyl glucuronide (X-Gluc) to glucuronic acid and 5-bromo-4-chloro-indoxyl. The latter is oxidized to the dark blue colour 5,5'-dibromo-4,4'-dichloro-indigo that is directly visible without further treatment (Kurzbauer 2008). So, if the promoter fragments are active, they should drive the GUS gene, leading to blue staining. These pCBK04 vectors, carrying different *AtCOM1* promoter fragments were used to transform plant cell suspension cultures via *Agrobacterium tumefaciens*. Soon it became apparent, that the E2F binding site is essential for promoter activity. Only promoter fragments containing the E2F site were able to drive GUS activity in cell culture, while cell cultures, transformed with fragments lacking the E2F binding site never showed staining, independent of fragment length. (Figure 15a and 15b)

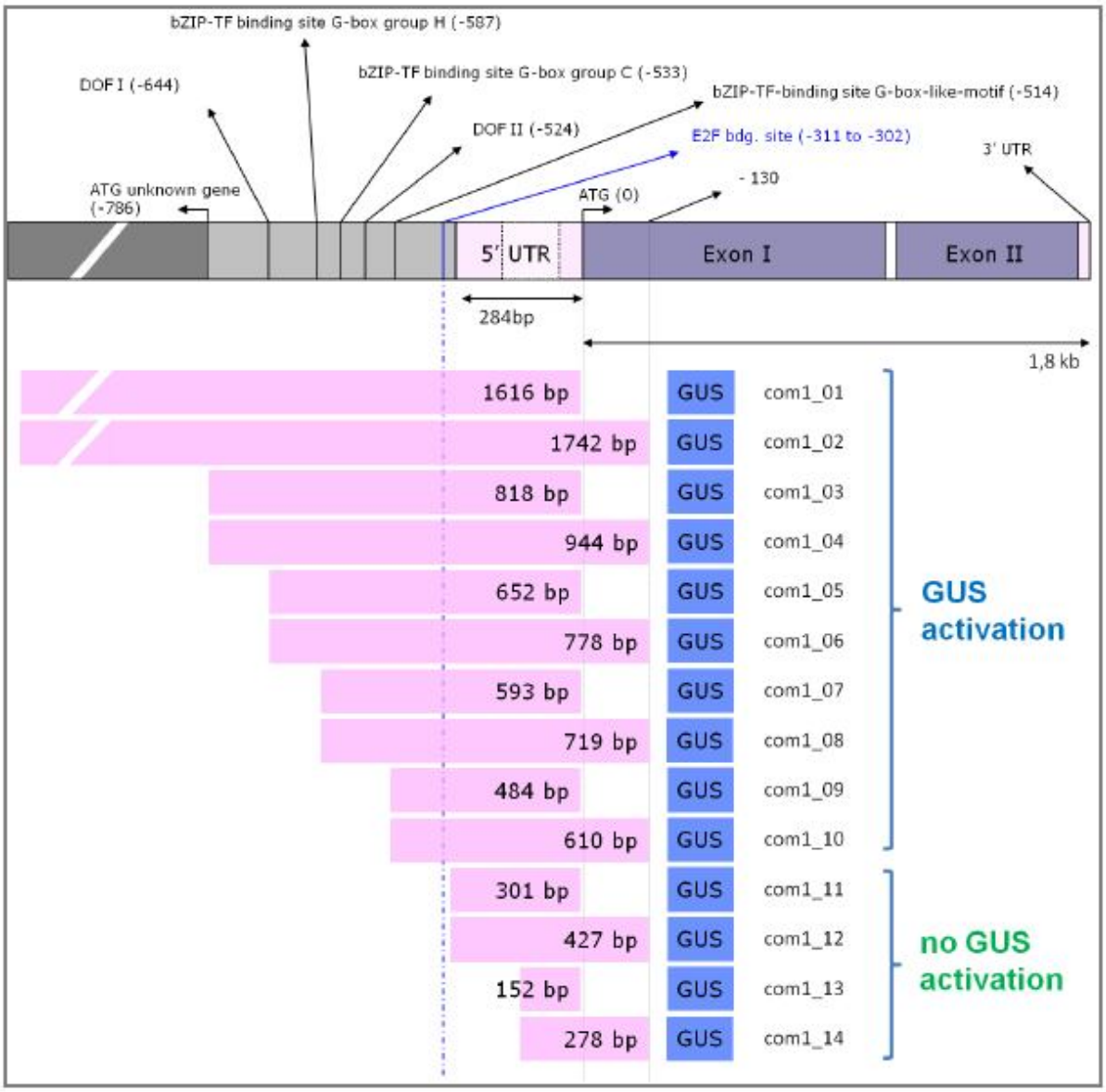


Figure 15a – Overview of the GUS assay with plant cell culture, transformed with vectors containing promoter fragments of different length, controlling the reporter GUS gene. Only promoter fragments containing the E2F transcription factor binding site activated the reporter gene (Kurzbauer 2008).

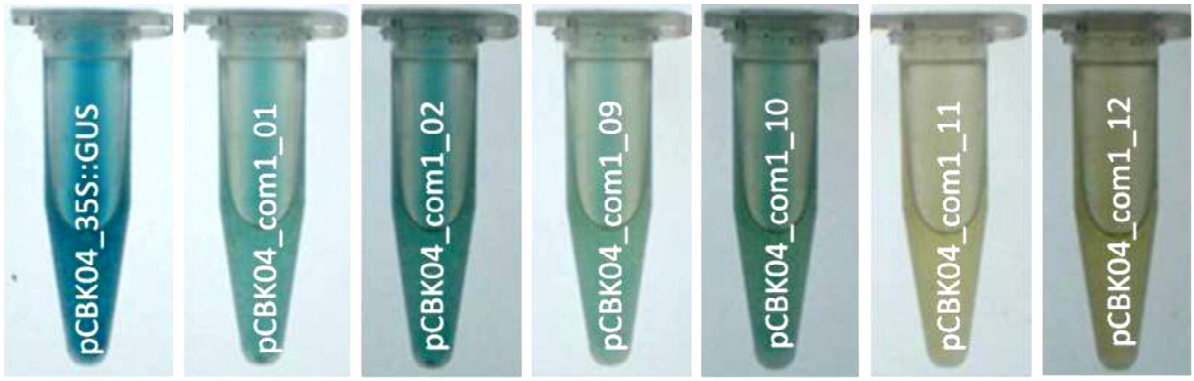


Figure 15b – GUS staining of cell suspension cultures. The original pCBK04 vector was used as a positive control (left). Fragments com1_11 and com1_12, that lack the E2F site could not activate GUS (right), while the other fragments, containing the E2F site did.

To further support the theory, that the E2F site is essential for *AtCOM1* expression, the E2F site of a promoter fragment (com1_10), that had activated GUS in the previous experiment was mutated. This mutation of the E2F binding sequence lead to a loss of promoter activity. These results provide strong evidence, that E2F transcription factors play an important role in controlling the *AtCOM1* promoter (Kurzbauer 2008).

3.10 Objectives

The aim of this thesis was to study the dynamics of cell cycle and tissue specific expression of *AtCOM1* and to identify cis and trans acting factors, that affect the activity of the *AtCOM1* promoter.

There were four main objectives for this project.

1.) The first objective was to verify the findings, that had been made previously in an *AtCOM1* promoter analysis by Marie-Therese Kurzbauer. It had been shown, that a specific E2F transcription factor binding site in the *AtCOM1* promoter is essential for promoter activity. These findings had been made in cell suspension culture, where most genes are upregulated. In study presented here the role of the E2F site for promoter activity was to be tested in intact plants, where expression of genes is generally anticipated to be tightly regulated, in contrast to cell suspension culture.

In order to determine the effect of the E2F transcription factor binding site on *AtCOM1* promoter activity a GUS assay was conducted. For this assay plant lines were used, that had been previously transformed with selected *AtCOM1* promoter-GUS fragments (Kurzbauer, 2008).

Another means to investigate the importance of the E2F site for promoter activity was to compare *AtCOM1* mRNA levels of an RBR RNAi line and wildtype plants, by quantitative real time PCR (qPCR). RBR is a repressor of E2F activity, so the *AtCOM1* mRNA level of the RBR RNAi was expected to be lower, than in wildtype, if E2F activity is important for *AtCOM1* promoter activity.

2.) The second objective was to determine in which tissues and at what developmental stages the *AtCOM1* promoter is active. In order to answer this question GUS assays were conducted with mature plants and seedlings carrying selected *AtCOM1* promoter-GUS fragments. Additionally, the *AtCOM1* mRNA level of specific tissues was determined by qPCR.

3.) The third objective was to analyze, which of the six *Arabidopsis* E2F transcription factors actually bind to the *AtCOM1* promoter. Therefore, we aimed, in collaboration with other laboratories, at demonstrating binding of E2F TFs to the *AtCOM1* promoter by Chromatin immunoprecipitation (ChIP).

4.) The last objective was to dissect the E2F dependent regulation of the *AtCOM1* promoter by analyzing the effects of the different E2F transcription factors on *AtCOM1* promoter activity. The chosen approach was to analyze the *AtCOM1* mRNA levels of different mutant and over-expressing *E2F* lines by qPCR.

4 Results

4.1 The E2F binding site is necessary for *AtCOM1* promoter activity in intact plants

To test, the dynamics of *AtCOM1* promoter activity in plants, GUS assays were performed using different transgenic plant lines, having been transformed with *AtCOM1* promoter fragments, that control the GUS gene. The fragments have been ligated into the plant vector pCBK04, replacing the constitutively active Cauliflower mosaic virus 35S promoter and transformed into wildtype plants, via *Agrobacterium tumefaciens* (Kurzbauer 2008). The GUS gene encodes a β-glucuronidase, that hydrolyzes 5-bromo-4-chloro-3-indolyl glucuronide (X-Gluc) to glucuronic acid and 5-bromo-4-chloro-indoxyl. The latter is then oxidized to the dark blue dye 5,5'-dibromo-4,4'-dichloro-indigo.

It had been shown in a previous *AtCOM1* promoter study, that a putative E2F transcription factor binding site in the *AtCOM1* promoter is necessary for promoter activity in plant cell suspension cultures (Kurzbauer 2008). A variety of promoter fragments had been tested for their potential to drive the GUS gene by transformation into plant cell suspension culture cells and GUS assays. Soon it had become apparent, that promoter fragments lacking the E2F site never activated the reporter gene (see Figures 15a and 15b). The objective of the first experiment of this thesis was to test, whether the presence of the putative E2F binding site has the same effect on GUS activation in plants, as it does in cell suspension culture cells. It had been shown, that *AtCOM1* is upregulated in response to ionizing radiation (Deveaux, Alonso et al. 2000; Ricaud, Proux et al. 2007; Kurzbauer 2008). So the effect of treatment with ionizing radiation on the GUS activity in the transformed plants was to be tested as well.

Three promoter fragments had been chosen for transformation of wild type plants, namely com1_2, com1_10 and com1_12 (Figure 16). com1_2 is the longest fragment (1742bp). It carries the full length wildtype promoter sequence, including predicted binding sites for other transcription factors (bZIP, DOF) besides E2F. A shorter fragment was com1_10 (610bp). It includes the putative E2F site, but does not

contain the other predicted TF binding sites. com1_12, the shortest fragment (427bp) lacks the putative E2F site and all of the other predicted TF binding sites. Wild-type plants were transformed, seeds harvested and positive transformants selected by using the BASTA resistance gene carried by pCBK04. The seeds of these selected transformants were sown on soil and selected for BASTA resistance again. For every promoter fragment, seeds of a single individual transformant of the generation T_1 were then used for the GUS assays. So the GUS assays were conducted with plants of the generation T_2.

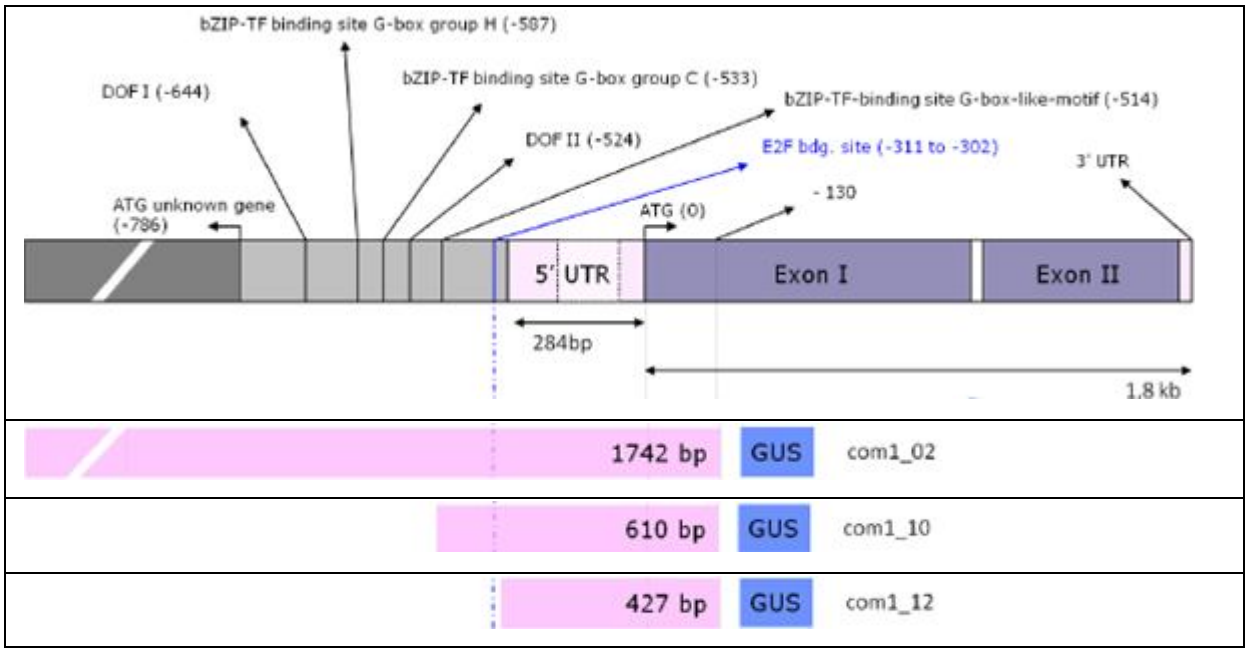


Figure 16 – The three promoter fragments, that were chosen for transformation and GUS assays. com1_2 is the longest fragment (1742bp). It contains the full length wildtype promoter and includes the 5´UTR and the first 130bp of Exon I of the *AtCOM1* gene. com1_10 is a shorter fragment (610bp), including the E2F site of the *AtCOM1* promoter, but none of the other predicted transcription factor binding sites. com1_12 is the shortest fragment used (427bp) and does not include the E2F site (picture modified from Kurzbauer, 2008).

In consideration of the results of the GUS assays in cell suspension culture, it was expected, that the two promoter fragments, including the putative E2F site (com1_2 and com1_10) would trigger GUS activity in plants. The expectation for the fragment, lacking the predicted E2F transcription factor binding site was, that it would not activate the reporter gene.

It was known, that *AtCOM1* is upregulated in response to ionizing radiation and that this upregulation depends on AtATM (Ricaud, Proux et al. 2007; Kurzbauer 2008). The effect of AtATM on the activity of the GUS reporter gene in the transformed plants was thus also to be tested. For this reason, plants of the generation T_2, carrying the pCBK04_com1_02 and 10 reporter constructs were crossed to *atm* mutant plants. These two constructs include the putative E2F site (Figure 16). Generation F_2 was screened for plants, being homozygous for *atm* and carrying the reporter gene construct (Kurzbauer 2008). These plants were included in the GUS assays. The expectation was, that plants carrying reporter gene constructs, containing the predicted E2F site would show GUS activity, and the promoter activity was expected to be lost in homozygous *atm* mutant plants.

Plants of five different genotypes were thus used for the GUS assays, namely com1_2, com1_10, com1_12 (see Figure 16) and com1_2 x *atm,* as well as com1_10 x *atm.* Plants of the generations T_2 and F_2 for the crossings, respectively were grown on soil and selected for BASTA resistance. After six weeks inflorescences, cauline leaves and mature rosette leaves were cut off, fixed in a formaldehyde solution and used for the GUS assays. Ten individual plants of each plant line were tested. Plants carrying the promoter fragment com1_2 (carrying the E2F site) showed GUS staining in the female reproductive organs (gynoecium) as well as at the cutting sites of inflorescences. Cauline leaves showed strong staining at the base of the leaf and there was a faint staining visible in the vascular tissue of mature rosette leaves (Figure 17). The identical staining pattern could be observed for plants carrying the promoter fragment com1_10 (carrying the E2F site) (Figure 17). Homozygous *atm* mutant plants, carrying the com1_2 promoter fragment were not stained in inflorescences and mature rosette leaves. They did however show GUS staining in the base of cauline leaves (Figure 17). The identical staining pattern was observed for homozygous *atm* plants, carrying the com1_10 promoter fragment (Figure 17). No staining could be observed in any of the tested tissues (inflorescences, cauline leaves, mature rosette leaves) of plants carrying the promoter fragment com1_12 (lacking the E2F site) (Figure17).

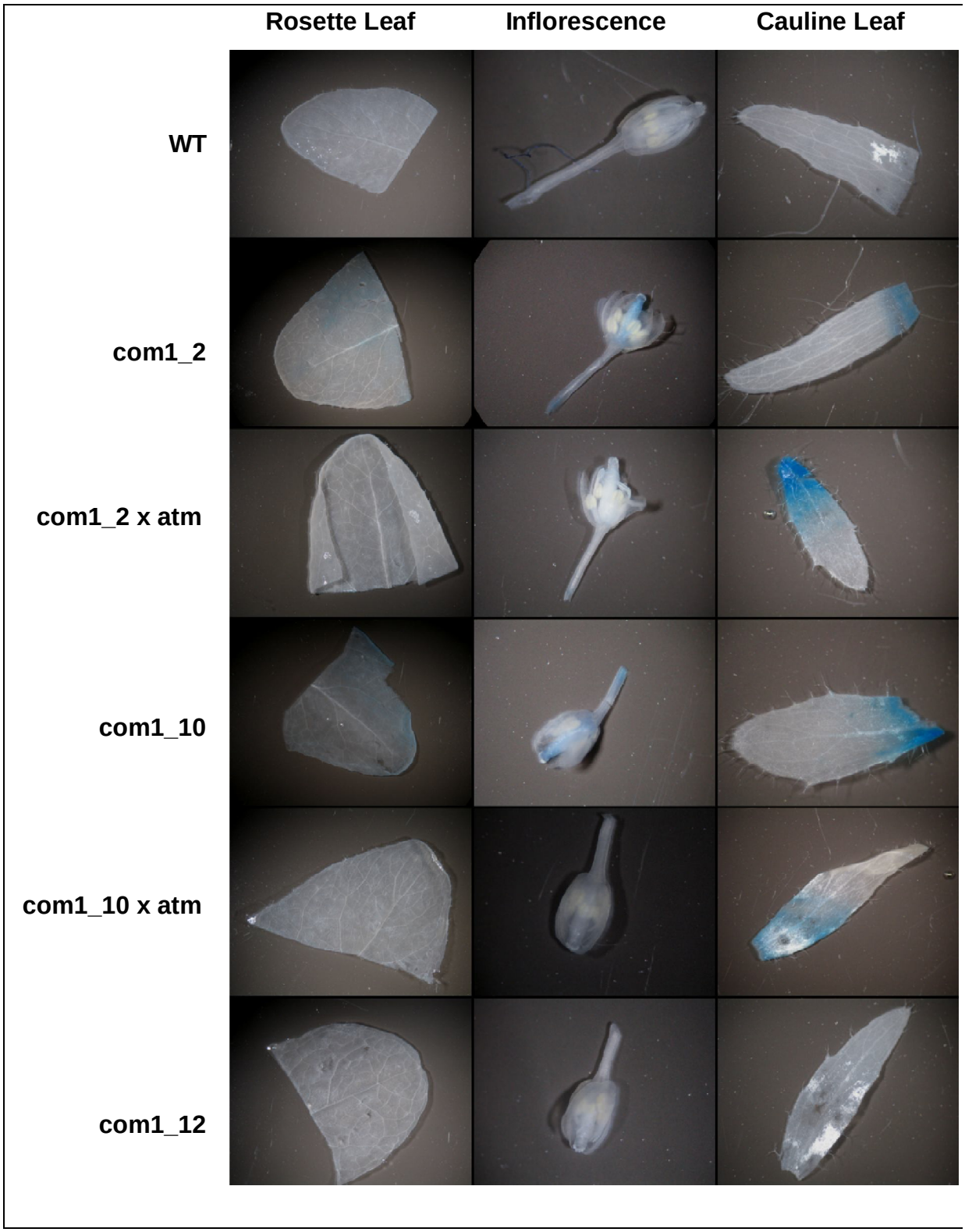


Figure 17 – GUS staining of plants, transformed with different *AtCOM1* promoter fragments (Figure 16). Only plants, transformed with a promoter fragment, including the E2F site (com1_2, com1_10) showed staining. Homozygous *atm* mutants, transformed with these fragments, were only stained at the base of cauline leaves.

The fact, that only plants of the line com1_12 (lacking E2F site; see Figure 16) did not show GUS staining in any of the tested plant parts (inflorescences, cauline leaves, mature rosette leaves) (Figure 17) suggests, that the E2F site plays an important role in controlling the *AtCOM1* promoter and is essential for the artificial promoter fragments to drive the expression of the GUS reporter gene. All plants, carrying one of the two promoter fragments, com1_2 or com1_10 (both including the E2F site; see Figure 16) showed at least faint GUS staining in all of the tested plant parts (inflorescences, cauline leaves, mature rosette leaves) (Figure 17). Activity of the GUS reporter gene was not observed in inflorescences and rosette leaves of homozygous *atm* mutant plants, carrying one of the two fragments, com1_2 and com1_10. There was however GUS staining visible at the base of cauline leaves of these plants (Figure 17). The GUS activity in the tips of the gynoecia, the cutting sites of inflorescences and the vascular tissue of mature rosette leaves of plants, carrying the promoter fragments com1_2 or com1_10, could be caused by oxidative stress. Oxidative damage is a major stress in plants, inducing the production of ROS (reactive oxygen species) and free radicals (Wise and Naylor 1987). These radicals can cause DNA damage (Tuteja, Ahmad et al. 2009). It has been shown, that *AtCOM1* is upregulated in response to treatment with H_2O_2 (Deveaux, Alonso et al. 2000). The GUS activity of plants, carrying the promoter fragments com1_2 or com1_10 is lost in the mentioned tissue types (tips of gynoecia, cutting sites of inflorescences, vascular tissue of mature rosette leaves) in a homozygous *atm* background (Figure 17). These findings suggest, that ATM is required for *AtCOM1* promoter activity in response to oxidative stress. The base of cauline leaves showed GUS activity in plants, carrying either one of the promoter fragments com1_2 or com1_10, irrespective of the ATM status (Figure 17). At the base of cauline leaves, the leaf meristem is situated, harbouring many dividing cells (Gudesblat and Russinova 2011). The gene *AtCOM1* is known to be expressed at basal levels in mitotically dividing cells (meristematic tissues and organ primordia) (Deveaux, Alonso et al. 2000). The finding, that *AtCOM1* promoter activity is not altered in dividing cells by mutation of *ATM* in our experimental setup suggests, that the basal *AtCOM1* promoter activity in dividing cells is independent of ATM.

The next step was to conduct the GUS assays with ten individual plants from each of the five transgenic plant lines, after exposure to 100 Gy of ionizing radiation. The

same parts of the plants (inflorescences, cauline leaves, mature rosette leaves), as in the previous GUS assays were cut and GUS stainings were performed, 45 minutes post irradiation, when the upregulation of *AtCOM1* in response to IR is strongest (Deveaux, Alonso et al. 2000). The assumption, that GUS staining would be enhanced after IR, which leads to a variety of DNA lesions, including DSBs, could not be confirmed, as the staining patterns and intensities of the different transgenic lines did not change significantly (Figure 18).

Perhaps a GUS assay is not the adequate method to compare expression levels. It is rather a qualitative method, to determine, whether a promoter is active or not. To determine, whether *AtCOM1* promoter activity is altered in response to IR, in the five transgenic plant lines, qPCR experiments, analyzing the expression level of GUS before and after irradiation, would probably give useful results.

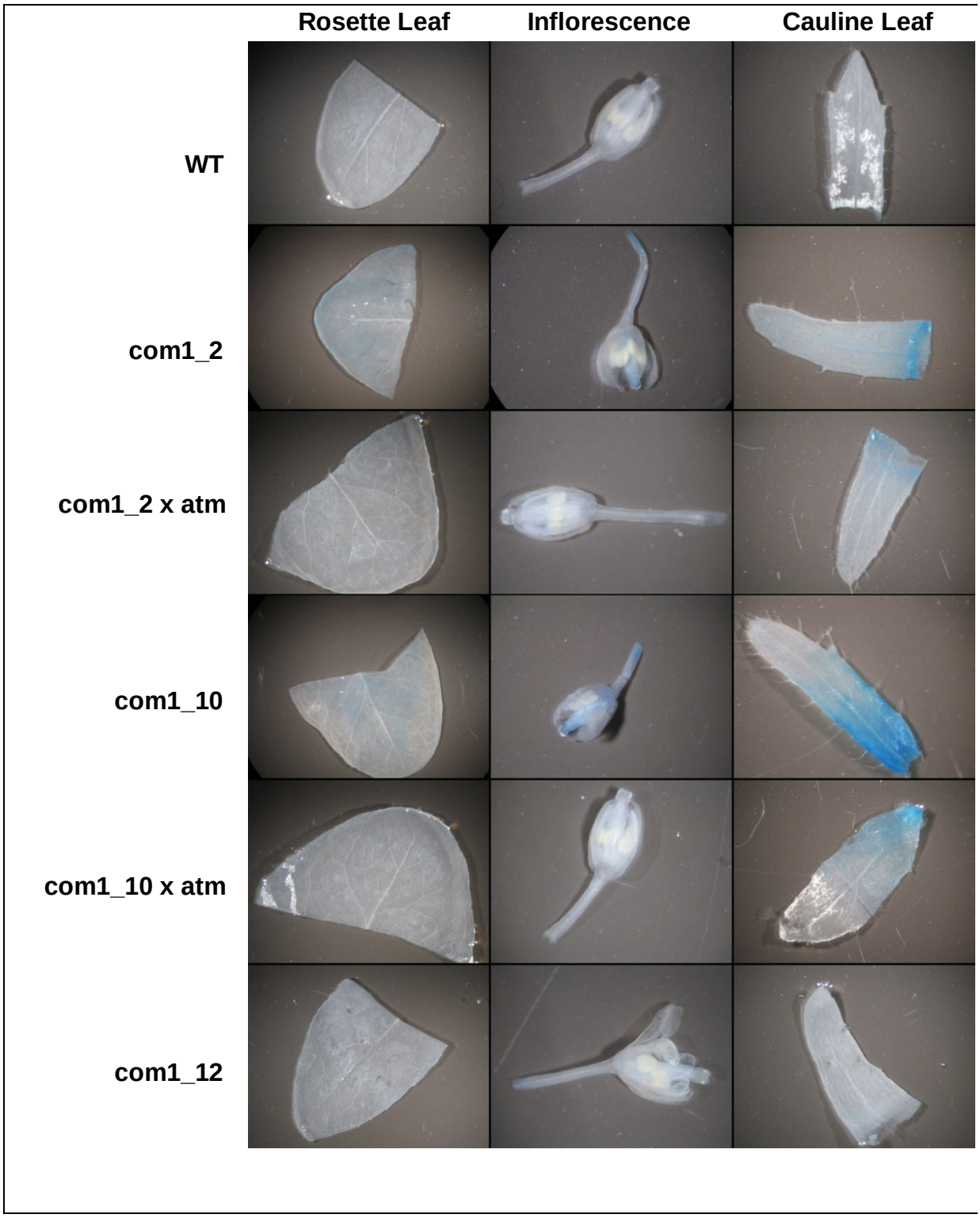


Figure 18 – GUS staining of plants, transformed with different *AtCOM1* promoter fragments after treatment with 100 Gy of IR. Staining patterns like in untreated counterparts (Figure 17). Only plants, transformed with a promoter fragment, including the E2F site (com1_2, com1_10) showed staining. Homozygous *atm* mutants, transformed with these fragments, were only stained at the base of cauline leaves.

4.2 The AtCOM1 promoter is active in dividing cells and this basal promoter activity is independent of ATM

In the previous GUS assays it could be shown, that the E2F site in the *AtCOM1* promoter is essential for promoter activity. It was also shown, that *AtCOM1* promoter activity in response to oxidative stress depends on ATM. A basal *AtCOM1* promoter activity in dividing cells was found to be independent of ATM.

In this experiment, the objective was to confirm the findings, that the *AtCOM1* promoter is active in dividing cells and that this basal promoter activity is independent of ATM. Again, GUS assays were conducted with plants of the five transgenic lines (Figure 16) com1_2, com1_10, com_12, com1_2 x *atm* and com1_10 x *atm.* To avoid tissue injury and resulting oxidative stress, as well as to study the activity of the promoter fragments in dividing cells, 8 day old, intact seedlings were used. Seedlings of the five transgenic lines were grown in liquid medium and 8 days after germination, the GUS assays were conducted. No staining could be observed in seedlings, carrying the promoter fragment com1_12 (lacking the E2F site; see Figure 16) (Figure 19). Seedlings, carrying either of the promoter fragments com1_2 or com1_10 (including E2F site; see Figure16) showed GUS staining in emerging true leaves, root tips and emerging side roots. These regions of the seedlings, containing a large number of rapidly dividing cells. The other parts of the seedlings, carrying the promoter fragments com1_2 or com1_10 were not stained (Figure 19). The identical staining pattern was observed in homozygous *atm* mutant seedlings, carrying one of the fragments com1_2 or com1_10 (Figure19). These results confirm the assumption, that the *AtCOM1* promoter is active in dividing cells under non-stress conditions. The basal activity of the *AtCOM1* promoter is independent of ATM. It was also shown again, that the E2F site is essential for *AtCOM1* promoter activity.

In the next step, the GUS assays were repeated with seedlings, that had been exposed to 100Gy of ionizing radiation. The assays were conducted 45 minutes post irradiation. Analogous to the previous experiment, the staining patterns and intensities could not be altered by radiation treatment in any of the five transgenic lines (Figure19).

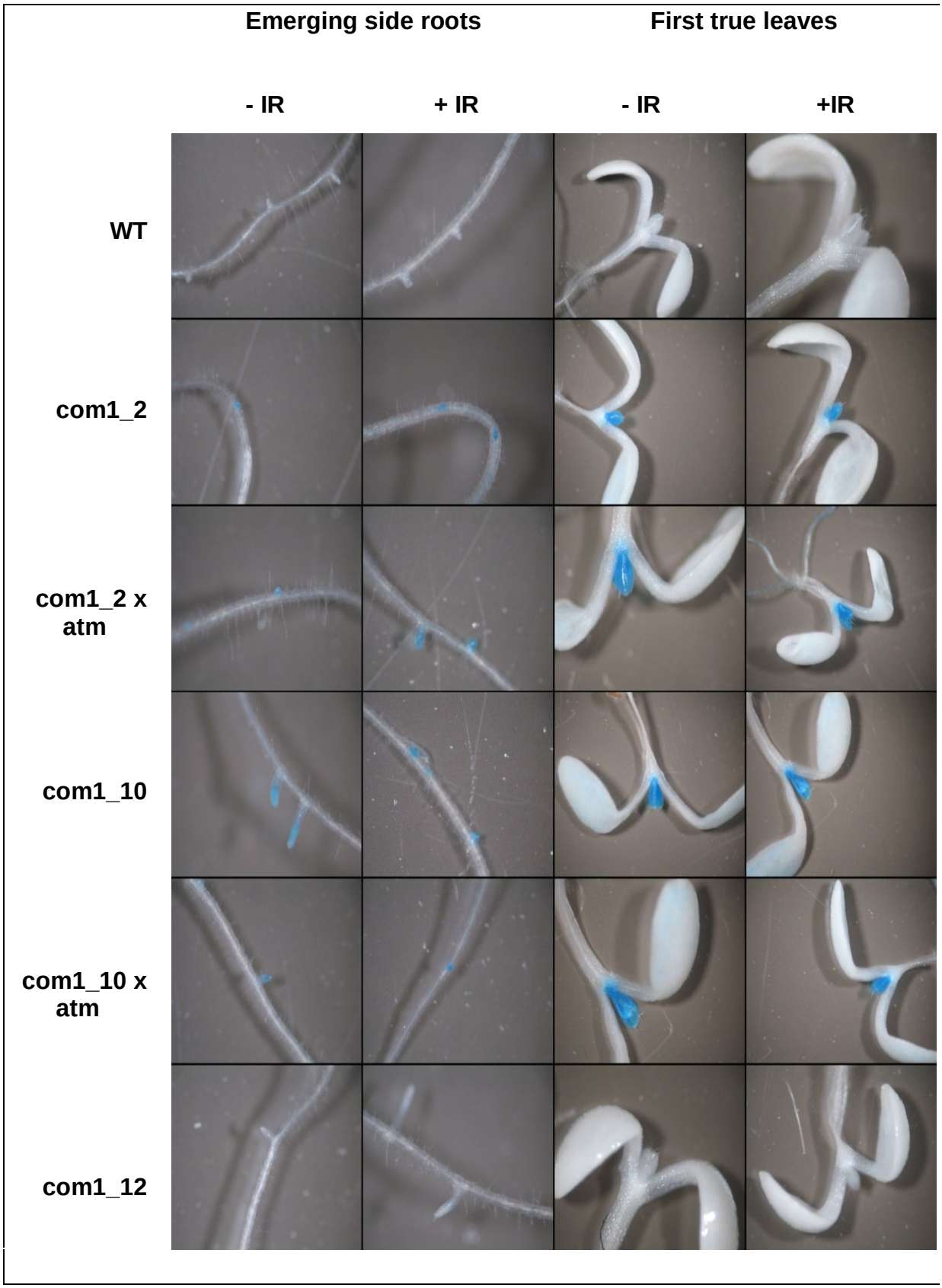


Figure 19 – GUS staining of transformed seedlings. Only the seedlings transformed with an *AtCOM1* promoter fragment, containing the E2F site (Figure 16) showed GUS staining. The staining was not altered by IR.

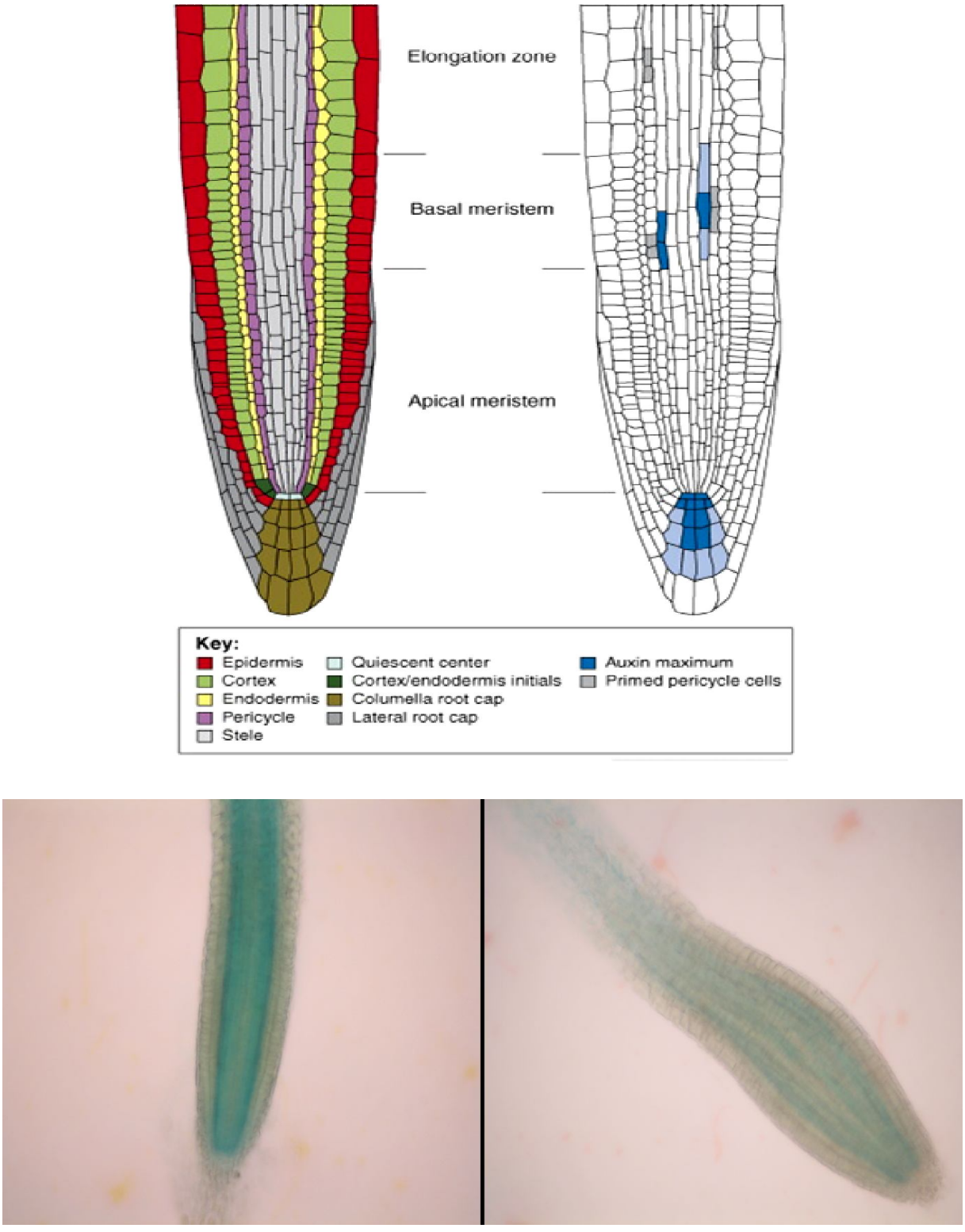


Figure 20 – Above: cell layers of the *Arabidopsis* root tip (Peret, De Rybel et al. 2009). Below left: GUS staining of a com1_2 root tip. Below right: GUS staining of a com1_10 root tip.

In order to verify, that the *AtCOM1* promoter is only active in mitotic cells, the next step was to repeat the GUS assays with longer formaldehyde fixation (1h instead of 30 min), thereby confining GUS staining to those cells, in which the promoter fragments are active and GUS is expressed. For these assays only seedlings, of the two lines, com1_2 and com1_10 (carrying E2F site; see Figure 16) were used. The results for both transgenic lines were identical. It could be shown, that GUS activity is restricted to root tip cells undergoing mitosis. Only endodermis and pericycle cells were stained (Figure 20).

4.3 Expression of *AtCOM1* in first true leaves

In the previous GUS assays, it was shown, that artificial *AtCOM1* promoter fragments show at least a basal activity and drive the GUS reporter gene in mitotic cells. This basal promoter activity depends on the presence of an E2F binding site in the promoter region, but not on AtATM. To differentiate requirements of basal AtCOM1 promoter activity from those under genotoxic stress conditions and to corroborate the earlier findings of cell cycle dependency we analysed the native AtCOM1 promoter in dividing cells. Cells in emerging first true leaves are rapidly dividing. Proportionally, the number of mitotically active cells is highest in young seedlings, with a peak six days post germination (DAG). Sixteen days after germination most cells in the two first emerging leaves have terminated dividing and further leaf growth is accomplished by cell expansion. In this experiment, *AtCOM1* expression levels in first true leaves at different time points post germination were compared. qPCR experiments were conducted, to test *AtCOM1* mRNA levels in wildtype first true leaves. The mRNA was extracted from tissues harvested and snap frozen in liquid nitrogen 6, 8, 10 and 16 days after germination (DAG). The RNA was then converted to cDNA with a cDNA synthesis kit containing random hexamer primers. To test whether the cDNA samples contained contaminations of genomic DNA, a PCR was conducted. For this PCR *ACTIN 2/7* primers were used. Because the two primers bound in different exons, it was possible to distinguish between cDNA and genomic DNA. The amplicon lengths were 180bp for cDNA and 278 bp for genomic DNA (Figure 21). Uncontaminated cDNA was used for the qPCRs.

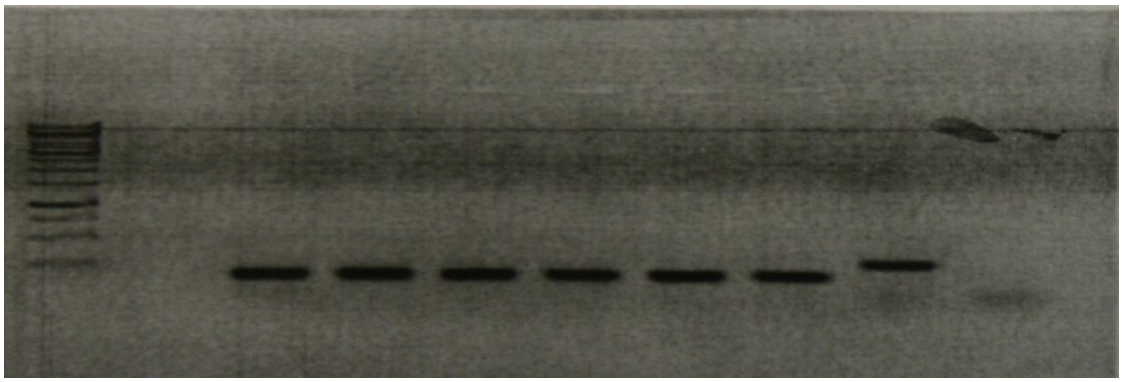


Figure 21 – Example of a control gel to eliminate the possibility of gDNA (genomic DNA) contaminations. The first six lanes contain cDNA samples. The higher band in the seventh lane is the amplicon of a gDNA control. In the last lane a non-template control was loaded.

The qPCRs were conducted, using primers for the *AtCOM1* gene and for the reference gene *ACTIN 2/7*. This is a so-called housekeeping gene, that is homogenously expressed in all tissues. All measured *AtCOM1* mRNA levels were normalized to *ACTIN 2/7* (see Materials and Methods section; Quantitative Real Time PCR for further details). The expression level of *AtCOM1*, 16 DAG, was taken as a reference point and set to "1". All other values are given relative to the control, in arbitrary units of normalized fold expression (Figure 22)

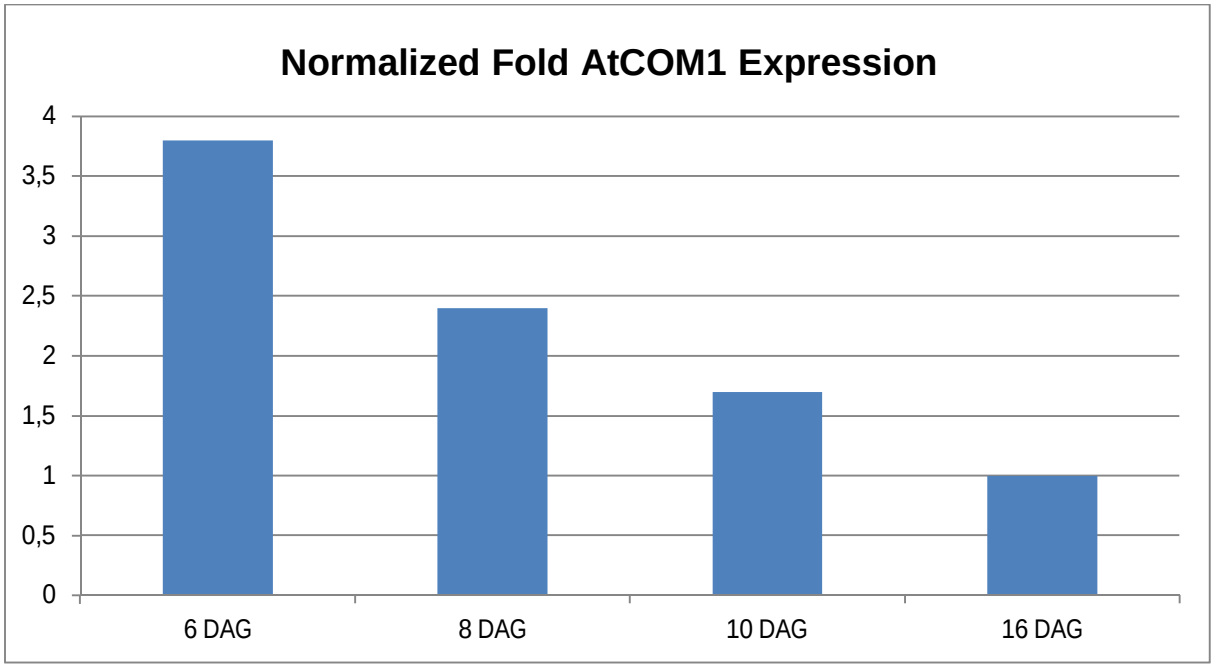


Figure 22 – Expression levels of *AtCOM1* in wildtype first true leaves at different time points. All values are given relative to the amount of expression at time point 16 DAG.

The highest level of *AtCOM1* expression was measured six days after germination. The transcription levels then decrease, as the leaves grow older. The lowest amount of *AtCOM1* mRNA was measured at the last time point, 16 days after germination. These results indicate, that *AtCOM1* is more prominently expressed in dividing cells. Together with the findings, that artificial *AtCOM1* promoters drive the GUS reporter gene exclusively in dividing cells (emerging true leaves, emerging side roots, root tips, base of cauline leaves), in unstressed seedlings, this data provides a strong indication for a role of AtCOM1, during cell division. Future experiments will be performed following this lead (see **5.4** Experimental outlook for details).

4.4 Reduction of *RBR* mRNA level increases *AtCOM1* expression

The retinoblastoma related protein (RBR) is a regulator of E2F activity (Figure 4). When it is bound to the E2F/DP heterodimer it represses the transcriptional activation of E2F responsive genes. Upon cell cycle dependent phosphorylation of RBR by CDKA, it releases the transcription factor dimer, which can then activate gene expression in its RBR free form.

As shown in earlier GUS-experiments (Kurzbauer 2008; sections **4.1** and **4.2** of this thesis) the presence of an E2F binding site is essential for *AtCOM1* promoter activity in transgenic plants, transformed with artificial promoter fragments. Another approach to correlate transcription of the native *AtCOM1* gene with E2F transcription factor activity, was to measure *AtCOM1* mRNA levels in plants with reduced *RBR* mRNA levels (*RBR* RNAi line; Borghi, Gutzat et al. 2010). The idea was, that if the *RBR* mRNA level is reduced, E2F activity is anticipated to be elevated, leading to increased transcription of E2F responsive genes, including *AtCOM1*.

In order to test this hypothesis, an *RBR* RNAi line was kindly provided by Wilhelm Gruissem (Borghi, Gutzat et al. 2010). This transgenic *Arabidopsis* line contains a DNA hairpin (RBRhp), targeting a siRNA against the first six exons of RBR, under the control of the OLexA promoter. This promoter can be induced by the constitutively expressed XVE chimeric transcription factor after ß-estradiol dependent translocation to the nucleus (Brand, Horler et al. 2006; Borghi, Gutzat et al. 2010).

The *AtCOM1* mRNA levels of *RBRi* plants and wildtype plants were to be compared 48h after treatment with a ß-estradiol solution. This was done with *RBRi* plants and

wildtype plants, that had been exposed to 100 Gy of ionizing radiation, as well as with non-irradiated plants.

Wildtype plants and *RBRi* plants were grown on plant medium plates for four weeks. After four weeks half of the wildtype plants and half of the *RBRi* plants were treated with a ß-estradiol solution. This was done to induce RNAi in *RBRi* plants and to have a ß-estradiol treated wildtype control. 48 hours after ß-estradiol application half of the ß-estradiol treated plants and half of the untreated plants were exposed to 100Gy of IR. 45 minutes after the treatment with ionizing radiation, the irradiated plants and their untreated counterparts were ground to a fine powder and snap-frozen in liquid nitrogen. 15-20 plants were used for each condition. Total RNA was isolated from the plant material and subsequently transcribed into cDNA. qPCR experiments, to investigate AtCOM1 expression levels, were conducted. The *AtCOM1* transcript levels of ß-estradiol treated and untreated, irradiated and non-irradiated RBRi plants were compared with wildtype plants (Figure 23). The uninduced *RBRi* line showed wildtype-like *AtCOM1* expression. This was the case for irradiated and non-irradiated individuals. Treatment of the wildtype with ß-estradiol lead to a slight (p=0,077) decrease of *AtCOM1* mRNA after exposure to 100 Gy of gamma-radiation. The induction of the *RBR* RNAi by ß-estradiol lead to a significant induction of *AtCOM1* expression in non-stressed plants (p= 0,001; 5µM ß-estradiol; 0Gy) and in irradiated *RBRi* plants (p=0,015; 5µM ß-estradiol; 100Gy). This effect could be elevated by increasing the ß-estradiol concentration (p= 0,017; 10µM ß-estradiol; 0Gy) (p=0,007; 10µM ß-estradiol; 100Gy).

As expected, the reduction of the *RBR* mRNA level leads to significantly increased *AtCOM1* expression. As RBR is an inhibitor of the transcription of E2F responsive genes, these results are further support for the assumption, that the expression of *AtCOM1* is regulated by E2F transcription factors.

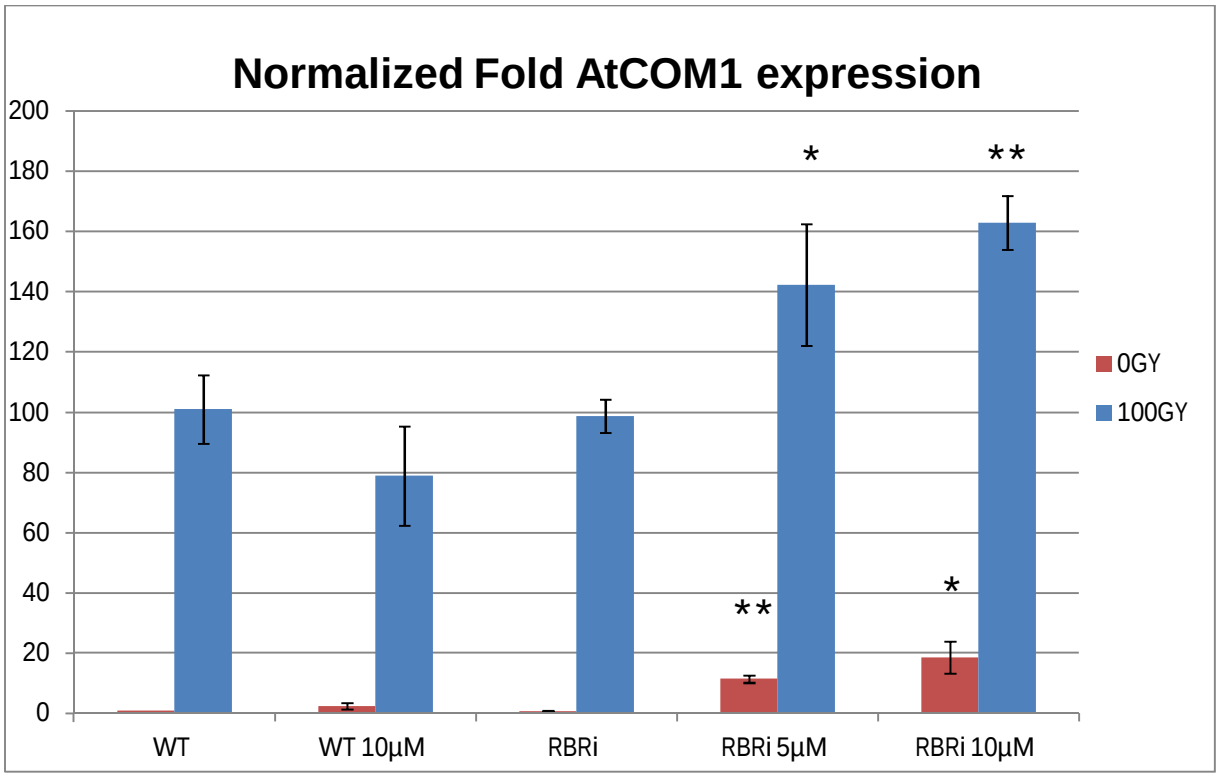


Figure 23 – AtCOM1 expression in different lines. Wildtype and uninduced *RBRi* line show similar level of transcription. Treatment with 10µM ß-estradiol leads to slight reduction of *AtCOM1* expression in radiated wildtype. Induction of *RBR* RNAi leads to increase of *AtCOM1* transcription. This effect depends on ß-estradiol concentration. ***** and ** Indicate statistically significant difference compared to ß-estradiol treated WT. * p <0,05; ** p<0,01.

4.5 E2Fa is enriched at the E2F binding site of the *AtCOM1* promoter

It has been shown in this thesis, that the E2F binding site of the *AtCOM1* promoter is essential for promoter activity. Basal and IR-dependent *AtCOM1* transcription is elevated, when the E2F repressor *RBR* is downregulated. These results provide strong support for the involvement of E2F transcription factors in the regulation of *AtCOM1* expression. There are however six E2F transcription factors and two dimerization partners (DPs) in *Arabidopsis*, that could all play a role in the regulation of *AtCOM1*. Therefore it was aimed at demonstrating binding of E2F TFs to the *AtCOM1* promoter by Chromatin Immunoprecipitation (ChIP), in collaboration with other laboratories. So far only one ChIP experiment has been conducted in the laboratory of our collaborator, Arp Schnittger. This experiment used whole wildtype

seedlings under non stress conditions, using a specific antibody against E2Fa (Figure 24).

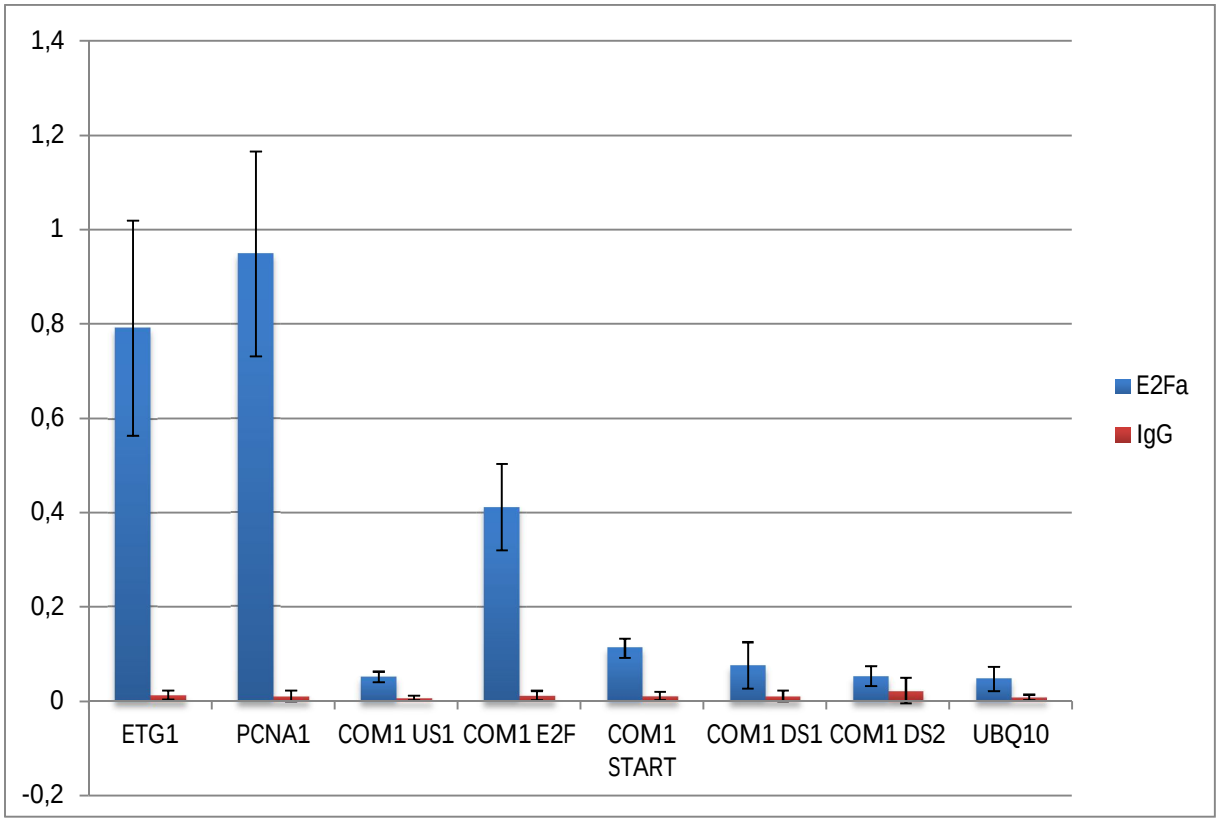


Figure 24 – ChIP data provided by Arp Schnittger. There is a clear enrichment of E2Fa at the E2F binding site of the *AtCOM1* promoter. No enrichment of E2Fa could be detected at a position of the promoter, upstream of the E2F binding site, at two downstream positions, as well as at the transcriptional start of the gene. ETG1 and PCNA1 are positive controls. UBQ1 is a fragment of the Ubiquitin 10 promoter and was used as a negative control (Arp Schnittger). An overview of the positions of the used primers is given in Figure 25.

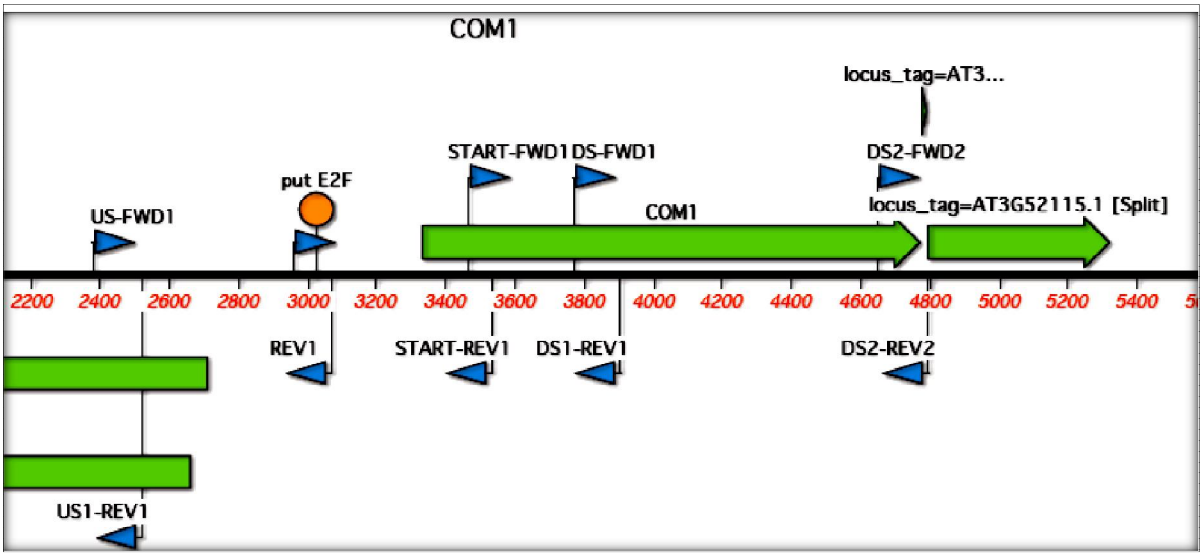


Figure 25 – Overview of the positions of the primers used for ChIP. In addition to the E2F site, one position of the *AtCOM1* promoter, upstream of the E2F site was tested. Three positions inside the gene itself were tested for E2Fa binding.

E2Fa stimulates S phase genes and is necessary and sufficient for both endoreduplication and proliferation. Within the meristems E2Fa is bound by RBR, preventing it from activating endocycle and S- phase genes. Proliferation is thus indirectly maintained by E2Fa/RBR within the meristems. Outside the meristems E2Fa promotes endocycle by activating S phase genes in its RBR-free form (Magyar, Horvath et al. 2012). A clear enrichment of the transcription factor E2Fa could be shown at the E2F binding site of the *AtCOM1* promoter in unstressed wildtype plants. This is however just one of six *Arabidopsis* E2Fs and it remains elusive how the regulation of *AtCOM1* expression is regulated by the E2F family. Further ChIP experiments will be done, to check, whether the other E2Fs are also found at the binding site of the promoter and whether there is a preference for a certain E2F factor. It will also be interesting to see, if E2F binding is altered by genotoxic stress, or if the preference for a specific E2F changes in different tissue types. Also a ChIP experiment will be conducted with an antibody against RBR, in different tissues and under genotoxic stress and non-stress conditions, to study how RBR is integrated in the E2F dependent regulation of *AtCOM1* expression.

4.6 *AtCOM1* expression in transgenic E2F lines

The effect of different E2F transcription factors on the expression of *AtCOM1* was investigated in the next experiment. The *AtCOM1* expression levels of various plant lines, with altered E2F levels were measured by qPCR. Homozygous mutant lines and overexpressing lines for each of the six E2Fs and the two DPs have been obtained for this cause. So far, only a small number of these lines could be tested. The lines, that have been tested, are: an *E2Fe/DEL1* OE line, as well as a *e2fe/del1* mutant line, both kindly provided by Lieven De Veylder (Vlieghe, Boudolf et al. 2005); a *DPa* OE line, kindly provided by Dirk Inze (De Veylder, Beeckman et al. 2002); and an *e2ff* mutant line, that had been kindly provided by Crisanto Gutierrez (Ramirez-Parra, Lopez-Matas et al. 2004). These plant lines were grown for four weeks on plant medium plates, before they were treated with 100 Gy of IR. RNA of the whole plants was extracted 45 minutes post radiation. This RNA was used for cDNA synthesis and subsequent qPCR analysis. The *AtCOM1* mRNA levels of the different irradiated plant lines were compared to their untreated counterparts and to irradiated and untreated wildtype plants. The expression level in the untreated wildtype was set as reference (value “1“) and all the other expression levels were given relative to this. There was no detectable difference between the *AtCOM1* expression levels of the different non-irradiated transgenic *E2F* lines and the non-irradiated wildtype (Figure 26). This is probably, because the whole 4 week old plants were used for RNA extraction. There are proportionally not many dividing cells in 4 week old plants. For the future, it will be meaningful to repeat the experiment with mitotic tissue (emerging first true leaves) and meiotic tissue (emerging buds) to compare *AtCOM1* expression in the different *E2F* backgrounds. It could however be shown, that altered *E2F* status changes *AtCOM1* expression in response to ionizing radiation (Figure 26). The levels of *AtCOM1* transcripts were also compared with those of homozygous mutants of the two kinases ATM and ATR. As shown before (Kurzbauer 2008), there was a strong, significant induction (p=0,007) of *AtCOM1* expression in the wildtype, that was abolished in the *atm* mutant. Mutation of *ATR* had no effect on *AtCOM1* expression. The mRNA levels were measured 45 minutes post irradiation. It is clear, that ATM directly responds to DNA damage (in more detail: DSBs) introduced by IR, but ATR is activated in response to ssDNA, an intermediate generated during DSB repair, and therefore later. As cells progress to S phase, lesions, that lead to replication blocks

are encountered and ATR is activated (Culligan, Robertson et al. 2006). It is thus possible, that a downregulation of *AtCOM1* could be detected at a later time point. Overexpression of *E2Fe/DEL1* lead to a reduction of *AtCOM1* transcription, that was not significant (p=0,151). The reduction of *AtCOM1* transcription was considerably stronger and significant (p=0,002) in the *DPa* OE line. The *e2ff/del3* and *e2fe/del1* mutants both had not significantly (e2fe/del1: p=0,185; e2ff/del3: p=0,144) increased AtCOM1 levels in response to 100Gy of gamma radiation (Figure 26).

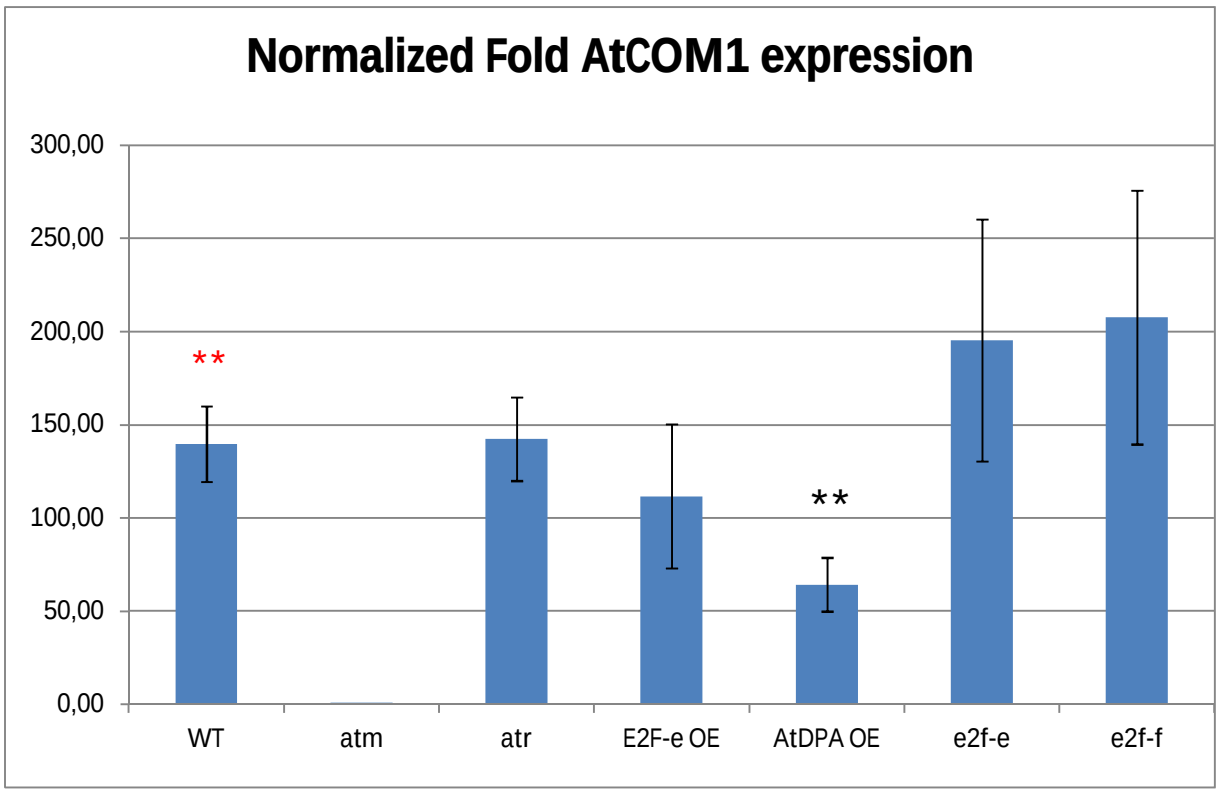


Figure 26 – AtCOM1 expression levels in different transgenic lines, 45 minutes after treatment with 100Gy of gamma radiation, normalized to untreated wildtype. In wildtype plants *AtCOM1* transcription is significantly (0,007) increased in response to IR. This increase in *AtCOM1* transcription is stronger, but not significant in e2fe (p=0,185) and e2ff (p=0,144) mutants. Compared to the irradiated WT, induction of *AtCOM1* expression is weaker in plants overexpressing E2Fe (p=0,0151; not significant) and significantly (p=0,002) weaker in DPa overexpressing plants. ** indicates statistically significant difference, compared to non-irradiated WT. ** indicates statistically significant difference, compared to irradiated WT.

There is a slight downregulation (p=0,151; not significant) of *AtCOM1* transcription in *E2Fe/DEL1* OE plants and an upregulation (p=0,185; not significant) in *e2fe/del1* mutants. E2Fe/DEL1 belongs to the second group of E2Fs, that do not have a

transactivation domain and can bind promoters, without forming a dimer with one of the two DPs. That is why it is considered as a transcriptional repressor in general. So, if *E2Fe/DEL1* is overexpressed, it probably competes with activating E2Fs for the binding site in the *AtCOM1* promoter, thereby repressing its activity. When it is mutated, it cannot inhibit expression of *AtCOM1*, which might be the reason for the upregulation of *AtCOM1* in *e2fe/del1* mutant plants. In addition, E2Fe/DEL1 is known to inhibit expression of genes, involved in the switch from the cell cycle to the endocycle in proliferating cells, thereby restraining their expression to endoreduplicating cells and inhibiting endocycle onset (Lammens, Boudolf et al. 2008). This could mean, that *AtCOM1* is directly, or indirectly repressed by E2Fe/DEL1, as *AtCOM1* is strongly enhanced in endoreduplication. Endoreduplication has been proposed to be switched on, in response to DNA damage (Adachi, Minamisawa et al. 2011). *e2ff/del3* mutants, like *e2fe/del1* mutants show an enhanced (p=0,144; not significant) expression of AtCOM1 in response to IR. A possible explanation is again, since E2Ff/DEL3 is considered to be a transcriptional repressor, that the absence of the protein facilitates the binding of activating E2Fs.

The most striking effect on *AtCOM1* expression was observed in plants, that overexpressed a stable form of DPa, the dimerisation partner, that is preferentially bound by E2Fa and E2Fb. It had been shown, that overexpression of *E2Fa/DPa* leads to a downregulation of endocycle genes, by elevated formation of the E2Fa/DPa-RBR complex (Magyar, Horvath et al. 2012). So, the fact, that *AtCOM1* is not as strongly upregulated (p=0,002; significant) in response to ionizing radiation in 35S::DPa plants, compared to wildtype plants, could be caused by an increased recruitment of RBR bound E2Fa/DPa to the promoter of *AtCOM1*. In its RBR-bound form the E2Fa/DPa still binds to target genes, but these genes cannot be activated, due to the transactivational repressor function of RBR.

5 Discussion

In this study we aimed at identifying cis and trans acting factors, that play a role in the regulation of *AtCOM1* expression. Another objective was to determine cell cycle and tissue specificity of *AtCOM1* promoter activity. We found, that E2F transcription factors play an essential role in the regulation of *AtCOM1* promoter activity. There is a basal level of ATM-independent *AtCOM1* expression in proliferating cells of the meristems, that is strongly upregulated in response to ionizing radiation, in an ATM-dependent manner.

5.1 *AtCOM1* is expressed in dividing cells

AtCOM1 is a DNA repair factor, that is essential for homologous recombination. In order to get a better insight into plant DNA repair, we analyzed the dynamics of *AtCOM1* promoter activity. It was one of the objectives of this thesis, to determine in which tissues and cell cycle stages the *AtCOM1* promoter is active.

To test the dynamics of *AtCOM1* promoter activity in plants, GUS assays were conducted with plants expressing GUS under the control of AtCOM1 promoter fragments. In two week old plants, that had been grown in liquid medium, GUS staining could only be observed in rapidly dividing tissues, namely emerging true leaves, emerging side roots and root tips. For root tips of unstressed plants, it was shown, by longer formaldehyde fixation, prior to the staining, that the colouring is limited to dividing cells. This GUS staining was not altered in the *atm* mutant background, indicating, that ATM is not needed for the basal expression of *AtCOM1* during cell division, under non-stress conditions.

It had been shown before (Kurzbauer 2008) and was again demonstrated in this work, that ATM is essential for a strong upregulation of *AtCOM1* in response to ionizing radiation. The protein kinase ATM is activated in response to DNA DSBs and recruited to sites of the breaks. It phosphorylates a number of downstream targets, like proteins involved in cell cycle checkpoint control, DNA repair and apoptosis, making ATM a major regulator of the cell cycle in response to DSBs.

A recent study has correlated DNA double strand breaks (DSBs) with the onset of endoreduplication. It is proposed, that DSB signals affect the expression of cell cycle regulators, such as CDK suppressors, thereby switching the mitotic cycle to the endocycle (Adachi, Minamisawa et al. 2011). Also it had been shown, that *AtCOM1* is

expressed at a strongly enhanced level in endoreduplicating cells (Deveaux, Alonso et al. 2000). *AtCOM1* is probably needed to repair stalled DSBs, resulting from the processing of stalled replication forks. The assumption is, that *AtCOM1* is expressed at basal levels in mitotic cells, independent of ATM, and that upon exposure to ionizing radiation, activation of ATM leads to endocycle entry of these cells. The altered gene expression levels of regulatory transcription factors in endoreduplicating cells then leads to enhanced expression of *AtCOM1*. This is done to ensure a higher availability of the repair protein, since there is a strong augmentation of replication activity during endoreduplication, compared to mitosis.

Expression of *AtCOM1* in mitotic cells was also shown by a qPCR experiment, comparing *AtCOM1* mRNA levels in emerging first true leaves of wildtype seedlings, at different time points. It could be shown, that a reduction of mitotic activity correlates with a proportional reduction of *AtCOM1* expression. The next step would be, to repeat this experiment with irradiated and non-stressed wildtype and *atm* mutant plants. If the non-stressed wildtype and atm mutant show similar *AtCOM1* expression, it would support the theory, that the basal transcription level in mitotic cells is independent of ATM. The *AtCOM1* expression would be expected to increase in the wildtype emerging true leaves, in response to IR, while it should not increase in the *atm* mutant. In addition, the ploidy level of the cells could be measured, to determine, whether exposure to ionizing radiation leads to endocycle onset in these cells. The ATM dependent increase of *AtCOM1* expression, in response to IR could then be correlated to endoreduplication.

5.2 *AtCOM1* expression is regulated by E2F transcription factors

It had been shown before in plant cell culture, that the E2F TF binding site of the AtCOM1 promoter is essential for activation of the promoter (Kurzbauer 2008). In this work, promoter fragments, controlling the GUS reporter gene, that had been transformed into *Arabidopsis* plants, were analyzed for their ability to drive GUS activity. In the transformed and regenerated, intact plants only promoter fragments containing the E2F site were able to activate GUS. This result shows, that the E2F binding site is also essential for *AtCOM1* promoter activity in plants. The E2F site of one promoter fragment, (com1_10), that had activated GUS in plant cell culture, had

been mutated (Figure 27). This mutation of the E2F site led to loss of promoter activity in plant cell culture, further supporting the statement, that this E2F site is essential for *AtCOM1* promoter activity (Kurzbauer 2008).

Figure 27 - Sequences of the natural E2F binding site found in the *AtCOM1* promoter and the mutated version. Mutation of the E2F site led to a loss of promoter activity.

This promoter fragment was transformed into plants via *Agrobacterium tumefaciens*, but stable transformants still have to be selected. Then, GUS stainings can be conducted, in order to obtain further evidence for the importance of the E2F site, for *AtCOM1* promoter activity.

E2F transcription factors are present in all higher eukaryotes and regulate genes, involved in proliferation, differentiation, development and apoptosis. They play a critical role in cell cycle progression, as they stimulate the expression of genes, required for the onset of S-phase and DNA replication (Rossignol, Stevens et al. 2002). Six *E2F* genes have been characterized in plants. They all bind to a highly conserved consensus sequence (Figure 2). The first three *Arabidopsis* E2Fs (E2Fa-c) share a common domain organization with their mammalian counterparts. They all contain a domain for the binding of cylinA, a DNA binding domain, a transcriptional activation domain, which includes a binding site for the retinoblastoma related protein (RBR), as well as a binding site for one of the two possible dimerization partners DPa and DPb (Figure 3). These E2Fs require dimerization with one of two DP proteins for successful DNA binding. The other three E2Fs, E2Fd, e and f, also known as DEL2, 1 and 3 possess two DNA binding sites, enabling them to bind promoter regions independent of DPs. They possess none of the other conserved domains, including the transactivation domain, which is why they are considered to be transcriptional repressors. The RBR protein controls the transcription factor activity of the E2F-DP dimer. When RBR is bound by these transcription factors, the ability to activate gene expression is suppressed (Figure 4).

A qPCR experiment was conducted, to verify the hypothesis, that E2F TFs play an important role in the regulation of *AtCOM1*. In this experiment the expression level of a transgenic plant line with reduced *RBR* mRNA level (RBR RNAi) was compared

with the expression level of *AtCOM1* in the wildtype. It was demonstrated, that *AtCOM1* expression is increased upon reduction of *RBR* mRNA level. This is true for plants, that had been exposed to 100Gy of ionizing radiation as well as for non-irradiated plants. Since RBR is a repressor of E2F transactivation activity, this result confirms the hypothesis of E2F TFs playing a role in *AtCOM1* regulation. The RBR/E2F/DP pathway comprises an interconnected gene regulatory network, regulating for example the balance between cellular proliferation and endoreduplication (Figure 6) (Magyar, Horvath et al. 2012). There are six different E2F transcription factors in plants. The first three, E2Fa, b and c can only bind DNA as a heterodimer with one of the two DP proteins and their ability to drive transcription of the bound gene is comprised by RBR binding of the dimer. The other three, E2Fd, e and f posses two DNA binding domains and no RBR binding domain. So they can bind DNA on their own and operate independently of RBR regulation. Since they also do not possess a transactivation domain, they are considered to be transcriptional repressors. E2Fe/DEL1 inhibits the expression of genes, involved in the switch from the cell cycle to the endocycle in proliferating cells, thereby inhibiting endocycle onset (Lammens, Boudolf et al. 2008). The expression of *DEL1* is antagonistically regulated by E2Fb and E2Fc. E2Fb induces transcription of *DEL1*, while E2Fc is a repressor of *DEL1* expression. E2Fc was also shown to repress genes in G2 control, thereby promoting endocycle (del Pozo, Diaz-Trivino et al. 2006). While E2Fb stimulates S phase and M phase genes, making it a driver of the cell cycle, E2Fa only stimulates S phase genes and is necessary and sufficient for both endoreduplication and proliferation. During cellular proliferation, E2Fa is bound by RBR. In its RBR-bound form E2Fa cannot activate expression of S phase genes. Within cells, that have entered the endocycle, E2Fa is released from RBR and thus activates S phase genes (Figure 6) (Magyar, Horvath et al. 2012). These findings all fit very well into the picture of *AtCOM1* being an E2F-regulated gene, that is active during S-phase, to repair DSBs, resulting from processing of stalled replication forks, with basal levels in mitosis and enhanced levels during endoreduplication.

To demonstrate the binding activity of the six E2Fs, two DPs and RBR at the specific E2F site on the *AtCOM1* promoter and to study the dynamics of *AtCOM1* regulation by these proteins, ChIP experiments will be conducted (in collaboration with L. Bögre and M. Zoltan). These experiments will be performed with specific antibodies for each

of the nine proteins, in wildtype plants and *atm* mutants, before and after exposure to 100Gy of ionizing radiation. These ChIPs are supposed to reveal which of these proteins preferentially bind the *AtCOM1* promoter in the unstressed plant and in response to IR, and how this is influenced by ATM. So far only one ChIP experiment was carried out, using an antibody against E2Fa. Wildtype seedlings were used, and the experiment was done in the laboratory of Arp Schnittger. This ChIP showed a definite enrichment of E2Fa at the E2F TF binding site of the *AtCOM1* promoter.

E2Fa is known to drive S phase genes and to stimulate cell division and inhibit endoreduplication in its RBR bound form. When it is released from RBR, it stimulates endocycle entry. For *AtCOM1* this could mean, that E2Fa-RBR binds the promoter during mitosis, repressing its expression. The basal *AtCOM1* transcription level in mitotic cells could be induced by E2Fb, which is an activator of S phase and M phase genes. While the E2Fb is released from RBR by CYCD during G1/S phase transition, the E2Fa-RBR complex stays stable, either because the E2Fa-RBR is not disrupted through phosphorylation by CYCD/CDKA, or because it is somehow protected from this phosphorylation (Magyar, Horvath et al. 2012).

It was shown in this study, that *AtCOM1* expression is altered in transgenic E2F lines, in response to IR. This was determined by qPCR experiments comparing *AtCOM1* mRNA levels 45 minutes after exposure to 100Gy of IR.

There is a slight downregulation of *AtCOM1* transcription in *E2Fe/DEL1* OE plants and an upregulation in *e2fe/del1* mutants. So, if *E2Fe/DEL1* is overexpressed, it probably competes with activating E2Fs for the binding site in the *AtCOM1* promoter, thereby repressing its activity. When it is mutated, it cannot inhibit expression of *AtCOM1*. In addition, E2Fe/DEL1 is known to inhibit expression of genes, involved in the switch from the cell cycle to the endocycle in proliferating cells, thereby restraining their expression to endoreduplicating cells and inhibiting endocycle onset (Lammens, Boudolf et al. 2008). This could mean, that *AtCOM1* is directly, or indirectly repressed by E2Fe/DEL1, as it is strongly enhanced in endoreduplication, that has been proposed to be switched on, in response to DNA damage (Adachi, Minamisawa et al. 2011). AtCOM1 is needed for DNA repair, which may occur more frequently during endoreduplication. It is also cogitable, that *AtCOM1* transcription is directly affected and not indirectly only after establishment of endoreduplication. Another result of the qPCR experiments was, that *e2ff/del3* mutants, like *e2fe/del1*

mutants show an enhanced expression of AtCOM1 in response to IR. A possible explanation is again, since E2Ff/DEL3 is considered to be a transcriptional repressor, that the absence of the protein facilitates the binding of activating E2Fs.

The most striking effect on *AtCOM1* expression was observed in plants, that overexpressed a stable form of DPa, the dimerisation partner, that is preferentially bound by E2Fa and E2Fb. It had been shown, that overexpression of *E2Fa/DPa* leads to a downregulation of endocycle genes, by elevated formation of the E2Fa/DPa-RBR complex. These genes were however upregulated in an overexpression line of a truncated E2Fa version, without RBR binding site (Magyar, Horvath et al. 2012). Since the RBR binding site is located inside the transactivation domain, the transactivation domain is also missing in the truncated version of E2Fa (35S::E2Fa$^{\Delta RB}$/DPa). These results show, that E2Fa/DPa actively represses the transcription of endocycle genes and thereby indirectly promotes proliferation in the RBR bound form (Magyar, Horvath et al. 2012). So, the fact, that *AtCOM1* is not effectively upregulated in response to ionizing radiation in 35S::DPa plants, could be caused by an increased recruitment of RBR bound E2Fa/DPa to the promoter of *AtCOM1*, thereby repressing transcription of the repair gene.

5.3 A model for the regulation of AtCOM1 expression

AtCOM1 is active in mitotic cells, at basal levels and is enhanced during endoreduplication. It is highly upregulated by ATM in response to ionizing radiation, whereas in the absence of genotoxic stress ATM does not seem to be involved in the regulation of *AtCOM1*. ATM induces onset of endoreduplication via the unique plant transcription factor SOG1 in response to DNA DSBs (Adachi, Minamisawa et al. 2011). The expression of *AtCOM1* is regulated by the RBR/E2F/DP network. This regulation is cell cycle dependent. During mitotic S phase E2Fa is bound to RBR and represses the transcription of S phase genes and genes, involved in endoreduplication. The other activator of S phase genes, E2Fb however is RBR free and facilitates replication and S phase progression. When cells enter the endocycle, E2Fa is released from RBR and activates genes necessary for S phase and endoreduplication. E2Fe/DEL1 is a repressor of endoreduplication and is activated

by E2Fb during proliferation, when E2Fc, an inhibitor of *E2Fe/DEL1* expression and thus activator of endoreduplication is repressed by RBR-E2Fa.

Therefore we hypothesize that *AtCOM1*, being an S phase gene is probably directly activated by E2Fb during mitosis and by E2Fa during endoreduplication. We hypothesize further, that the ATM/SOG1 dependent induction of endocycle entry is responsible for the strong increase in *AtCOM1* expression, in response to IR-induced DSBs. This increase in *AtCOM1* expression is hypothesized to be mediated by E2Fa, which is released from RBR upon endocycle onset and thus activates transcription of *AtCOM1.* The fact, that AtCOM1 promoter activity is restricted to the meristems, even after treatment with IR (as shown in **4.2**) supports the hypothesis, that *AtCOM1* transcription is enhanced, via ATM/SOG1 dependent endocycle onset. An alternative hypothesis would be, that *AtCOM1* expression is upregulated by ATM via a different, unknown pathway. The strongly enhanced expression of *AtCOM1* in endocycling cells could be an indirect effect. It could be caused by the enhanced occurrence of DNA damage, as a result of augmented replication events in the endocycle. Figure 28 shows a model of cell cycle and DNA damage dependent control of transcription of the DNA repair gene *AtCOM1*.

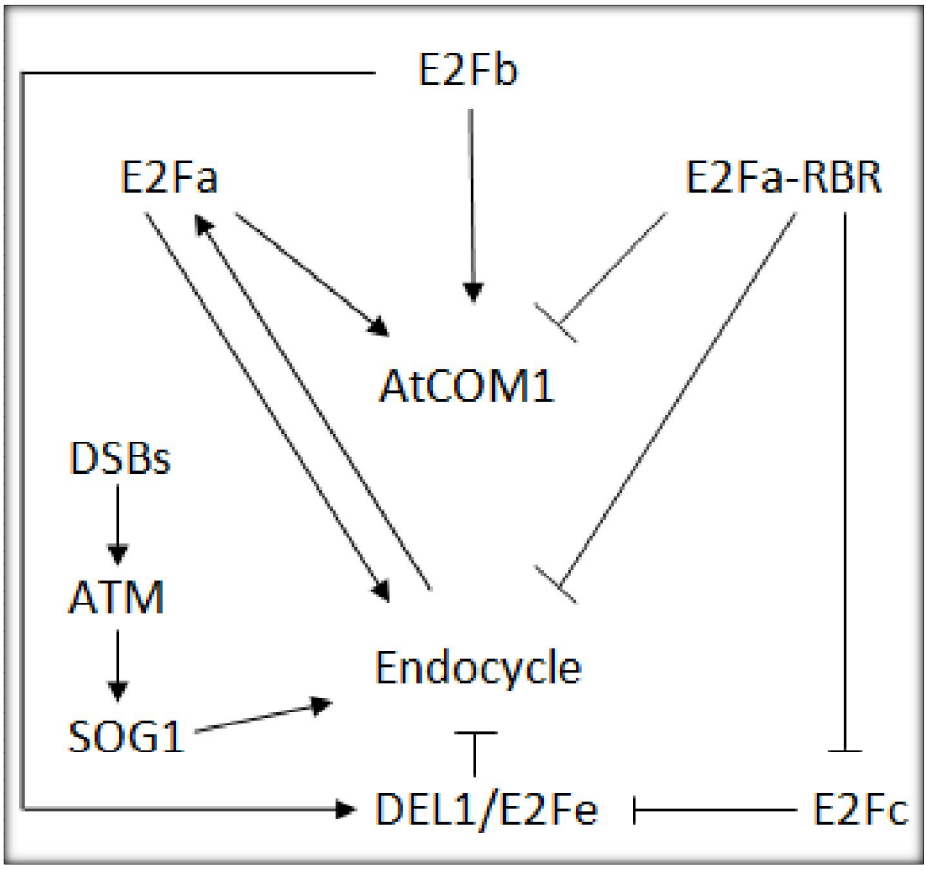


Figure 28 – Model for the regulation of *AtCOM1*. *AtCOM1* is expressed at basal levels in mitotic cells and at strongly enhanced levels in endoreduplication. DNA DSBs lead to activation of ATM, which induces endocycle onset via SOG1, by an unknown mechanism. In endoreduplicating cells *AtCOM1* is upregulated by E2Fa, in its RBR-free form. During mitotic S phase *AtCOM1* is activated by E2Fb, while it is also partly repressed by RBR-E2Fa. E2Fa promotes endocycle in its RBR-free form and represses endocycle in its RBR bound form. DEL1 is an inhibitor of the endocycle, that is antagonistically regulated by E2Fb and E2Fc.

5.4 Experimental outlook

To verify the model of *AtCOM1* regulation and to answer several remaining open questions, a number of experiments will have to be conducted.

Further ChIP experiments

The enrichment of E2Fa at the E2F site of the *AtCOM1* promoter has been shown for non-stressed wildtype plants. To elucidate binding properties and preferences of all the proteins involved in the RBR/E2F/DP pathway and to get more insight in *AtCOM1* regulation, it will be necessary to conduct ChIP experiments with antibodies against each of the nine proteins. Further ChIP experiments will be done, to check, whether the other E2Fs are also found at the binding site of the promoter and whether there is a preference for a certain E2F factor. It will also be interesting to see, if E2F protein abundance is altered by genotoxic stress, or if the preference for a specific E2F changes in different tissue types. Also a ChIP experiment will be conducted with an antibody against RBR, in different tissues and under genotoxic stress and non-stress conditions, to study how RBR is integrated in the E2F dependent regulation of *AtCOM1* expression. The ChIP experiments will be conducted with non-stressed and irradiated wildtype and *atm* mutant plants.

Further qPCR experiments

We have shown, that reduction of *RBR* mRNA level (*RBRi*) leads to increase of *AtCOM1* transcription in four week old plants. In the future it will be interesting to determine, whether this effect is specific for a certain cell cycle stage. This could be done by comparing *AtCOM1* expression of mitotic cells (first true leaves; 6DAG) and G0/G1 cells (mature leaves) of *RBRi* plants with that of WT plants.

For all of the six E2Fs and two DPs, OE lines and T-DNA insertion mutants have been obtained. They will be used to conduct further qPCRs to compare *AtCOM1* expression levels in non-stressed and irradiated plants. This will be done with four week old plants, as in **4.6** and also with first true leaves at different time points, as in **4.2**. Also, these qPCRs will be done with sog1 mutant plants, to check, whether the ATM signal is transduced by SOG1. The *AtCOM1* transcription level of atr mutant

plants could be measured at later timepoints, than 45 minutes after irradiation, to see, if ATR leads to *AtCOM1* induction as a long-term response.
To investigate, whether E2Fa regulates expression of *AtCOM1*, it will be necessary to conduct qPCR experiments with an E2F OE line, that has been crossed to the *RBR* RNAi line. Investigation of the *AtCOM1* expression level of the E2Fa x *RBRi* line will reveal, whether E2Fa regulates *AtCOM1* expression. A plant line overexpressing a truncated version of E2Fa will also be used for these qPCRs. The truncated version of E2Fa lacks the RBR binding domain, as well as the transactivation domain. The protein can still bind DNA, but it has lost its capability to repress activity of the bound gene promoter, as well as to activate expression of the bound gene. These qPCR experiments will reveal, whether *AtCOM1* expression is really inhibited by the E2Fa/RBR complex in non-stressed plants and upregulated by RBR-free E2Fa in response to IR.

Immunostaining of root tips

So far it has been shown, that the *AtCOM1* promoter is active in dividing cells (GUS assay) and that *AtCOM1* is expressed in first true leaves. Immunostaining of root tip cells, with an AtCOM1 specific antibody could demonstrate, that the corresponding AtCOM1 protein is actually present and associated to DNA in mitotic cells. This could be done with roots of wildtype and atm, atr, atm/atr mutants, as well as with an *Atcom1* mutant as a negative control, again before and after exposure to 100Gy of IR.

6 Materials and methods

6.1 Media

ARA medium for plants

4,3g/l MURASHIGE & SKOOG basal salt; 0,5g/l MES; 15g/l Sucrose; 1x Gamborg´s vitamin solution; pH 5,8 (calibrated with 1M KOH);6g/l plant agar; autoclave

2xTY medium for bacteria

16g/l tryptone; 10g/l yeast extract; 5g/l NaCl; pH 7,0 (calibrated with 1M NaOH); 15g/l agar; autoclave

6.2 Plant work

Plant lines and growth conditions

All *Arabidopsis thaliana* lines used were of the “Columbia“ ecotype (Col-o). They were grown under long day conditions (16h of light; 8 h of darkness; 21°C; 60-80% humidity, 5800 LUX, 4x Philips TLD 36W and 2x Sylvana GroLUX 36W). Before being sown on soil, seeds were kept at 4°C in water for pre-germination for 2 days.

Seed sterilization

5g $Ca(OCl)_2$ and 20µl Triton X-100 were added to 100ml dH_2O. This solution was agitated for 2h. The next day about 50 seeds per *Arabidopsis* line were covered with 1ml of the sterilization solution in Eppendorf tubes and agitated for 20 min at room temperature. They were washed twice with 1ml of sterile water and dried overnight, before they were transferred to ARA-plates. These plates were kept at 4°C for 2 days before being transferred to long day conditions.

Stable transformation of *Arabidopsis thaliana*

3ml of 2xTY medium, supplemented with 50g/l Gentamycin and 25g/l Kanamycin were inoculated with a single colony of *Agrobacterium tumefaciens* (strain GV3101) and incubated overnight at 28°C under constant shaking. The next day 500ml of 2xTY medium were inoculated with the overnight culture and again incubated at 28°C over night under constant shaking. The overnight culture was centrifuged (25 min; 300rpm; RT) and the harvested cells were then washed with 5% sucrose by centrifugation (10 min; 300 rpm; RT). After discarding the supernatant, the cells were resuspended in 300ml of a 5% sucrose solution supplemented with 0,02% Silwet L-77. *Arabidopsis* inflorescences were dipped into the bacterial suspension and the plants were kept in a box covered with a light-transmissive foil for two days. Then the

plants were transferred to normal long day growth conditions. The seeds of these plants were harvested, sown on soil and selected for stable transformation.

Selection of stable transformants via BASTA resistance on soil

Seeds of transformed plants were grown on soil and after approximately one week, when the first true leaves had developed, the seedlings were sprayed with 150µg/ml BASTA (glufosinate ammonium). Spraying was repeated three to four times, every other day. Only plants, that had stably integrated the BASTA resistance gene survived this treatment.

GUS staining of plant material

Plant material was harvested and covered with ice-cold fixing solution (2% formaldehyde; 1mM EDTA; 0,1% Triton X-100) in multiwell culture plates. Then a short vacuum was applied and the plates were kept at 4°C for 30 minutes for fixing. The material was then washed twice with 0,1M Na-phosphate buffer (pH7) and covered with GUS-staining solution (100mM Na-phosphate buffer pH7; 10mM EDTA; 1mM X-Gluc; 0,1% Triton X-100). The material was incubated overnight at 37°C and washed with dH_2O the next day. The chlorophyll was removed from the plant material with 70% EtOH at room temperature.

6.3 DNA work

Isolation of DNA from *E.coli*

1,5ml of an overnight culture of *E.coli* were spinned down in an Eppendorf tube (1 minute; 14000rpm; RT), the supernatant discarded and the pelleted cells resuspended in 200µl of mini prep solution 1 (50mM glucose; 25mM Tris-Cl pH8; 10mM EDTA). 200µl of mini prep solution 2 (0.2N NaOH; 1% SDS) were added and the tube was inverted 3-4 times. After 5 minutes 200µl of mini prep solution 3 (3M KoAc; 11.5% acetic acid) were added and the tube was again inverted 3-4 times. The solution was spinned down (5 min; 14000 rpm; RT) and 500µl of isopropanol were added to 500µl of the supernatant. The mixture was centrifuged (10 min;14000 rpm; RT) and the resulting DNA pellet was then washed with 500µl of 70% EtOH (5 min; 14000 rpm; RT). The pellet was air-dried and resuspended in 50µl 1xTE (10mM Tris-Cl pH8; 1mM EDTA pH8).

Extraction of DNA from *A. thaliana*

Young leaves of about 1cm length were transferred to TissueLyser tubes. To each tube a small stainless steel bead and 400µl of plant DNA extraction buffer (200mM Tris pH 7,5; 250mM NaCl; 0,5% SDS; 25mM EDTA pH 8,0) were added. The tubes were equally distributed to two tube racks and the plant material was then disrupted by high speed shaking of the tubes in the TissueLyser (Qiagen). The tubes were centrifuged (10 min; 4000 rpm; RT) and 200µl of the supernatant was mixed with 200µl of isopropanol. After 10 minutes of incubation, another centrifugation step followed to precipitate the DNA (5 min; 14000 rpm; RT). The resulting pellet was washed with 200µl of 70% EtOH, air-dried and resuspended in 50µl sterile water or 1xTE. The tubes were shaken at 65°C for 10 minutes, before they were stored at -20°C.

Transformation of chemically competent *E.coli*

50µl of chemically competent *E.coli* (strains: DH5α or XL1-Blue) were thawn on ice and 10ng of plasmid DNA were added. After 10 minutes of incubation on ice, a heat shock was carried out (1,5 min; 42°C). The cells were put back on ice for 5 minutes, before 1ml of 2xTY medium was added. One hour incubation at 37°C followed and the cells were then plated on 2xTY medium plates supplemented with the required antibiotics.

Transformation of electrocompetent *Agrobacterium tumefaciens*

100µl of the electro-competent *Agrobacteria* strain GV3101 were thawn on ice and 1ng of plasmid DNA was added. Then the cells were electroporated in a pre-cooled 1mm electroporation cuvette at 400Ω, 25µF, 2,5kV. Immediately after electroporation 1ml of 2xTY medium was added to the bacterial cells and they were incubated for 1h at room temperature. 100µl of the cell suspension was then spread on 2xTY plates, supplemented with 50mg/l Kanamycin and 50mg/l Gentamycin, and incubated for 1-2 days at 28°C.

PCR

The standard PCR mix for one reaction consisted of 2µl 10x DreamTaq™ buffer (includes 20mM $MgCl_2$), 200µM dNTPs, 25pM of each primer, 0,1µl of DreamTaq™ DNA Polymerase(supplied in 20 mM Tris-HCl pH 8.0; 1 mM DTT; 0.1 mM EDTA; 100 mM KCl; stabilizing agent and 50% (v/v) glycerol), 1ng of template DNA an dH_2O

to a final volume of 20µl. The standard PCR programme consisted of 1 cycle of 95°C for 1 minute, 40 cycles, which were comprised of 95°C for 1 minute of melting, 30 seconds of the required annealing temperature, and 72°C for 30 seconds of elongation, followed by a final cycle of 72°C for 5 minutes

DNA Gel – Electrophoresis

For standard gel-electrophoresis of DNA, gels with 1% Standard Electrophoresis Agarose in 1xTAE buffer (50X: 242g/l Tris, 57,1ml/l glacial acetic acid, 100ml/l 0,5M EDTA) were used. For visualization of the DNA fragments 5µl of an EtBr solution (7mg/ml) were added to 100ml of agarose gel.

6.4 RNA work

Extraction of total RNA from *A. thaliana*

Plant material was harvested and snap frozen in liquid nitrogen. Then it was ground to a fine powder in liquid nitrogen and stored at -80°C. For isolation of total RNA, the Promega SV Total RNA Isolation System was used according to the manufacturer´s protocol. 30mg of ground plant tissue were added to 175µl of RNA Lysis Buffer, before 350µl of RNA Dilution Buffer were added. After mixing by inversion, 10 min of centrifugation at 14000 rpm, at RT followed. The cleared lysate was transferred to a fresh microcentrifuge tube and 200µl of 95% EtOH were added. The solution was mixed by pipetting 3-4 times. The mixture was transferred to a Spin Column Assembly and centrifuged for 1 min at 14000 rpm. The liquid in the Collection Tube was discarded and 600µl of RNA Wash Solution were added to the Spin Column Assembly. One minute of centrifugation at 14000 rpm followed. The Collection tube was emptied and 50µl of freshly prepared DNase incubation mix (40µl Yellow Core Buffer, 5µl 0.09M MnCl2 and 5µl of DNase I enzyme) were applied directly to the membrane inside the Spin Basket. After 30 min of incubation 200µl of DNase Stop Solution were added and the Spin Column Assembly was centrifuged for 1 min at 14000rpm. 600µl of RNA Wash solution ere added and another centrifugation step of 1 min at 14000 rpm followed. Next, the Collection Tube was emptied and 250µl of RNA Wash Solution were added before the Column was centrifuged for 2 min at 14000 rpm. The Spin Basket was removed from the Collection Tube and transferred

to an Elution Tube. 100µl of Nuclease Free Water were added and the RNA was eluted by centrifugation for 1 min at 14000 rpm. The RNA was stored at -80°C.
The average yields of RNA were between 3 and 5 µg.

RNA Gel – Electrophoresis

RNA quality was tested on a denaturing 1,4% agarose RNA gel. 0,7g of Standard Electrophoresis Agarose were dissolved in 50ml of DEPC (Diethylpyrocarbonate)-treated water (add 450µl DEPC to 1L of dH_2O; incubate 1h at RT; autoclave). After cooling down 5,58ml 10xMOPS (41,8g MOPS; 16,6ml 3M NaOAc pH4,8; 20ml 0,5M EDTA; ad 1000ml DEPC.H2O) and 1,75 ml formaldehyde were added. EtBr was added directly to the RNA sample before loading.

Preparation of RNA samples for Gel-Electrophoresis

Before loading the RNA onto the gel, 2,4µl of 10xMOPS, 4,5µl formaldehyde, 12µl formamide, 0,75µl loading dye and 0,75µl EtBr were added to 5µl RNA solution. This mixture was heated to 95°C for 5 minutes, then kept on ice for a few minutes. After short centrifugation, the samples were loaded onto the gel.

DNase Treatment

In order to remove genomic DNA from RNA preparations they were incubated with DNase I. To 1µg of RNA 1µl 10x DNase I reaction buffer (with $MgCl_2$), 1µl DNase I and 10µl DEPC-treated water were added. The reaction mixture was incubated at 37°C for 30 minutes. Then 1µl 50mM EDTA was added and 10 minutes of incubation at 65°C followed. The DNA-free RNA could now be used for cDNA synthesis.

cDNA Synthesis

For synthesis of cDNA the BioRad iScript cDNA Synthesis Kit was used. 1µg of RNA was mixed with 4µl of 5x iScript Reaction Mix and1µl of iScript Reverse Transcriptase. Nuclease-free water was added to a final volume of 20µl. This mixture was then incubated for 5 minutes at 25°C, 30 minutes at 42°C and 5 minutes at 85°C. To test whether the cDNA sample contained contamination of genomic DNA, a PCR (1x 95°C, 1 min; 40x (95°C, 1 min, 60°C, 30 sec, 72°C, 30 sec)) was conducted with primers for *ACTIN 2/7*. Because the two primers bound in different exons, it was possible to distinguish between cDNA and genomic DNA. The amplicon lengths were

180bp for cDNA and 278 bp for genomic DNA. The uncontaminated cDNA was used for qPCR.

Quantitative Real Time PCR (qPCR)

For preparation of the reaction mix, the IQ SYBR Green Supermix [BioRad] was used according to the manufacturer´s manual, with minor modifications (only the half amount of the reagents described in the manual were used) . A total reaction volume of 25µl was chosen (12,5µl 2x reaction mix, 2,5µl of each primer; 2,5µl template and 5µl dH2O). The standard PCR program was used (1 x 1min 95°C; 50 x (1 min 95°C, 30sec 60°C, 30 sec 72°C), with 50 cycles and 60°C annealing temperature, as the qPCR primers for *AtCOM1* and *ACTIN2/7* were both designed according to the recommendations of the used reaction kit (60°C; amplicon length 100-300bp). The final amplicon lengths were 194bp for *AtCOM1* and 180bp for *ACTIN2/7*. Reactions were performed in the BioRad iQ5 Cycler and results were calculated with the gene expression analysis tool of the BioRad iQ5 software. The critical value for the calculation is the cycle threshold (C_t). This is the number of cycles needed to reach. the fluorescence detection threshold. The C_t value depends on the number of templates present at the start of the PCR (Lafarge and Montane 2003). Every sample was analyzed twice in parallel, so an average C_t value was calculated for every sample. *ACTIN2/7* was used as a reference gene. Relative quantification was done using the comparative C_t method. The ΔC_t value is the substraction of the average C_t value of the reference gene from the average C_t value of the sample. This can be compared to the ΔC_t value of a control (for example: unirradiated wildtype). The $\Delta\Delta C_t$ is the substraction of the ΔC_t of the control from that of the sample. The amount of target, determined by normalization to the reference gene and relative to the control, is $2^{-\Delta\Delta Ct}$ (Lafarge and Montane 2003). An example of the calculation is given in the supplements. A technical repeat, as well as a biological repeat was performed for each experiment and an average target amount was calculated.

Calculation of statistical significance

For calculations of statistical significance the program SPSS 15.0 was used. A paired t-test with confidence level = 0,95 was performed.

7 Supplementary Data

7.1 qPCR

For all qPCRs the same program was used:

Cycle1: 1x 95°C for 3 min
Cycle2: 50x
    Step1: 95°C for 1 min
    Step2: 60°C for 30 sec
    Step3: 72°C for 30 sec
Cycle3: 1x 95°C for 1 min
Cycle4: 1x 60°C for 1 min
Cycle5: 72x
    60-95°C for 30 sec

In Cycle5 the temperature was increased by 0,5°C every 30 seconds, starting from 60°C. This was done to establish a melting curve.

Example for a qPCR run:

Figure 29 shows an amplification curve of the exemplary qPCR experiment. In this experiment *COM1* mRNA levels of different samples were compared. *ACTIN2/7* was used as a reference.

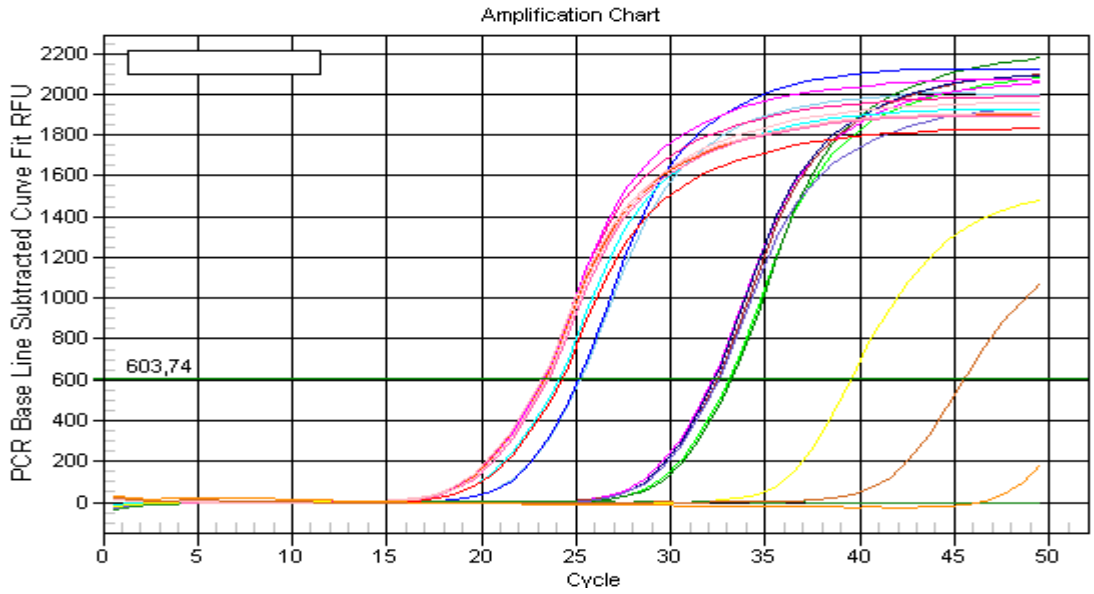


Figure 29 - The graph shows amplification curves of different samples exceeding the fluorescence detection threshold at different PCR cycles. These cycle numbers are the C_t values of the samples (Figure 30).

Gene	Condition	Ctrl	Ct SD	Mean CT
ACTIN	NTC-2		0,00	45,46
COM1	NTC-1		0,00	39,54
COM1	wt-0Gy	*	0,10	33,14
ACTIN	wt-0Gy	*	0,11	24,16
COM1	wt-100Gy		0,06	25,21
ACTIN	wt-100Gy		0,08	23,24
COM1	atm-0Gy		0,17	32,32
ACTIN	atm-0Gy		0,23	23,39
COM1	atm-100Gy		0,19	32,40
ACTIN	atm-100Gy		0,17	23,29

Figure 30 – A list of mean C_t values of different samples of the qPCR experiment

COM1 wt-0Gy, which has a mean C_t value of 33,14 is the control in this example. ACTIN wt-0Gy, with a mean C_t value of 24,16 is the reference of COM1 wt-0Gy. Since ΔC_t is the substraction of the C_t of the reference from that of the sample, ΔC_t of the control is 8,98. The target in this example, COM1 wt-100Gy has a mean C_t of 25,21 and its reference, ACTIN wt-0Gy has a mean C_t of 23,24. The ΔC_t of the target is thus 1,97. The substraction of the ΔC_t of the control from that of the target is $\Delta\Delta C_t$. In this example it is -7,01. The normalized amount of the target (COM1 wt-100Gy) is $2^{-\Delta\Delta Ct}$ and thus 128,89. This is shown in Figure 31.

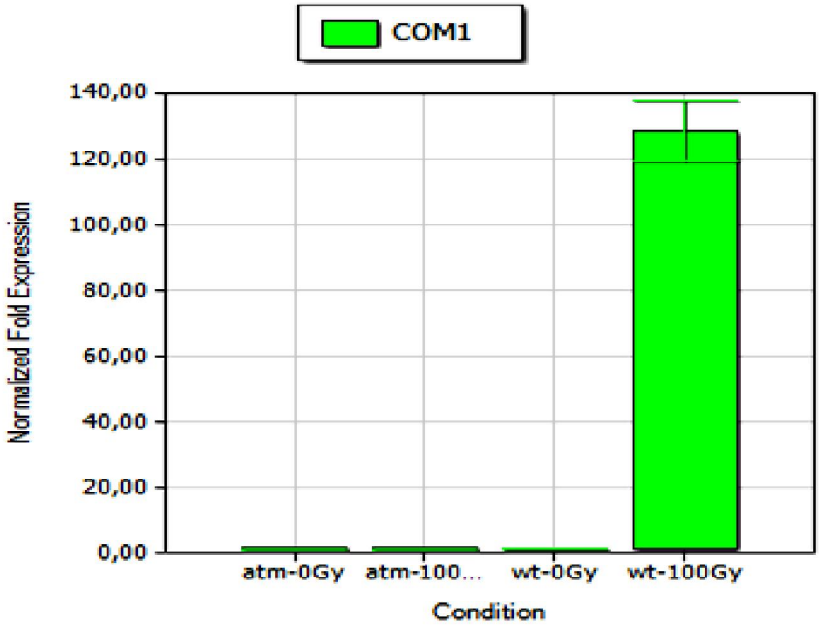
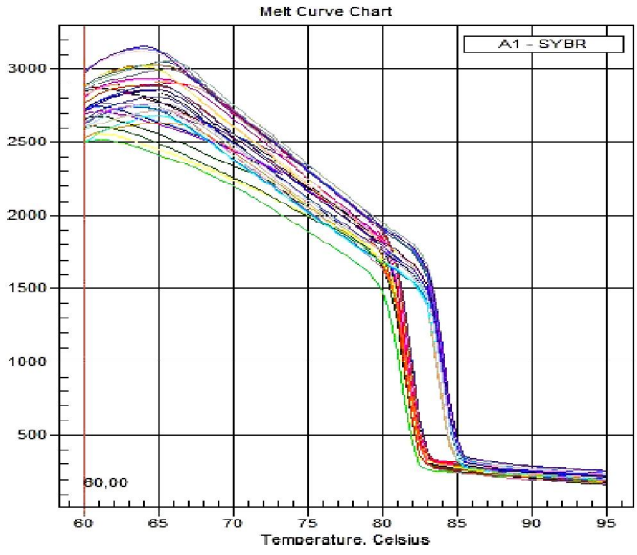


Figure 31 – The graph shows the normalized fold expression of *COM1* in different samples. The calculated value for wt-100Gy is 128,89

The according melting curves of the samples used for this qPCR are shown in the next figure.



The ACTIN2/7 amplicons are melted at a temperature of about 83°C, while the amplicons are melted at a temperature of about 85°C. If another DNA fragment had been amplified during the qPCR, it would appear as an additional curve, that reaches its minimum at a different temperature.

7.2 Primers

All primers are in 5´ to 3´ orientation

qPCR

#395: actin_ampl3_dn: TTGCTGACCGTATGAGCAAAGA
#396: actin_ampl3_up: TCGATGGACCTGACTCATCGT

#397: com1_ampl1_dn: TTCACCAAAGCAGCCTTGAG
#398: com1_ampl1_up: GGAAGTGATAGGTGTCTGCACTG

#1665: E2FA_dn: TTCCCCAGTGAGATTGGTTT
#1666: E2FA_up: TTGTCGAGATGGGTGTTTGA

#1663: E2FB_dn: TTCTAAGCGGCAGCTTCATC
#1664: E2FB_up: CAGAAGCTAGCGTTCCAGACT

#1669: E2FC_dn: TCAAACTCAGGCGAAGATCC
#1670: E2FC_up: CATTCGTTTCCCAGCCTTTA

#1661: DEL1_dn: TGATGATGAGGATGATGATGAAG
#1662: DEL1_up: TCAGAGCAAATAAAGAGTTTGATAAAG

#1659: DEL3_dn: CCTTCCCTTTCCGACTTTGT
#1660: DEL3_up: CAAATGTATTTGCCTCGATGA

#1667: DPA_dn: GCAATGCAAGAACTGGATGA
#1668: DPA_up: AAACCCTCACGCAGTAGTCG

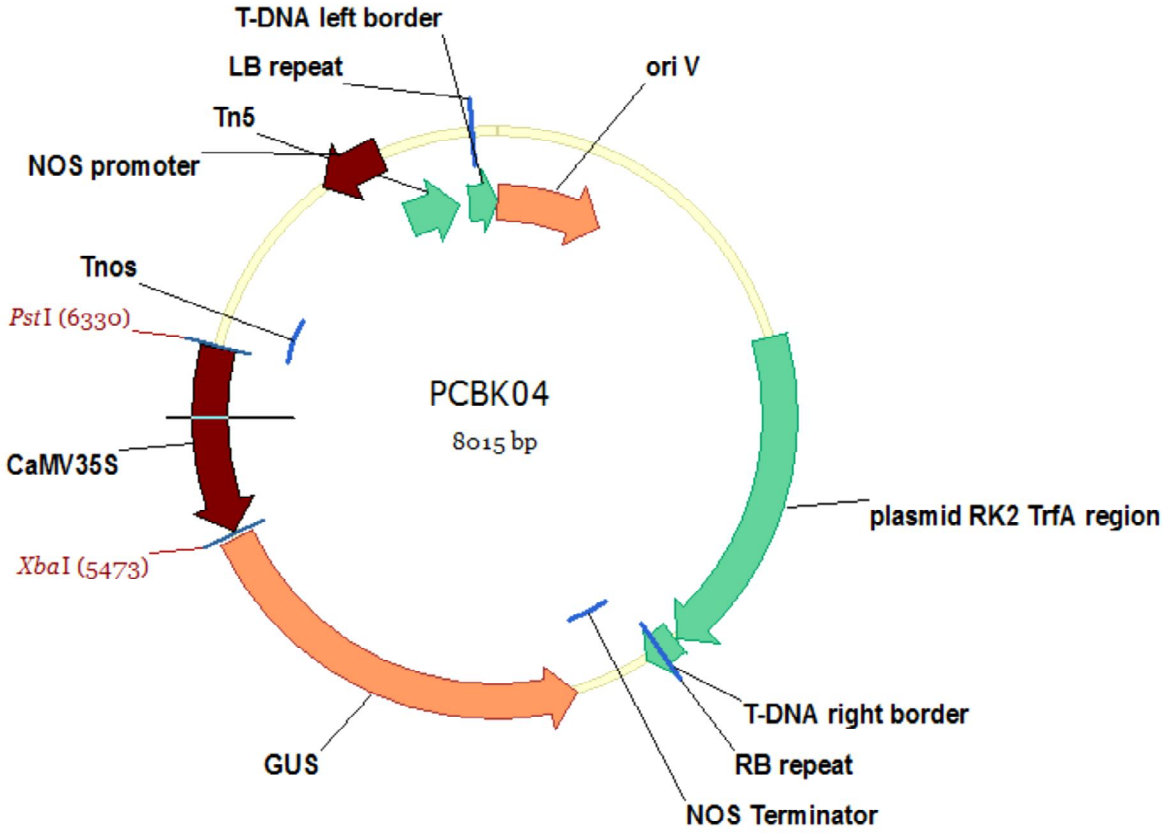
Conventional PCR

#395: actin_ampl3_dn: TTGCTGACCGTATGAGCAAAGA
#396: actin_ampl3_up: TCGATGGACCTGACTCATCGT

PCR program: 1x 95°C for 1min; 40x 95°C for 1min, 60°C for 30 sec, 72°C for 30 sec

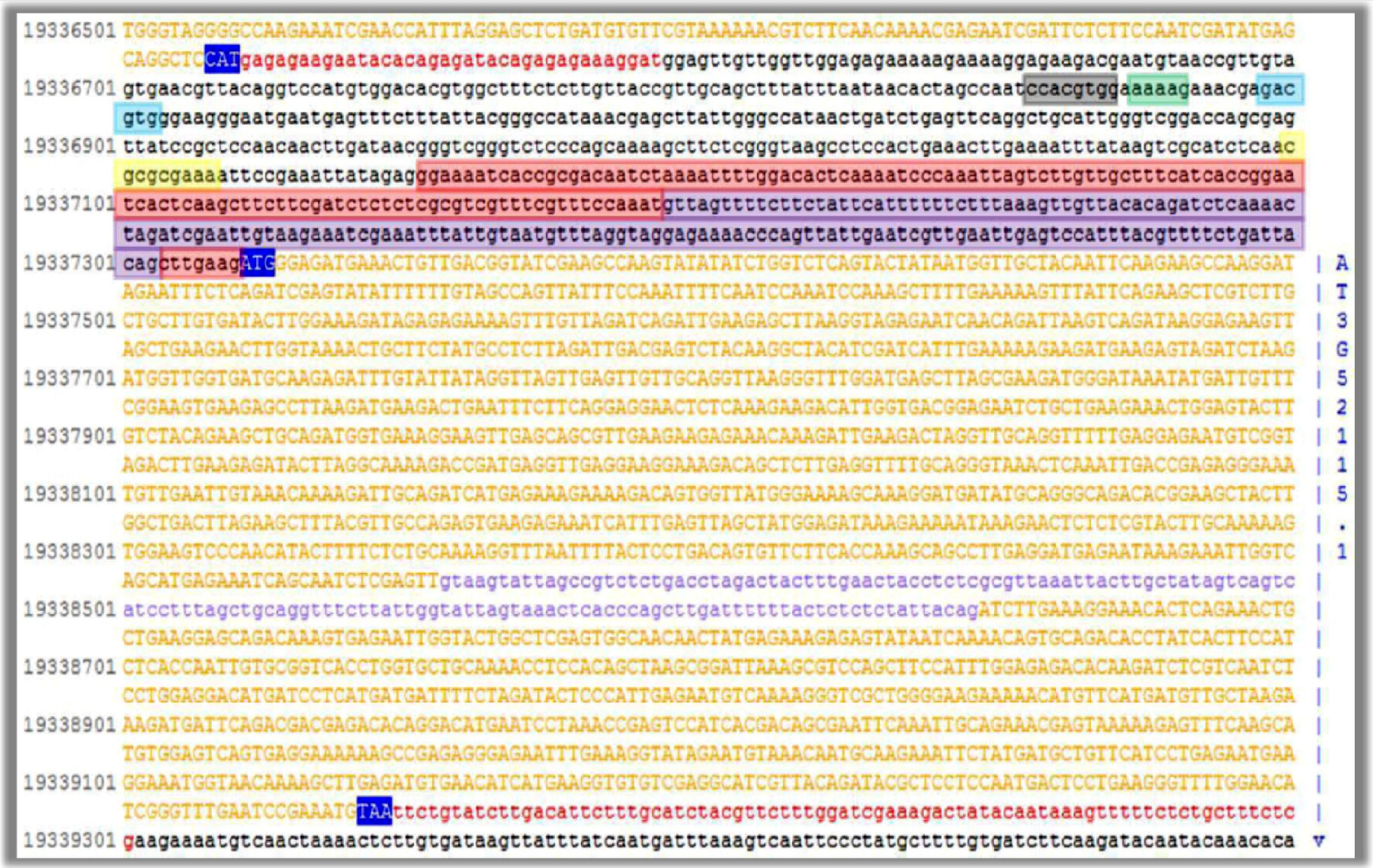
7.3 pCBK04 vector

This binary vector was kindly provided by Karel Riha



7.4 Sequence of AtCOM1

The sequence of the *AtCOM1* gene (At3g52115), full-length genomic, according to the TAIR database (http://www.arabidopsis.org). Picture was taken from Uanschou, 2008.


19336501 TGGGTAGGGGCCAAGAAATCGAACCATTTAGGAGCTCTGATGTGTTCGTAAAAAACGTCTTCAACAAAACGAGAATCGATTCTCTTCCAATCGATATGAG
CAGGCTCCATgagagaagaatacacagagatacagagagaaaggatggagttgttggttggagagaaaaagaaaaggagaagacgaatgtaaccgttgta
19336701 gtgaacgttacaggtccatgtggacacgtggctttctcttgttaccgttgcagctttatttaataacactagccaatccacgtggaaaaagaaacgagac
gtgggaagggaatgaatgagtttctttattacgggccataaacgagcttattgggccataactgatctgagttcaggctgcattgggtcggaccagcgag
19336901 ttatccgctccaacaacttgataacgggtcgggtctcccagcaaaagcttctcgggtaagcctccactgaaacttgaaaatttataagtcgcatctcaac
gcgcgaaaattccgaaattatagaggaaaatcaccgcgacaatctaaaattttggacactcaaaatcccaaattagtcttgttgctttcatcaccggaa
19337101 tcactcaagcttcttcgatctctctcgcgtcgtttcgtttccaaatgttagttttcttctattcatttttctttaaagttgttacacagatctcaaaac
tagatcgaattgtaagaaatcgaaatttattgtaatgtttaggtaggagaaaacccagttattgaatcgttgaattgagtccatttacgttttctgatta
19337301 cagcttgaagATGGAGATGAAACTGTTGACGGTATCGAAGCCAAGTATATATCTGGTCTCAGTACTATAATGGTTGCTACAATTCAAGAAGCCAAGGAT | A
AGAATTTCTCAGATCGAGTATATTTTTTGTAGCCAGTTATTTCCAAATTTTCAATCCAAATCCAAAGCTTTTGAAAAAGTTTATTCAGAAGCTCGTCTTG | T
19337501 CTGCTTGTGATACTTGGAAAGATAGAGAGAAAAGTTTGTTAGATCAGATTGAAGAGCTTAAGGTAGAGAATCAACAGATTAAGTCAGATAAGGAGAAGTT | 3
AGCTGAAGAACTTGGTAAAACTGCTTCTATGCCTCTTAGATTGACGAGTCTACAAGGCTACATCGATCATTTGAAAAAGAAGATGAAGAGTAGATCTAAG | G
19337701 ATGGTTGGTGATGCAAGAGATTTGTATTATAGGTTAGTTGAGTTGTTGCAGGTTAAGGGTTTGGATGAGCTTAGCGAAGATGGGATAAATATGATTGTTT | 5
CGGAAGTGAAGAGCCTTAAGATGAAGACTGAATTTCTTCAGGAGGAACTCTCAAAGAAGACATTGGTGACGGAGAATCTGCTGAAGAAACTGGAGTACTT | 2
19337901 GTCTACAGAAGCTGCAGATGGTGAAAGGAAGTTGAGCAGCGTTGAAGAAGAGAAACAAAGATTGAAGACTAGGTTGCAGGTTTTTGAGGAGAATGTCGGT | 1
AGACTTGAAGAGATACTTAGGCAAAAGACCGATGAGGTTGAGGAAGGAAAGACAGCTCTTGAGGTTTTGCAGGGTAAACTCAAATTGACCGAGAGGGAAA | 1
19338101 TGTTGAATTGTAAACAAAAGATTGCAGATCATGAGAAAGAAAAGACAGTGGTTATGGGAAAAGCAAAGGATGATATGCAGGGCAGACACGGAAGCTACTT | 5
GGCTGACTTAGAAGCTTTACGTTGCCAGAGTGAAGAGAAATCATTTGAGTTAGCTATGGAGATAAAGAAAAATAAAGAACTCTCTCGTACTTGCAAAAAG | .
19338301 TGGAAGTCCCAACATACTTTTCTCTGCAAAAGGTTTAATTTTACTCCTGACAGTGTTCTTCACCAAAGCAGCCTTGAGGATGAGAATAAAGAAATTGGTC | 1
AGCATGAGAAATCAGCAATCTCGAGTTgtaagtattagccgtctctgacctagactactttgaactacctctcgcgttaaattacttgctatagtcagtc |
19338501 atcctttagctgcaggtttcttattggtattagtaaactcacccagcttgatttttactctctctattacagATCTTGAAAGGAAACACTCAGAAACTG |
CTGAAGGAGCAGACAAAGTGAGAATTGGTACTGGCTCGAGTGGCAACAACTATGAGAAAGAGAGTATAATCAAAACAGTGCAGACACCTATCACTTCCAT |
19338701 CTCACCAATTGTGCGGTCACCTGGTGCTGCAAAACCTCCACAGCTAAGCGGATTAAAGCGTCCAGCTTCCATTTGGAGAGACACAAGATCTCGTCAATCT |
CCTGGAGGACATGATCCTCATGATGATTTTCTAGATACTCCCATTGAGAATGTCAAAAGGGTCGCTGGGGAAGAAAAACATGTTCATGATGTTGCTAAGA |
19338901 AAGATGATTCAGACGACGAGACACAGGACATGAATCCTAAACCGAGTCCATCACGACAGCGAATTCAAAATTGCAGAAACGAGTAAAAAGAGTTTCAAGCA |
TGTGGAGTCAGTGAGGAAAAAAGCCGAGAGGGAGAATTTGAAAGGTATAGAATGTAAACAATGCAAGAAATTCTATGATGCTGTTCATCCTGAGAATGAA |
19339101 GGAAATGGTAACAAAAGCTTGAGATGTGAACATCATGAAGGTGTGTCGAGGCATCGTTACAGATACGCTCCTCCAATGACTCCTGAAGGGTTTTGGAACA |
TCGGGTTTGAATCCGAAATGTAAttctgtatcttgacattctttgcatctacgttctttggatcgaaagactatacaataaagtttttctctgctttctc |
19339301 gaagaaaatgtcaactaaaactcttgtgataagttatttatcaatgatttaaagtcaattccctatgcttttgtgatcttcaagatacaatacaaacaca v

The gene consists of two exons (orange), that are separated by an intron (violet). The start-and stopcodons are marked in blue and the 5´ and 3´UTRs of *AtCOM1* are marked in red. An intron, situated inside the 5´UTR is depicted in violet. The two putative bZIP TF binding sites are coloured in grey and light blue, respectively and the DOF binding site is coloured in green. The E2F site is depicted in yellow. Upstream of the *AtCOM1* promoter lies the promoter of another gene (At3g52110), whose 5´UTR is marked in red

7.5 Abbreviations

At	Arabidopsis thaliana
ATM	Ataxia telangiectasia mutated
ATR	ATM and Rad3 related
bp	base pairs
CARE	*cis* acting regulatory elements
CDK	cyclin dependant kinase
cDNA	copy DNA
ChIP	chromatin immunoprecipitation
CKI	cyclin-dependant kinase inhibitor
CO	crossover
COM	Completion of Meiosis
DAG	days after germination
DDR	DNA damage response
DEL	DP-E2F-like
DNA-PK	DNA-dependent protein kinase
DP	dimerization partner
DSB	double strand break
DSBR	double-strand break repair
GR	gamma response

GTF	general transcription factor
GUS	ß-glucuronidase
HR	homologous recombination
HU	Hydroxyurea
IR	ionizing radiation
kb	kilobases
MMC	Mitomycin C
MMS	methyl methane sulfonate
MRE	meiotic recombination
mRNA	messenger RNA
NBS	Nijmegen Breakage Syndrome
NCO	non-crossover
NHEJ	non-homologous end joining
OE	overexpressor
Os	Oryza sativa
PCD	programmed cell death
PCR	polymerase chain reaction
PKcs	catalytic subunit of DNA-PK
Pol	polymerase
qPCR	quantitative real time PCR
RAD	Radiation sensitive
RBR	retinoblastoma related
RPA	replication protein A
SAE	sporulation in the absence of spo eleven
SC	synaptonemal complex
SDSA	synthesis-dependent strand annealing
SOG	suppressor of gamma response
SSB	single strand break
ssDNA	single-stranded DNA
TF	transcription factor
TSS	transcription start site
UTR	untranslated region
X-Gluc	5-bromo-4-chloro-3-indolyl glucuronide

8 References

Abraham, R. T. (2001). "Cell cycle checkpoint signaling through the ATM and ATR kinases." Genes Dev **15**(17): 2177-2196.

Adachi, S., K. Minamisawa, et al. (2011). "Programmed induction of endoreduplication by DNA double-strand breaks in Arabidopsis." Proc Natl Acad Sci U S A **108**(24): 10004-10009.

Amiard, S., C. Charbonnel, et al. (2010). "Distinct roles of the ATR kinase and the Mre11-Rad50-Nbs1 complex in the maintenance of chromosomal stability in Arabidopsis." Plant Cell **22**(9): 3020-3033.

Barlow, J. H., M. Lisby, et al. (2008). "Differential regulation of the cellular response to DNA double-strand breaks in G1." Mol Cell **30**(1): 73-85.

Bennett, C. B., A. L. Lewis, et al. (1993). "Lethality induced by a single site-specific double-strand break in a dispensable yeast plasmid." Proc Natl Acad Sci U S A **90**(12): 5613-5617.

Beranek, D. T. (1990). "Distribution of methyl and ethyl adducts following alkylation with monofunctional alkylating agents." Mutat Res **231**(1): 11-30.

Berckmans, B. and L. De Veylder (2009). "Transcriptional control of the cell cycle." Curr Opin Plant Biol **12**(5): 599-605.

Berckmans, B., T. Lammens, et al. (2011). "Light-dependent regulation of DEL1 is determined by the antagonistic action of E2Fb and E2Fc." Plant Physiol **157**(3): 1440-1451.

Bergerat, A., B. de Massy, et al. (1997). "An atypical topoisomerase II from Archaea with implications for meiotic recombination." Nature **386**(6623): 414-417.

Bernstein, K. A. and R. Rothstein (2009). "At loose ends: resecting a double-strand break." Cell **137**(5): 807-810.

Bird, A. (2007). "Perceptions of epigenetics." Nature **447**(7143): 396-398.

Bisova, K., D. M. Krylov, et al. (2005). "Genome-wide annotation and expression profiling of cell cycle regulatory genes in Chlamydomonas reinhardtii." Plant Physiol **137**(2): 475-491.

Bleuyard, J. Y., M. E. Gallego, et al. (2005). "Differing requirements for the Arabidopsis Rad51 paralogs in meiosis and DNA repair." Plant J **41**(4): 533-545.

Bleuyard, J. Y., M. E. Gallego, et al. (2006). "Recent advances in understanding of the DNA double-strand break repair machinery of plants." DNA Repair (Amst) **5**(1): 1-12.

Borde, V. (2007). "The multiple roles of the Mre11 complex for meiotic recombination." Chromosome Res **15**(5): 551-563.

Borghi, L., R. Gutzat, et al. (2010). "Arabidopsis RETINOBLASTOMA-RELATED is required for stem cell maintenance, cell differentiation, and lateral organ production." Plant Cell **22**(6): 1792-1811.

Brand, L., M. Horler, et al. (2006). "A versatile and reliable two-component system for tissue-specific gene induction in Arabidopsis." Plant Physiol **141**(4): 1194-1204.

Britt, A. B. and G. D. May (2003). "Re-engineering plant gene targeting." Trends Plant Sci **8**(2): 90-95.

Campbell NA, R. J. (2009). Biology, 8th edn. San Francisco, Pearson Benjamin Cummings.

Carlson, J. M., A. Chakravarty, et al. (2007). "SCOPE: a web server for practical de novo motif discovery." Nucleic Acids Res **35**(Web Server issue): W259-264.

Casimiro, I., T. Beeckman, et al. (2003). "Dissecting Arabidopsis lateral root development." Trends Plant Sci **8**(4): 165-171.

Charbonnel, C., E. Allain, et al. (2011). "Kinetic analysis of DNA double-strand break repair pathways in Arabidopsis." DNA Repair (Amst) **10**(6): 611-619.

Charbonnel, C., M. E. Gallego, et al. (2010). "Xrcc1-dependent and Ku-dependent DNA double-strand break repair kinetics in Arabidopsis plants." Plant J **64**(2): 280-290.

Chen, P. L., F. Liu, et al. (2005). "Inactivation of CtIP leads to early embryonic lethality mediated by G1 restraint and to tumorigenesis by haploid insufficiency." Mol Cell Biol **25**(9): 3535-3542.

Chen, Z., H. Yang, et al. (2008). "Mechanism of homologous recombination from the RecA-ssDNA/dsDNA structures." Nature **453**(7194): 489-484.

Cimprich, K. A. and D. Cortez (2008). "ATR: an essential regulator of genome integrity." Nat Rev Mol Cell Biol **9**(8): 616-627.
Clerici, M., D. Mantiero, et al. (2006). "The Saccharomyces cerevisiae Sae2 protein negatively regulates DNA damage checkpoint signalling." EMBO Rep **7**(2): 212-218.
Coelho, C. M., R. A. Dante, et al. (2005). "Cyclin-dependent kinase inhibitors in maize endosperm and their potential role in endoreduplication." Plant Physiol **138**(4): 2323-2336.
Cramer, P., D. A. Bushnell, et al. (2001). "Structural basis of transcription: RNA polymerase II at 2.8 angstrom resolution." Science **292**(5523): 1863-1876.
Critchlow, S. E. and S. P. Jackson (1998). "DNA end-joining: from yeast to man." Trends Biochem Sci **23**(10): 394-398.
Culligan, K., A. Tissier, et al. (2004). "ATR regulates a G2-phase cell-cycle checkpoint in Arabidopsis thaliana." Plant Cell **16**(5): 1091-1104.
Culligan, K. M. and A. B. Britt (2008). "Both ATM and ATR promote the efficient and accurate processing of programmed meiotic double-strand breaks." Plant J **55**(4): 629-638.
Culligan, K. M., C. E. Robertson, et al. (2006). "ATR and ATM play both distinct and additive roles in response to ionizing radiation." Plant J **48**(6): 947-961.
Czornak, K., S. Chughtai, et al. (2008). "Mystery of DNA repair: the role of the MRN complex and ATM kinase in DNA damage repair." J Appl Genet **49**(4): 383-396.
De Veylder, L., T. Beeckman, et al. (2002). "Control of proliferation, endoreduplication and differentiation by the Arabidopsis E2Fa-DPa transcription factor." EMBO J **21**(6): 1360-1368.
De Veylder, L., T. Beeckman, et al. (2001). "Functional analysis of cyclin-dependent kinase inhibitors of Arabidopsis." Plant Cell **13**(7): 1653-1668.
del Pozo, J. C., M. B. Boniotti, et al. (2002). "Arabidopsis E2Fc functions in cell division and is degraded by the ubiquitin-SCF(AtSKP2) pathway in response to light." Plant Cell **14**(12): 3057-3071.
del Pozo, J. C., S. Diaz-Trivino, et al. (2006). "The balance between cell division and endoreplication depends on E2FC-DPB, transcription factors regulated by the ubiquitin-SCFSKP2A pathway in Arabidopsis." Plant Cell **18**(9): 2224-2235.
Deveaux, Y., B. Alonso, et al. (2000). "Molecular cloning and developmental expression of AtGR1, a new growth-related Arabidopsis gene strongly induced by ionizing radiation." Radiat Res **154**(4): 355-364.
Dewitte, W., S. Scofield, et al. (2007). "Arabidopsis CYCD3 D-type cyclins link cell proliferation and endocycles and are rate-limiting for cytokinin responses." Proc Natl Acad Sci U S A **104**(36): 14537-14542.
Di Laurenzio, L., J. Wysocka-Diller, et al. (1996). "The SCARECROW gene regulates an asymmetric cell division that is essential for generating the radial organization of the Arabidopsis root." Cell **86**(3): 423-433.
Dissmeyer, N., M. K. Nowack, et al. (2007). "T-loop phosphorylation of Arabidopsis CDKA;1 is required for its function and can be partially substituted by an aspartate residue." Plant Cell **19**(3): 972-985.
Doonan, J. and T. Hunt (1996). "Cell cycle. Why don't plants get cancer?" Nature **380**(6574): 481-482.
Downs, J. A. and S. P. Jackson (2004). "A means to a DNA end: the many roles of Ku." Nat Rev Mol Cell Biol **5**(5): 367-378.
Edlinger, B. and P. Schlögelhofer (2011). "Have a break: determinants of meiotic DNA double strand break (DSB) formation and processing in plants." J Exp Bot **62**(5): 1545-1563.
Fulcher, N. and R. Sablowski (2009). "Hypersensitivity to DNA damage in plant stem cell niches." Proc Natl Acad Sci U S A **106**(49): 20984-20988.
Furler, R. (2012). "Why is Glucose Metabolized Differently Throughout the Cell Cycle?" Microbiology, Immunology & Molecular Genetics.
Furukawa, T., M. J. Curtis, et al. (2010). "A shared DNA-damage-response pathway for induction of stem-cell death by UVB and by gamma irradiation." DNA Repair (Amst) **9**(9): 940-948.

Galbraith, D. W., K. R. Harkins, et al. (1991). "Systemic Endopolyploidy in Arabidopsis thaliana." Plant Physiol **96**(3): 985-989.
Garcia, V., H. Bruchet, et al. (2003). "AtATM is essential for meiosis and the somatic response to DNA damage in plants." Plant Cell **15**(1): 119-132.
Garcia, V., S. E. Phelps, et al. (2011). "Bidirectional resection of DNA double-strand breaks by Mre11 and Exo1." Nature **479**(7372): 241-244.
Gasch, A. P., M. Huang, et al. (2001). "Genomic expression responses to DNA-damaging agents and the regulatory role of the yeast ATR homolog Mec1p." Mol Biol Cell **12**(10): 2987-3003.
Gottlieb, T. M. and S. P. Jackson (1993). "The DNA-dependent protein kinase: requirement for DNA ends and association with Ku antigen." Cell **72**(1): 131-142.
Grafi, G. and Y. Avivi (2004). "Stem cells: a lesson from dedifferentiation." Trends Biotechnol **22**(8): 388-389.
Gravel, S., J. R. Chapman, et al. (2008). "DNA helicases Sgs1 and BLM promote DNA double-strand break resection." Genes Dev **22**(20): 2767-2772.
Grawunder, U., M. Wilm, et al. (1997). "Activity of DNA ligase IV stimulated by complex formation with XRCC4 protein in mammalian cells." Nature **388**(6641): 492-495.
Greenberg, R. A., B. Sobhian, et al. (2006). "Multifactorial contributions to an acute DNA damage response by BRCA1/BARD1-containing complexes." Genes Dev **20**(1): 34-46.
Grelon, M., D. Vezon, et al. (2001). "AtSPO11-1 is necessary for efficient meiotic recombination in plants." EMBO J **20**(3): 589-600.
Gudesblat, G. E. and E. Russinova (2011). "Plants grow on brassinosteroids." Curr Opin Plant Biol **14**(5): 530-537.
Hamant, O., H. Ma, et al. (2006). "Genetics of meiotic prophase I in plants." Annu Rev Plant Biol **57**: 267-302.
Harper, J. W. and S. J. Elledge (2007). "The DNA damage response: ten years after." Mol Cell **28**(5): 739-745.
Hartley, K. O., D. Gell, et al. (1995). "DNA-dependent protein kinase catalytic subunit: a relative of phosphatidylinositol 3-kinase and the ataxia telangiectasia gene product." Cell **82**(5): 849-856.
Hartung, F. and H. Puchta (2000). "Molecular characterisation of two paralogous SPO11 homologues in Arabidopsis thaliana." Nucleic Acids Res **28**(7): 1548-1554.
Hays, J. B. (2002). "Arabidopsis thaliana, a versatile model system for study of eukaryotic genome-maintenance functions." DNA Repair (Amst) **1**(8): 579-600.
Holliday, R. (1990). "Mechanisms for the control of gene activity during development." Biol Rev Camb Philos Soc **65**(4): 431-471.
Holt, K. E., A. H. Millar, et al. (2006). "ModuleFinder and CoReg: alternative tools for linking gene expression modules with promoter sequences motifs to uncover gene regulation mechanisms in plants." Plant Methods **2**: 8.
Huang, J. and W. S. Dynan (2002). "Reconstitution of the mammalian DNA double-strand break end-joining reaction reveals a requirement for an Mre11/Rad50/NBS1-containing fraction." Nucleic Acids Res **30**(3): 667-674.
Huertas, P., F. Cortes-Ledesma, et al. (2008). "CDK targets Sae2 to control DNA-end resection and homologous recombination." Nature **455**(7213): 689-692.
Inze, D. and L. De Veylder (2006). "Cell cycle regulation in plant development." Annu Rev Genet **40**: 77-105.
Jackson, S. P. (2002). "Sensing and repairing DNA double-strand breaks." Carcinogenesis **23**(5): 687-696.
Jasinski, S., C. Perennes, et al. (2002). "Comparative molecular and functional analyses of the tobacco cyclin-dependent kinase inhibitor NtKIS1a and its spliced variant NtKIS1b." Plant Physiol **130**(4): 1871-1882.

Ji, J., D. Tang, et al. (2012). "The role of OsCOM1 in homologous chromosome synapsis and recombination in rice meiosis." Plant J.
Johnson, R. D. and M. Jasin (2000). "Sister chromatid gene conversion is a prominent double-strand break repair pathway in mammalian cells." EMBO J **19**(13): 3398-3407.
Kanoh, Y., K. Tamai, et al. (2006). "Different requirements for the association of ATR-ATRIP and 9-1-1 to the stalled replication forks." Gene **377**: 88-95.
Kasamatsu, H., D. L. Robberson, et al. (1971). "A novel closed-circular mitochondrial DNA with properties of a replicating intermediate." Proc Natl Acad Sci U S A **68**(9): 2252-2257.
Keeney, S., C. N. Giroux, et al. (1997). "Meiosis-specific DNA double-strand breaks are catalyzed by Spo11, a member of a widely conserved protein family." Cell **88**(3): 375-384.
Kinner, A., W. Wu, et al. (2008). "Gamma-H2AX in recognition and signaling of DNA double-strand breaks in the context of chromatin." Nucleic Acids Res **36**(17): 5678-5694.
Klapstein, K., T. Chou, et al. (2004). "Physics of RecA-mediated homologous recognition." Biophys J **87**(3): 1466-1477.
Kondorosi, E., F. Roudier, et al. (2000). "Plant cell-size control: growing by ploidy?" Curr Opin Plant Biol **3**(6): 488-492.
Kosugi, S. and Y. Ohashi (2003). "Constitutive E2F expression in tobacco plants exhibits altered cell cycle control and morphological change in a cell type-specific manner." Plant Physiol **132**(4): 2012-2022.
Kovesdi, I., R. Reichel, et al. (1986). "Identification of a cellular transcription factor involved in E1A trans-activation." Cell **45**(2): 219-228.
Krejci, L., V. Altmannova, et al. (2012). "Homologous recombination and its regulation." Nucleic Acids Res **40**(13): 5795-5818.
Krogh, B. O. and L. S. Symington (2004). "Recombination proteins in yeast." Annu Rev Genet **38**: 233-271.
Kurzbauer, M.-T. (2008). Analysis of the AtCOM1/GR1 Promoter. universität wien. wien, university of vienna. **mag. re. nat.:** 71.
Kurzbauer, M. T., C. Uanschou, et al. (2012). "The recombinases DMC1 and RAD51 are functionally and spatially separated during meiosis in Arabidopsis." Plant Cell **24**(5): 2058-2070.
Lafarge, S. and M. H. Montane (2003). "Characterization of Arabidopsis thaliana ortholog of the human breast cancer susceptibility gene 1: AtBRCA1, strongly induced by gamma rays." Nucleic Acids Res **31**(4): 1148-1155.
Lamarche, B. J., N. I. Orazio, et al. (2010). "The MRN complex in double-strand break repair and telomere maintenance." FEBS Lett **584**(17): 3682-3695.
Lammens, T., V. Boudolf, et al. (2008). "Atypical E2F activity restrains APC/CCCS52A2 function obligatory for endocycle onset." Proc Natl Acad Sci U S A **105**(38): 14721-14726.
Larkins, B. A., B. P. Dilkes, et al. (2001). "Investigating the hows and whys of DNA endoreduplication." J Exp Bot **52**(355): 183-192.
Lees-Miller, S. P. and K. Meek (2003). "Repair of DNA double strand breaks by non-homologous end joining." Biochimie **85**(11): 1161-1173.
Lengsfeld, B. M., A. J. Rattray, et al. (2007). "Sae2 is an endonuclease that processes hairpin DNA cooperatively with the Mre11/Rad50/Xrs2 complex." Mol Cell **28**(4): 638-651.
Lieber, M. R. (2010). "The mechanism of double-strand DNA break repair by the nonhomologous DNA end-joining pathway." Annu Rev Biochem **79**: 181-211.
Limbo, O., C. Chahwan, et al. (2007). "Ctp1 is a cell-cycle-regulated protein that functions with Mre11 complex to control double-strand break repair by homologous recombination." Mol Cell **28**(1): 134-146.
Lisby, M., J. H. Barlow, et al. (2004). "Choreography of the DNA damage response: spatiotemporal relationships among checkpoint and repair proteins." Cell **118**(6): 699-713.
Lloyd, A. H., D. Wang, et al. (2012). "Single molecule PCR reveals similar patterns of non-homologous DSB repair in tobacco and Arabidopsis." PLoS One **7**(2): e32255.

Lobachev, K. S., D. A. Gordenin, et al. (2002). "The Mre11 complex is required for repair of hairpin-capped double-strand breaks and prevention of chromosome rearrangements." Cell **108**(2): 183-193.

Longhese, M. P., D. Bonetti, et al. (2009). "DNA double-strand breaks in meiosis: checking their formation, processing and repair." DNA Repair (Amst) **8**(9): 1127-1138.

Longhese, M. P., D. Bonetti, et al. (2010). "Mechanisms and regulation of DNA end resection." EMBO J **29**(17): 2864-2874.

Lukaszewicz, A., R. A. Howard-Till, et al. (2010). "MRE11 and COM1/SAE2 are required for double-strand break repair and efficient chromosome pairing during meiosis of the protist Tetrahymena." Chromosoma **119**(5): 505-518.

Lundin, C., M. North, et al. (2005). "Methyl methanesulfonate (MMS) produces heat-labile DNA damage but no detectable in vivo DNA double-strand breaks." Nucleic Acids Res **33**(12): 3799-3811.

Ma, H. (2006). "A molecular portrait of Arabidopsis meiosis." Arabidopsis Book **4**: e0095.

Ma, Y., K. Schwarz, et al. (2005). "The Artemis:DNA-PKcs endonuclease cleaves DNA loops, flaps, and gaps." DNA Repair (Amst) **4**(7): 845-851.

Magyar, Z., B. Horvath, et al. (2012). "Arabidopsis E2FA stimulates proliferation and endocycle separately through RBR-bound and RBR-free complexes." EMBO J **31**(6): 1480-1493.

Maloisel, L., J. Bhargava, et al. (2004). "A role for DNA polymerase delta in gene conversion and crossing over during meiosis in Saccharomyces cerevisiae." Genetics **167**(3): 1133-1142.

Manfrini, N., I. Guerini, et al. (2010). "Processing of meiotic DNA double strand breaks requires cyclin-dependent kinase and multiple nucleases." J Biol Chem **285**(15): 11628-11637.

McKee, A. H. and N. Kleckner (1997). "A general method for identifying recessive diploid-specific mutations in Saccharomyces cerevisiae, its application to the isolation of mutants blocked at intermediate stages of meiotic prophase and characterization of a new gene SAE2." Genetics **146**(3): 797-816.

McKinnon, P. J. and K. W. Caldecott (2007). "DNA strand break repair and human genetic disease." Annu Rev Genomics Hum Genet **8**: 37-55.

McMahill, M. S., C. W. Sham, et al. (2007). "Synthesis-dependent strand annealing in meiosis." PLoS Biol **5**(11): e299.

Meek, K., S. Gupta, et al. (2004). "The DNA-dependent protein kinase: the director at the end." Immunol Rev **200**: 132-141.

Melo, J. and D. Toczyski (2002). "A unified view of the DNA-damage checkpoint." Curr Opin Cell Biol **14**(2): 237-245.

Mimitou, E. P. and L. S. Symington (2009). "DNA end resection: many nucleases make light work." DNA Repair (Amst) **8**(9): 983-995.

Mladenov, E. and G. Iliakis (2011). "Induction and repair of DNA double strand breaks: the increasing spectrum of non-homologous end joining pathways." Mutat Res **711**(1-2): 61-72.

Moggs, J. G., D. E. Szymkowski, et al. (1997). "Differential human nucleotide excision repair of paired and mispaired cisplatin-DNA adducts." Nucleic Acids Res **25**(3): 480-491.

Nakagami, H., M. Sekine, et al. (1999). "Tobacco retinoblastoma-related protein phosphorylated by a distinct cyclin-dependent kinase complex with Cdc2/cyclin D in vitro." Plant J **18**(3): 243-252.

Neale, M. J. and S. Keeney (2006). "Clarifying the mechanics of DNA strand exchange in meiotic recombination." Nature **442**(7099): 153-158.

Neale, M. J., J. Pan, et al. (2005). "Endonucleolytic processing of covalent protein-linked DNA double-strand breaks." Nature **436**(7053): 1053-1057.

Nimonkar, A. V., A. Z. Ozsoy, et al. (2008). "Human exonuclease 1 and BLM helicase interact to resect DNA and initiate DNA repair." Proc Natl Acad Sci U S A **105**(44): 16906-16911.

Nottke, A., M. P. Colaiacovo, et al. (2009). "Developmental roles of the histone lysine demethylases." Development **136**(6): 879-889.

Osakabe, K., T. Yoshioka, et al. (2002). "Molecular cloning and characterization of RAD51-like genes from Arabidopsis thaliana." Plant Mol Biol **50**(1): 71-81.
Osman, K., J. D. Higgins, et al. (2011). "Pathways to meiotic recombination in Arabidopsis thaliana." New Phytol **190**(3): 523-544.
Paull, T. T. and M. Gellert (1999). "Nbs1 potentiates ATP-driven DNA unwinding and endonuclease cleavage by the Mre11/Rad50 complex." Genes Dev **13**(10): 1276-1288.
Paull, T. T., E. P. Rogakou, et al. (2000). "A critical role for histone H2AX in recruitment of repair factors to nuclear foci after DNA damage." Curr Biol **10**(15): 886-895.
Pavesi, G., P. Mereghetti, et al. (2004). "Weeder Web: discovery of transcription factor binding sites in a set of sequences from co-regulated genes." Nucleic Acids Res **32**(Web Server issue): W199-203.
Penkner, A., Z. Portik-Dobos, et al. (2007). "A conserved function for a Caenorhabditis elegans Com1/Sae2/CtIP protein homolog in meiotic recombination." EMBO J **26**(24): 5071-5082.
Peret, B., B. De Rybel, et al. (2009). "Arabidopsis lateral root development: an emerging story." Trends Plant Sci **14**(7): 399-408.
Pines, J. (1995). "Cyclins and cyclin-dependent kinases: a biochemical view." Biochem J **308 (Pt 3)**: 697-711.
Porceddu, A., H. Stals, et al. (2001). "A plant-specific cyclin-dependent kinase is involved in the control of G2/M progression in plants." J Biol Chem **276**(39): 36354-36360.
Prinz, S., A. Amon, et al. (1997). "Isolation of COM1, a new gene required to complete meiotic double-strand break-induced recombination in Saccharomyces cerevisiae." Genetics **146**(3): 781-795.
Ramirez-Parra, E., M. A. Lopez-Matas, et al. (2004). "Role of an atypical E2F transcription factor in the control of Arabidopsis cell growth and differentiation." Plant Cell **16**(9): 2350-2363.
Reik, W. (2007). "Stability and flexibility of epigenetic gene regulation in mammalian development." Nature **447**(7143): 425-432.
Ricaud, L., C. Proux, et al. (2007). "ATM-mediated transcriptional and developmental responses to gamma-rays in Arabidopsis." PLoS One **2**(5): e430.
Riechmann, J. L. (2002). "Transcriptional regulation: a genomic overview." Arabidopsis Book **1**: e0085.
Rosidi, B., M. Wang, et al. (2008). "Histone H1 functions as a stimulatory factor in backup pathways of NHEJ." Nucleic Acids Res **36**(5): 1610-1623.
Rossignol, P., R. Stevens, et al. (2002). "AtE2F-a and AtDP-a, members of the E2F family of transcription factors, induce Arabidopsis leaf cells to re-enter S phase." Mol Genet Genomics **266**(6): 995-1003.
Rozan, L. M. and W. S. El-Deiry (2007). "p53 downstream target genes and tumor suppression: a classical view in evolution." Cell Death Differ **14**(1): 3-9.
Samach, A., C. Melamed-Bessudo, et al. (2011). "Identification of plant RAD52 homologs and characterization of the Arabidopsis thaliana RAD52-like genes." Plant Cell **23**(12): 4266-4279.
San Filippo, J., P. Sung, et al. (2008). "Mechanism of eukaryotic homologous recombination." Annu Rev Biochem **77**: 229-257.
Sartori, A. A., C. Lukas, et al. (2007). "Human CtIP promotes DNA end resection." Nature **450**(7169): 509-514.
Schaeper, U., T. Subramanian, et al. (1998). "Interaction between a cellular protein that binds to the C-terminal region of adenovirus E1A (CtBP) and a novel cellular protein is disrupted by E1A through a conserved PLDLS motif." J Biol Chem **273**(15): 8549-8552.
Schnittger, A., U. Schobinger, et al. (2002). "Ectopic D-type cyclin expression induces not only DNA replication but also cell division in Arabidopsis trichomes." Proc Natl Acad Sci U S A **99**(9): 6410-6415.

Schwacha, A. and N. Kleckner (1997). "Interhomolog bias during meiotic recombination: meiotic functions promote a highly differentiated interhomolog-only pathway." Cell **90**(6): 1123-1135.
Seeliger, K., S. Dukowic-Schulze, et al. (2012). "BRCA2 is a mediator of RAD51- and DMC1-facilitated homologous recombination in Arabidopsis thaliana." New Phytol **193**(2): 364-375.
Shingu, Y., T. Mikawa, et al. (2010). "A DNA-binding surface of SPO11-1, an Arabidopsis SPO11 orthologue required for normal meiosis." FEBS J **277**(10): 2360-2374.
Smith, G. C. and S. P. Jackson (1999). "The DNA-dependent protein kinase." Genes Dev **13**(8): 916-934.
Sognier, M. A. and W. N. Hittelman (1986). "Mitomycin-induced chromatid breaks in HeLa cells: a consequence of incomplete DNA replication." Cancer Res **46**(8): 4032-4040.
Sorrell, D. A., A. Marchbank, et al. (2002). "A WEE1 homologue from Arabidopsis thaliana." Planta **215**(3): 518-522.
Sozzani, R., C. Maggio, et al. (2006). "Interplay between Arabidopsis activating factors E2Fb and E2Fa in cell cycle progression and development." Plant Physiol **140**(4): 1355-1366.
Stacey, N. J., T. Kuromori, et al. (2006). "Arabidopsis SPO11-2 functions with SPO11-1 in meiotic recombination." Plant J **48**(2): 206-216.
Steward, F. C. (1970). "From cultured cells to whole plants: the induction and control of their growth and morphogenesis." Proc R Soc Lond B Biol Sci **175**(38): 1-30.
Struhl, K. (1999). "Fundamentally different logic of gene regulation in eukaryotes and prokaryotes." Cell **98**(1): 1-4.
Sugimoto-Shirasu, K. and K. Roberts (2003). ""Big it up": endoreduplication and cell-size control in plants." Curr Opin Plant Biol **6**(6): 544-553.
Tansey, W. P. (2001). "Transcriptional activation: risky business." Genes Dev **15**(9): 1045-1050.
Thorslund, T. and S. C. West (2007). "BRCA2: a universal recombinase regulator." Oncogene **26**(56): 7720-7730.
Tuteja, N., P. Ahmad, et al. (2009). "Genotoxic stress in plants: shedding light on DNA damage, repair and DNA repair helicases." Mutat Res **681**(2-3): 134-149.
Uanschou, C. (2009). Characterization of the meiotic and DNA repair- associated gene AtCOM1 from Arabidopsis thaliana. university of vienna. vienna, university of vienna. **Dr. rer. nat.:** 143.
Uanschou, C., T. Siwiec, et al. (2007). "A novel plant gene essential for meiosis is related to the human CtIP and the yeast COM1/SAE2 gene." EMBO J **26**(24): 5061-5070.
Vandepoele, K., J. Raes, et al. (2002). "Genome-wide analysis of core cell cycle genes in Arabidopsis." Plant Cell **14**(4): 903-916.
Vandepoele, K., K. Vlieghe, et al. (2005). "Genome-wide identification of potential plant E2F target genes." Plant Physiol **139**(1): 316-328.
Vlieghe, K., V. Boudolf, et al. (2005). "The DP-E2F-like gene DEL1 controls the endocycle in Arabidopsis thaliana." Curr Biol **15**(1): 59-63.
Wang, H., L. C. Fowke, et al. (1997). "A plant cyclin-dependent kinase inhibitor gene." Nature **386**(6624): 451-452.
Wang, H., Z. C. Zeng, et al. (2001). "Genetic evidence for the involvement of DNA ligase IV in the DNA-PK-dependent pathway of non-homologous end joining in mammalian cells." Nucleic Acids Res **29**(8): 1653-1660.
Wise, R. R. and A. W. Naylor (1987). "Chilling-Enhanced Photooxidation : The Peroxidative Destruction of Lipids during Chilling Injury to Photosynthesis and Ultrastructure." Plant Physiol **83**(2): 272-277.
Wray, G. A., M. W. Hahn, et al. (2003). "The evolution of transcriptional regulation in eukaryotes." Mol Biol Evol **20**(9): 1377-1419.
Yoshiyama, K., P. A. Conklin, et al. (2009). "Suppressor of gamma response 1 (SOG1) encodes a putative transcription factor governing multiple responses to DNA damage." Proc Natl Acad Sci U S A **106**(31): 12843-12848.

Yu, X. and J. Chen (2004). "DNA damage-induced cell cycle checkpoint control requires CtIP, a phosphorylation-dependent binding partner of BRCA1 C-terminal domains." Mol Cell Biol **24**(21): 9478-9486.

Yu, X., S. Fu, et al. (2006). "BRCA1 ubiquitinates its phosphorylation-dependent binding partner CtIP." Genes Dev **20**(13): 1721-1726.

Zamble, D. B. and S. J. Lippard (1995). "Cisplatin and DNA repair in cancer chemotherapy." Trends Biochem Sci **20**(10): 435-439.

Zhang, M. Q. (2007). "Computational analyses of eukaryotic promoters." BMC Bioinformatics **8 Suppl 6**: S3.

Zhao, X., H. Harashima, et al. (2012). "A General G1/S-Phase Cell-Cycle Control Module in the Flowering Plant Arabidopsis thaliana." PLoS Genet **8**(8): e1002847.

Zickler, D. and N. Kleckner (1999). "Meiotic chromosomes: integrating structure and function." Annu Rev Genet **33**: 603-754.

9 Acknowledgements

First of all i want to thank my supervisor Dr. Peter Schlögelhofer for giving me the opportunity to work on this project and to gain knowledge about science and about life in general. I especially enjoyed our weekly discussions, which helped me a lot, to comprehend difficult scientific questions.

I also want to thank all my lab members Bernd, Clemens, David, Edith, Ines, Manuel, Maxl, Michael, Mona and Tesi for the nice working atmosphere. A special thank goes to Tesi for teaching me new methods and for helping me get the project started. I also want to specially mention Michael, who always took the time, to patiently share his knowledge and expertise and who gave me many fruitful advice. Finally i want to thank my family for always supporting me.

10 Curriculum vitae

Stefan Bailey
Franzensbrückenstraße 24/8
A-1020 Vienna

Date of birth:	25.01.1983
Place of birth:	Klagenfurt, Austria
Nationality:	Austria
Marital status:	Single

Education:

06/2011 – 08/2012	Diploma Thesis at the Department of Chromosome Biology of the University of Vienna (Laboratory "Meiotic recombination in the model plant Arabidopsis thaliana"; Dr. Peter Schlögelhofer)
Since 10/2005	University of Vienna: Molecular Biology Specializations: Genetics, Biochemistry and Plant Biology
10/2002 – 06/2005	University of Vienna: Pharmacy
06/2001	A – levels (Matura) BG/BRG St.Martin (High School), Villach, Austria