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Analysis of the *AtCOM1/GR1* Promoter

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2 Summary

The stability of the genome is fundamental for all living organisms. Therefore, DNA within the cells of higher organisms has to be constantly scrutinized by a multitude of proteins to sense and eliminate the damage that occurs randomly, due to environmental impacts or due to endogenous processes. One of these proteins, which are involved in DNA repair in somatic and meiotic cells, is COM1 that is highly conserved from yeast (COM1/SAE2) to humans (CtIP). The plant homologue, AtCOM1, confers resistance against the potent intrastrand crosslinker and N-alkylating agent Mitomycin C, is up-regulated after DNA damage by ionizing radiation and genotoxic substances and is furthermore essential for meiotic progression. *Atcom1* mutants are sterile due to DNA fragmentation and defects in chromosome pairing. *AtCOM1* expression depends on the checkpoint-kinase AtATM.

This study presents data of a deletion analysis of the *AtCOM1* promoter to identify *cis*-regulatory elements. These data suggest differential control of somatic and meiotic expression. Evidence is provided that a potential E2F binding site is crucial for *AtCOM1* promoter activity and that one or more of the six known AtE2F proteins could be responsible for the regulation of *AtCOM1*. Somatic activation of the *AtCOM1* promoter is only seen in promoter fragments containing the E2F site, whereas fragments without, show exclusive and strong activation in post-meiotic stages.

In the future, it is planned to develop artificial promoters, specifically up-regulated upon genotoxic stress or specific for meiotic expression. Ultimately these promoters will be used in conjunction with marker genes to produce screening tools for genotoxic hazards and cells undergoing meiosis, respectively.

3 Zusammenfassung

Die Stabilität des Genoms ist äußerst wichtig für sämtliche Lebewesen. Die Zellen höherer Organismen besitzen aus diesem Grund eine Vielzahl an Proteinen, die ständig damit beschäftigt sind, die DNS auf Schäden zu untersuchen, diese zu erkennen und zu reparieren.

Die DNS kann durch verschiedenste endogene Prozesse oder das Einwirken ionisierender Strahlung oder genotoxischer Substanzen Schaden erleiden. Eines der Proteine zur Reparatur von dadurch möglichen Doppelstrangbrüchen, den gefährlichsten aller DNS-Läsionen, ist COM1, ein von Hefe (COM1/SAE2) bis zum Menschen (CtIP) konserviertes Protein.

AtCOM1 ist an somatischer und meiotischer DNA-Reparatur beteiligt, wird nach DNA-Schädigung verstärkt exprimiert und ist unerlässlich für die Resistenz gegen Mitomycin C, ein genotoxisches Agens, das zwischen zwei DNA-Strängen interkaliert. Des Weiteren wird *AtCOM1* von der Checkpoint-Kinase AtATM reguliert und ist essentiell für den normalen Ablauf der Meiose. Durch starke DNA-Fragmentierung und Defekte in der Paarung der homologen Chromosomen sind *Atcom1* Mutanten steril.

Diese Studie präsentiert Resultate einer Deletionsanalyse des Promotors von *AtCOM1* zur Analyse *cis*-regulatorischer Elemente. Die Daten weisen auf eine differentielle Regulation in somatischem und meiotischem Kontext hin. Es wird außerdem gezeigt, dass eine potentielle E2F Transkriptionsfaktor-Bindestelle entscheidend für die Promotor-Aktivität ist und eines der sechs bekannten E2F Proteine für die Regulation von *AtCOM1* verantwortlich sein könnte.

Somatische Aktivierung des Promotors wurde nur bei jenen Promotor-Fragmenten, die die E2F site beinhalten, beobachtet, während Fragmente ohne diese Transkriptionsfaktor-Bindestelle ausschließliche und starke post-meiotische Aktivität aufwiesen.

In Zukunft sollen mit Hilfe der Erkenntnisse aus dieser Studie artifizielle Promotoren erzeugt werden, die spezifisch durch genotoxischen Stress oder meiotische Ereignisse aktiviert werden. So könnten sie in Verbindung mit Reportergenen dazu genutzt werden, Umweltgefahren, die dem Genom schaden können, sichtbar zu machen aber auch, um meiotische Zellen zu markieren.

4 Introduction

4.1 Promoters and Transcriptional Regulation

The DNA of every cell carries all the information an organism needs to evolve, develop and survive. In order to make this information accessible, each gene has to be "read" – transcribed – into messenger RNA (mRNA) that is then further translated into proteins that put together the organism and maintain it.

There are also stretches on the DNA molecule that do not code for proteins but act as regulators and determine the expression of genes at a certain time during the life cycle of a cell, in response to environmental stimuli. These *cis*-regulatory and promoter regions turn on or off genes by combining the input of various protein factors that interact with them (Riechmann, 2002). In animals, these regulatory sequences – the promoters - can extend over tens of kilobases (kb), whereas in plants, where gene density is higher, they are usually shorter, often less than one or two kb. Isolated *Arabidopsis* 5' promoter sequences often show the same regulatory characteristics as the native promoters when analyzed in transgenic plants by reporter gene fusions. But it is also possible for regulatory elements to be localized downstream of the transcription start site in introns, in the 5' untranslated region (UTR) or in 3' sequences (Sieburth, *et al.*, 1997).

Development and survival is based on this capability of cells for differential gene expression, allowing for the regulation of metabolic pathways and the response to pathogens, environmental cues and stresses (Scott, 2000). 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). Here, the DNA is tightly packed into chromatin, which blocks constant recognition of promoters by the transcription machinery. This machinery is composed of four different functional groups: (1) the basic transcription apparatus and intrinsic associated factors; (2) large multi-subunit coactivators and other cofactors; (3) sequence-specific DNA-binding transcription factors that act together in a combinatorial mechanism to selectively regulate the expression of tens of thousands of genes by a reduced number of factors using transcription factor binding sites of only 5-10 base pairs – those of prokaryotes are often longer than 12 base pairs; and (4) chromatin related proteins. In eukaryotes, the first functional group of the transcription machinery consists of RNA polymerases (Pol), that are responsible for the synthesis of

ribosomal RNA (Pol I), messenger RNA (Pol II) and transfer RNA, 5S rRNA, and other small RNA molecules (Pol III) (Riechmann, 2002). Pol II, necessary for the synthesis of mRNA, is a multi-subunit complex that requires accessory factors to recognize promoter sequences and to accurately initiate transcription *(Cramer, et al.,* 2001). These general transcription factors (GTFs) carry out a variety of functions from promoter recognition to correct positioning of the polymerase or unwinding the DNA (Veenstra, *et al.*, 2001). These GTFs and Pol II can interact with a very heterogeneous class of regulatory proteins, the large multi-subunit coactivators, and cofactors that bind sequence specific transcription factors. This kind of proteins thereby serves as a modular adapter to regulate transcription initiation, dependent on the presence of certain activators or repressors. These could in turn originate from the third functional group of proteins of the transcription machinery: the sequence-specific DNA-binding transcription factors. They can bind DNA in a sequence-specific manner and positively or negatively influence the expression of their specific target genes. The specificity of gene regulation relies on these *trans*-acting factors and they are themselves very often transcribed in a stimulus, cell type or tissue dependent modality. Transcription factors are modular proteins with distinct domains, like DNA-binding or transactivation domains, according to whose they are grouped into several families, like the *MYB, MADS, WRKY, E2F-DP, bZIP* and *DOF* families, to name a few from *A. thaliana* (Riechmann, 2002; Qu, *et al.,* 2006). As stated above, transcription factors can directly interact with different components of the general transcription machinery, with coactivators and chromatin remodeling complexes. The latter belong to the functional group of chromatin-related proteins. This group includes proteins capable of covalently modifying histones (histone acetylases and deacetylases) and remodeling complexes that hydrolyze ATP for the reorganization of chromatin structure. Acetylation of histones loosens chromatin and is therefore characteristic for transcribed chromosome regions, whereas deacetylation is associated with repression of transcription (Riechmann, 2002). Chromatin structure alternates between these two conformations according to arriving signals that might emanate from cell cycle regulators.

4.2 The Cell Cycle

Stress, DNA damage of any kind and developmental signals strongly influence gene expression via checkpoint proteins that are variably active throughout the cell cycle. The cell cycle consists of four different phases: In Gap phase 1 (G1), cells grow and prepare for the synthesis of DNA in S phase (S). In Gap phase 2

(G2), cells prepare for mitosis that takes places in M phase (M) (Figure 1). Transition from one phase to the other should only occur if all necessary processes of the last phase have been completed successfully. This is ensured by checkpoint proteins that – once activated by a DNA damage signal, for example – regulate the proteins that are described in the following in order to slow down or even stop cell cycle progression.

The cell cycle in all eukaryotes is governed by cyclins and cyclin-dependent kinases (CDKs). 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, *et al.*, 2001; Hemerly, *et al.*, 1995).

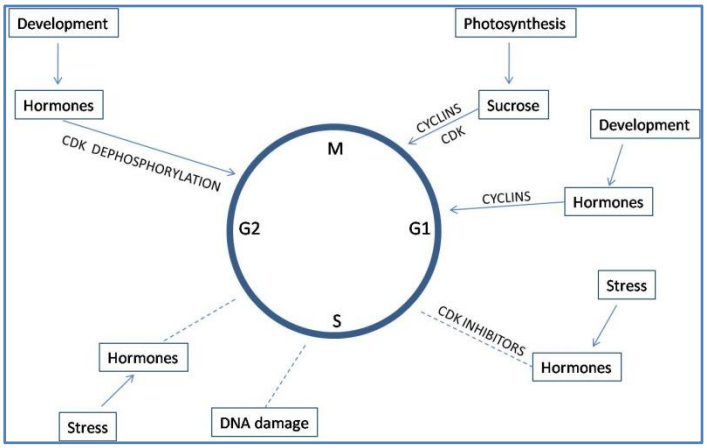


Figure 1- Schematic view of the plant cell cycle and the various developmental and environmental signals acting on it. Cyclin-dependent kinases (CDKs) and cyclins regulate progression through the cell cycle with the help of different interacting proteins (adapted from Inzé, 2005; Vandepoele, *et al*., 2002).

The second group of key factors controlling the cycle is the group of cyclins. Plants contain many more than previously described in other organisms - *Arabidopsis thaliana* contains at least 32 cyclins with a putative role in cell cycle progression. The plant cyclin nomenclature is based on the functional similarity with their mammalian counterparts. In general, D cyclins are thought to regulate the G1/S transition and appear to act as integrators of various signals; A cyclins are of importance for the S-to-M phase control, whereas B cyclins generally play a role in the G2/M transition and intra-M phase control and the H cyclin is part of the CDK-activating kinase. However, the precise role of cyclins in the cell cycle, their stability throughout the cell cycle and their subcellular location are poorly understood. Similarly to that in yeasts and animals, the activity of plant CDK/cyclin complexes is regulated by phosphorylation, dephosphorylation and the interaction with regulatory proteins. There are also CDK inhibitors found in plants that specifically regulate cell cycle arrests according to the impact of environmental signals like various kinds of physiological stress (Inzé, 2005).

4.3 The Protein Kinases ATM and ATR – Key Players in the Response to DNA Damage

ATM (ataxia telangiectasia mutated) and ATR (ataxia telangiectasia and Rad3 related) are checkpoint protein kinases that are known to be involved in a variety of responses to DNA damage in many organisms (yeast homologues are called Tel1 and Mec1, resepectively). Plants with null alleles of *AtATM* are viable but show reduced fertility, whereas *Atatr* mutants are fertile. In humans, ATR is generally thought to be activated by stalled replication forks or repair intermediates like single-stranded DNA (ssDNA) while ATM is activated by double-strand breaks (DSBs) (Cuadrado, *et al.,* 2006). They play partially redundant roles in the induction of cell-cycle responses to DNA damage but ATR is suggested to be specially required for the long-term maintenance of the response by maintaining the phosphorylated state of the downstream targets (Culligan, *et al.,* 2006).

The term checkpoint kinase means that the proteins coordinate the timely progression throughout the cell cycle. This is fundamental to ensure that for example DNA replication and chromosome segregation are completed accurately before cell division takes place. The integrity of these processes is verified both at the G1/S and G2/M transition boundaries as well as during S phase (these are the so-called checkpoints). In response to DNA DSBs, cell cycle checkpoints are activated that delay cell cycle progression until the breaks are repaired (Elledge, 1996; Rouse, *et al.*, 2002).

ATM is an essential checkpoint protein that is exclusively activated by DSBs and not by single-strand breaks (SSBs) (Ismail, *et al.,* 2005). It mediates cell cycle checkpoints to give cells enough time to repair DSBs by homologous recombination (HR) or non-homologous end-joining (NHEJ), depending on the structure of the DSB and on the cell cycle at the time of damage (Kurz, *et al.,* 2004). From yeast and humans there is striking evidence that ATM is recruited to the sites of DSBs via NBS1 by the MRE11 RAD50 NBS1 (MRN) protein complex, the direct sensor of DSB ends (Dupré, *et al.*, 2006). After its recruitment, ATM phosphorylates a large number of downstream targets containing proteins involved in DNA-repair, checkpoint control and apoptosis, making it the major regulator of G1/S, intra-S and G2/M transitions (Shiloh, 2006) (Figure 2).

AtATM was first characterized in 2000 and later described as a gene essential for meiosis and somatic responses to DNA damage in plants. It is transcribed

ubiquitously and its expression is not induced by ionizing radiation (Garcia, *et al*., 2000; Garcia, *et al.*, 2003).

ATM is related to another kinase of the phosphatidyl inositol 3-kinase-like family: ATR. This protein is essential for early mammalian development and plays a pivotal role in the checkpoint response to replicative stress and DNA damage caused by alkylating agents or UV-induced DNA lesions (Shechter, *et al.*, 2004). ATR is known to sense ssDNA and, like ATM, to activate downstream effectors of cell cycle progression and DNA repair by phosphorylation. It is primarily activated by agents that block the progression of replication forks and phosphorylates the serine/threonine-protein kinase CHK1 in order to initiate a cell cycle arrest in G2. This regulation of the G2 checkpoint was observed in mammals, fungi and plants (Culligan, *et al.*, 2004; Melo, *et al.*, 2002).

In plants, *Atatr* mutants are viable, fertile and phenotypically wild-type under non-stress conditions.

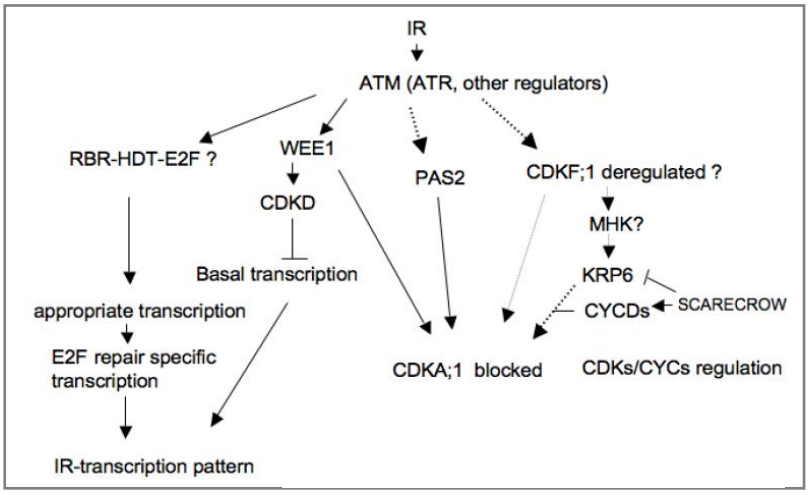


Figure 2 – Putative hallmark regulation steps in transcription response after ionizing radiation (IR) in plants. After IR, both the CDKF;1 pathway and WEE1 are regulated leading to inactive CDKA;1. WEE1, in addition to halting the basal transcription while disrupting and thereby activating in particular RBR/E2F complexes might specifically drive appropriate transcription as in human cells. In addition, CDKF;1 is possibly deregulated, resulting in CDKA;1 inactivation. This might be promoted by stabilizing KRP6 (a CDK inhibitor) that might interact with D cyclins (CYCDs). This results in the cell cycle being severely delayed after activating ATM-mediated DNA damage checkpoints by numerous inhibitors and regulators of proliferation. Cell growth and repair is enabled by the upregulation of KRP6 and CYCB1;1, which regulate specific activities of CDKs. Transcriptional, translational, and/or posttranslational regulation of E2Fs and other transcription factors in coordination with similar regulation of chromatin modifiers results in transcription patterns that are specific for IR. In irradiated *atm* mutants, transcription control might be lost due to misregulation of WEE1 and RBR. After ectopic division due to checkpoint abrogation, *atm* stem cells accumulated high amounts of unrepaired DNA and/or small amounts of DNA lesions that require ATM-related DNA repair. Dashed arrows indicate unknown regulators or pathways (modified from Ricaud *et al*., 2007).

4.4 E2F transcription factors

E2F transcription factors play an important role in cell cycle control and were originally identified as cellular factors stimulating the expression of the adenovirus promoter E2. Subsequently it was shown that they regulate the activity of a variety of genes, including cell cycle and regulatory genes which are required for

the expression of genes implicated in DNA replication and repair and genes encoding structural proteins of chromatin, like histones.

E2F transcription factors are key components of cell cycle control in higher eukaryotes that bind to highly conserved consensus sequences (Figure 3).

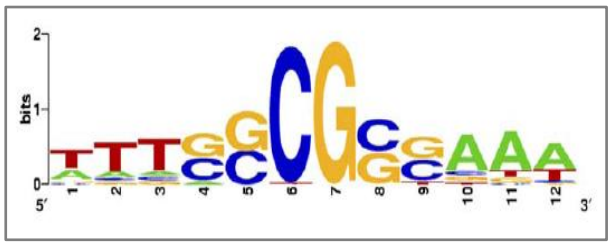


Figure 3 - Sequence logo of the overrepresented motif found in numerous putative plant E2F target genes. The overall height of each stack indicates the sequence conservation at that position, whereas the height of symbols within the stack reflects the relative frequency of the corresponding nucleic acid at that position. The consensus sequence of E2F transcription factor binding sites is highly conserved (Vandepoele, *et al*., 2005).

In humans, some mutations in E2F coding regions lead to uncontrolled cell proliferation and tumor formation. Multiple E2F binding sites are found in the promoter regions of cell cycle genes active in S-phase. E2F regulation involves interaction with pocket proteins - mainly the Retinoblastoma protein (Rb) (RBR in plants). Rb represses transcription by binding E2F transcription factors and thereby shielding the E2F transactivation domain. Furthermore, Rb recruits histone deacetylase that deacetylates nucleosomes and leads to tighter chromatin condensation. CDK-dependent phosphorylation of Rb in mid to late G1 disrupts Rb-E2F binding, thereby relieving repression of E2F regulated genes. There is another factor that is essential for E2F function: animal E2F proteins heterodimerize with members of a protein family called dimerization partners (DP). This interaction is necessary for efficient DNA binding and two DP genes have also been identified in plants.

In contrast to humans, where seven E2F genes are known (E2F1-7), there are only six E2F genes (E2Fa-f) found in *A. thaliana*. E2Fa, b and c share conserved domains with animal E2Fs. They have a DNA-binding domain, a Leucine zipper dimerization domain, an Rb binding domain (pocket), a “marked box” motif and a transactivation domain; and are generally regarded as transcriptional activators. E2Fd, e and f contain a duplicated DNA binding domain and none of the other conserved E2F domains. That is why they appear to function as repressors of E2F-regulated genes (Eckardt, 2002; Kosugi, *et al.*, 2002; Vandepoele, *et al.*, 2002).

As mentioned above (Figure 2), E2F transcription factors are part of the ATM-dependent signalling cascade that is activated after DNA damage, thereby initiating damage response and DNA repair.

4.5 Somatic DNA double-strand break repair

Every organism is defined by its genome: a huge number of nucleotide pairs, arranged as polymers in the DNA double helix and organized into chromosomes that are contained in the nucleus of every cell and identically passed on to generations of daughter cells during numerous cell divisions. Genomic stability is of great importance to ensure survival at the level of the individual organism by preventing DNA sequence changes that might alter genetic information, by protecting the structural integrity of chromosomes and their orderly transmission to progeny cells (Hays, 2002). One of the major threats to genomic stability are double-strand breaks (DSBs) that are generally regarded as the most toxic of all DNA lesions – a single unrepaired DSB can lead to cellular death (Bennett, *et al.*, 1993). They may occur all over the genome by a number of different means, including exposure to ionizing radiation (IR) or genotoxic agents and after the collapse of replication forks when the replication machinery encounters single-stranded breaks in the template DNA (Figure 4). If regular and precise DNA repair mechanisms are impaired, these lesions can lead to chromosome arm loss, small insertions and deletions, as well as chromosomal translocations (Valerie, *et al*., 2003).

The functions of the genomic stability "caretaking" machinery include activities associated with cell-cycle checkpoint and programmed-cell-death pathways and genome-maintenance activities, including DNA repair in general and DSB repair in special. In eukaryotes there are two major mechanisms of DSB repair: non-homologous end-joining (NHEJ) and homologous recombination (HR), both of which are conserved from yeast to humans (Hays, 2002).

Arabidopsis encodes orthologues of most proteins used by other eukaryotes to maintain genomic integrity (Arabidopsis Genome Initiative, 2000).

These orthologues include proteins that directly repair DNA lesions without cleaving base-sugar or phosphodiester bonds, glycosylases that initiate base excision repair, apurinic/apyrimidinic (AP) endonucleases that recognize abasic sites generated by glycosylases or spontaneous depurination/depyrimidination, components of mismatch repair pathways, DNA replication/repair polymerases, DNA ligases and proteins of the DSB repair machinery, including the NHEJ and HR pathways.

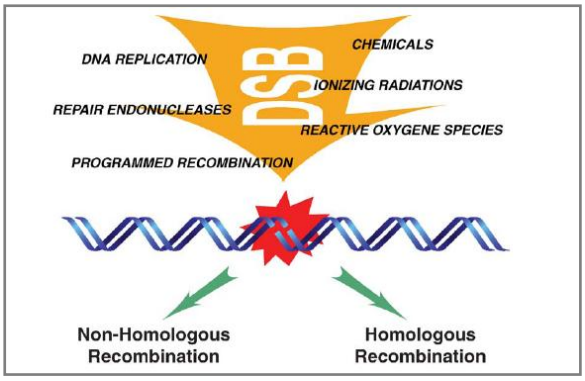


Figure 4 - Multiple origins of DNA double-strand breaks and principle repair pathways. DSBs are of both endogenous and exogenous origin. DNA replication, repair endonucleases, cellular metabolism by-products (such as reactive oxygen species) and programmed recombination events constitute the main endogenous origins of DSBs, while chemicals and radiations constitute the main exogenous. Repair of a DSB may involve, or not, the use of homology between the recombining molecules, defining the two main repair processes: homologous recombination and non-homologous end-joining (taken from Bleuyard *et al*., 2006).

Non-Homologous End-Joining

NHEJ was first described in mammals – where it is the predominant mechanism for DSB repair - and usually involves the rejoining of blunt ends or ends with a few overlapping bases without regard for absolute DNA sequence accuracy (Hays, 2002). In general, this repair pathway 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. NHEJ begins with the recognition and the juxtaposition of the broken ends. In mammals, this step is promoted by the DNA-dependent protein kinase (DNA-PK), a complex composed of the KU heterodimer and the DNA-PK catalytic subunit (DNA-PKcs). The Artemis protein and the Xrcc4/DNA ligase IV heterodimer are then recruited, with Artemis involved in the maturation of the DSB ends and the Xrcc4/DNA ligase IV complex catalyzing the resealing of the ends (Lees-Miller, *et al.*, 2003; Meek, *et al*., 2004; Bleuyard, *et al.,* 2006).

The KU heterodimer is composed of Ku70 and Ku80 and is involved in recognition, protection and juxtaposition of the ends of a DSB (Walker, *et al.,* 2001). DNA-PKcs is recruited to the DSB site via its interactions with the Ku/DNA complex. It is a kinase and phosphorylates five of the six proteins identified in the mechanisms of NHEJ: Ku70, Ku80, Artemis, Xrcc4 and DNA-PKcs itself (Gottlieb, *et al.*, 1993).

Artemis is the most recently identified NHEJ component. It possesses both exo- and endonuclease activities and performs ATM-regulated maturation of the DSB ends as it cleaves DNA hairpins and other DNA structures (Ma, *et al.*, 2005).

The final step, consisting of the ligation of broken ends, is carried out by the Xrcc4/DNA ligase IV heterodimer, which is recruited by DNA-PK. The MRN complex, composed of the proteins Mre11, Rad50 and Nbs1, stimulates this ligase activity in vitro and is also implicated in the juxtaposition of the ends of the break (Grawunder, *et al.*, 1997; Huang, *et al.*, 2002).

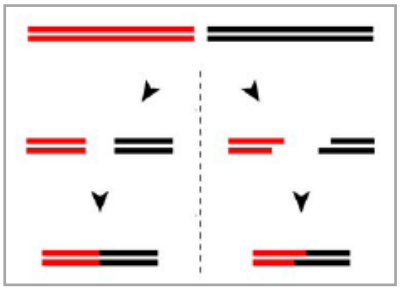


Figure 5 – Model for DSB repair via non homologous end joining: the ends of the break are juxtaposed, processed and then ligated. Overlaps of a few bases might be created. (Bleuyard, *et al.,* 2006)

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, *et al.*, 2006).

Homologous recombination

Homologous recombination is a sequence conserving pathway in the repair of DNA double-strand breaks. Its initiation involves the localization of the Mre11-Rad50-Xrs2 (MRX) complex to the DSB site in *S. cerevisiae*. This complex bridges the two ends and recruits a 5' to 3' nuclease that resects the exposed 5' ends of each DSB end, forming a stretch of single-stranded DNA (ssDNA). In addition, the MRX complex recruits Tel1 (the yeast homologue of ATM) that phosphorylates a large number of downstream targets like Rad9, Rad17, Rad53, Rpa1, Xrs1 and Exo1 that are involved in DNA-repair, checkpoint control and apoptosis (Shiloh, 2006). Among these target proteins there also is Com1/Sae2 that in yeast participates in DSB repair by ensuring both resection and association of the broken ends – together with the MRX complex (Clerici, *et al.*, 2005).

In *S.cerevisiae*, the remaining single-stranded DNA ends are coated by replication protein A (RPA), thereby stimulating the recruitment of the Rad52 epistasis group proteins (Rad51, Rad52, Rad54, Rdh54/Tid1, Rad55 and Rad57) that promote the invasion of the homologous DNA molecule (Symington, 2004) and activation of Mec1, the yeast homologue of ATR. The strand invasion protein Rad51 mediates this step. Following strand invasion, the 3' end serves as a starting site for new DNA synthesis, using the intact strand of the sister chromatid as a template. This is succeeded by branch migration and resolution of the Holliday junction that is formed during the process (Bleuyard, *et al.*, 2006).

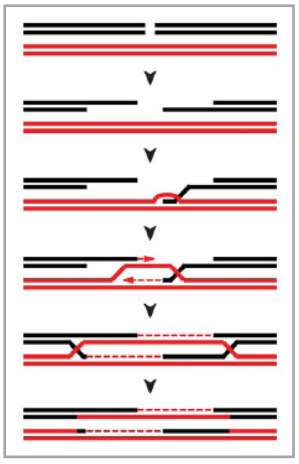


Figure 6 – Model for DSB repair via homologous recombination. The ends of the break are processed into ssDNA. One of these single-stranded ends invades the homologous sequence, creating two Holliday junctions that are later resolved (Bleuyard, *et al*., 2006).

In plants, the major protein complex acting in homologous recombination is – analogous to mammals - the MRN complex, consisting of MRE11, RAD50 and NBS1, the homologue of *S.cerevisiae* Xrs2. Even the RAD52 epistasis group seems well preserved among eukaryotes and orthologues have been identified in vertebrates and plants, except Rad59 that seems absent and Rad55/Rad57, which are replaced by five Rad51 paralogues (RAD51B, RAD51C, RAD51D, XRCC2, and XRCC3) (Symington, 2002). There is no obvious Rad52 counterpart in *Arabidopsis* but other proteins that are related to the mammalian HR machinery (Bleuyard, *et al*., 2006), depicting the close relation of the repair processes in plants and mammals. For example *Arabidopsis*, but not yeast, encodes proteins with striking similarity to the human repair protein Brca2 (Hays, 2002).

4.6 Meiotic DNA double-strand break repair

Although DSBs are potentially lethal DNA lesions, there is at least one physiological process in plants where they are absolutely desired and intentionally introduced, namely meiosis. Meiosis is a special type of cell division in sexually reproducing organisms. In contrast to mitosis, where DNA replication is followed by one round of nuclear division, which separates sister chromatids, in meiosis, a single round of DNA replication is followed by two rounds of nuclear division (meiosis I and meiosis II) leading to the generation of four haploid gametes. As during meiosis I ploidy is reduced by half by segregation of homologous chromosomes, it is often called reductional division. Meiosis II proceeds similar to mitosis in that sister chromatids are segregated – it is also called equational division. Both meiosis I and II are further divided into four phases: prophase, metaphase, anaphase, and telophase. Prophase of meiosis I (prophase I) is the longest and most complex stage of the division and is further divided into five sub-stages according to cytological features of the chromosomes: leptotene, zygotene, pachytene, diplotene, and diakinesis (Ma, 2006).

Homologous chromosomes condense during leptotene and pair during zygotene. In early leptotene, DSBs are introduced and repaired in zygotene/pachytene stages. At pachytene stage, repair and synapsis are completed and homologous chromosomes are closely paired. The repair processes establish physical connections between the homologues (crossovers; chiasmata) around which the synaptonemal complex (SC), the proteinaceous structure needed for synapsis starts to form (Zickler, *et al.*, 1999). Crossovers are the sites where homologous recombination happens that contributes to genetic diversity by combining maternal and paternal parts of the genetic material in new combinations.

The SC is disassembled in diplotene. Nevertheless, the homologous chromosomes are held together as bivalents until metaphase I by chiasmata, the physical connections mentioned above (Hamant, *et al.*, 2006). This is critical for reproductive success because it maintains homologue association until the metaphase I/anaphase I transition and allows correct disjunction of homologues at anaphase I. In telophase I, chromosomes have reached the opposite poles of the cell and decondense. Meiosis II then takes place comparable to mitosis as sister chromatids are separated. At the end of meiosis II, four clusters of chromosomes are formed and become four new nuclei. Cell membranes and wall materials are transported to the space in between and cytokinesis occurs to generate four haploid microspores or megaspores that further develop into male and female gametes, respectively (Ma, 2006).

At the population and species levels, meiotic recombination that occurs during prophase I, facilitates the redistribution of genetic material between generations, and increases genetic diversity among individuals in a population. This has great evolutionary implications, making meiosis and particularly meiotic recombination an important genetic mechanism that may have contributed to the success of eukaryotes (Ma, 2006).

There are two different classes of meiotic recombination events, both of them arising from a DSB. If chromosome arms on opposite sides of the recombination initiation event swap partners, a reciprocal crossover (CO) is created. If the original configuration of chromosome arms is retained, the event is designated a non-crossover (NCO) (see Figure 7), which is called gene conversion, but only CO form the chiasmata needed for chromosome segregation (McMahill, *et al.,* 2007).

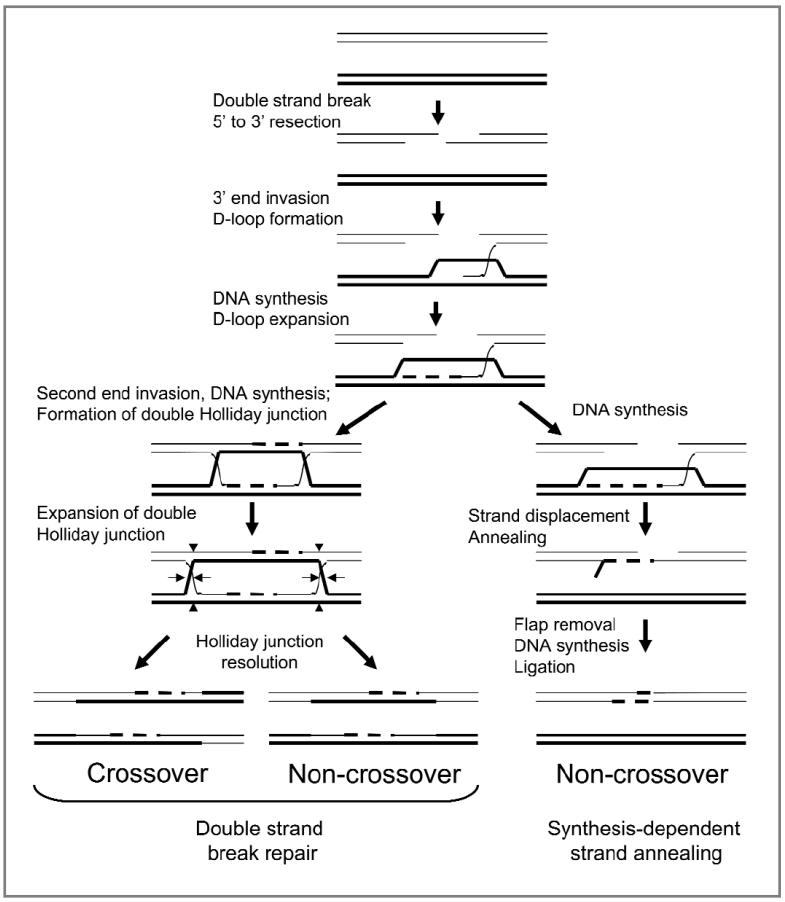


Figure 7 – Models for homologous recombination in plants: The double strand break repair model (DSBR) (left) and the synthesis-dependent strand annealing model (SDSA) (right). Both start with a DSB, 5' to 3' resection to 3' single strands, the invasion of one end into the intact recombination partner to form a D-loop, and some DNA synthesis. In the traditional DSBR model, the second end invades, leading to the formation of the double Holliday junction (dHJ). Resolution of the dHJ in the opposite sense leads to a crossover event whereas cleavage in the same sense results in a non-crossover event. Recent studies in yeast indicate that double Holliday junctions usually resolve into crossovers. In addition, a number of results support the SDSA model, where the invading end branches off following D-loop formation. This alternative model can generate noncrossovers, but not crossovers (taken from Ma, 2006; McMahill, *et al.*, 2007).

Meiotic recombination in yeast starts with the introduction of DSBs by the topoisomerase type II protein Spo11 that remains covalently bound to the DSB site until it is removed with the help of the Mre11 Rad50 Xrs2 (MRX) complex together with Com1/Sae2 allowing further processing of the break. *AtSPO11-1* (there are three Spo11 homologues in *A.thaliana*) is the functional plant homologue of yeast Spo11 (Prinz, *et al.*, 1997; Hamant, *et al*., 2006; Grelon, *et al.,* 2001).

The now free 5' ends are resected by an unknown nuclease activity, potentially supported or exhibited by the yeast MRX complex (Mre11-Rad50-Nbs1 complex in mammals and plants) leaving 3' DNA overhangs that are believed to be asymmetrically loaded with the RecA like proteins Rad51 and Dmc1 that possess single-stranded DNA-binding ability and DNA-dependent ATPase activity. These two proteins then engage in the search for a homologous template with a strong preference towards the homologous chromosome rather than the sister chromatid (Schwacha, *et al.*, 1997; Neale, *et al*., 2006; Bishop, *et al.,* 1992; Hamant, *et al.,* 2006). Homologues of both have been identified in *Arabidopsis*,

maize, lily and rice. Interestingly, RAD51 functions in mitosis as well as in meiosis, whereas DMC1 is meiosis-specific. As A*trad51* and *Atdmc1* mutant plants show different phenotypes regarding chromosome fragmentation (only observed in *Atrad51*) it is suggested that the two proteins act together but are functionally distinct (Li, *et al.*, 2004; Couteau, *et al.*, 1999; Hamant, *et al.*, 2006).

After loading of RAD51 and DMC1, the strand invasion process can follow one of two pathways: the double-strand break repair (DSBR) pathway or the synthesis-dependent strand-annealing (SDSA) pathway (McMahill, *et al.*, 2007). During DSBR, DNA-Synthesis from the invading strand and ligation yield the double Holliday junction (dHJ). Its resolution by cutting at alternate strands results in products with either crossovers or noncrossovers (Figure 7). A paralogue of Rad51, Xrcc3, participates in this dHJ resolution in vertebrate cells and mutation of *AtXRCC3* leads to gametophytic lethality in *Arabidopsis* (Brenneman, *et al.*, 2002; Bleuyard, *et al.,* 2004).

In the SDSA pathway, the D-loop formed after strand invasion is disrupted by the displacement of the elongated invading strand. The strand is annealed to its prior partner and the result is a noncrossover event (McMahill, *et al*., 2007).

4.7 COM1/SAE2 – A Repair Protein: Conserved from Yeast to Humans

COM1/SAE2 was first described as a new gene required to complete meiotic double strand break induced recombination in *S. cerevisiae* (Prinz, *et al.*, 1997; Keeney, *et al.,* 1995). In yeast mitotic cells, Com1/Sae2 is essential for the repair of hairpin-capped DSBs and for holding DNA ends in close proximity after DSB formation thereby preventing chromosome rearrangements (Clerici, *et al.*, 2005; Lengsfeld, *et al.*, 2007; Lobachev, *et al.,* 2002). It exhibits endonuclease activity, is phosphorylated by the checkpoint kinases Mec1 and Tel1 (yeast homologues of ATR and ATM) and interferes with DNA replication checkpoints (Clerici, *et al.,* 2006; Lengsfeld, *et al.*, 2007). Com1/Sae2 is one of the first proteins found at somatic DSB sites, right after Mre11 and Tel1 (Lisby, *et al.*, 2004).

Com1/Sae2 was long thought to be fungal specific but homologues in all eukaryotic kingdoms could be identified (Uanschou, *et al.,* 2007; Penkner, *et al.*, 2007; Limbo, *et al.,* 2007; Sartori, *et al.*, 2007). Quite recently, *COM1* was characterized in *C.elegans*, where its functions in DNA repair and meiotic recombination (Penkner, *et al.*, 2007) are similar to those in other organisms.

Recent studies have revealed similar functions for Ctp1, the *S.pombe* homologue of Com1. It could be shown that it collaborates with the MRN complex in DSB repair, is essential for homologous recombination and also required for meiosis. If it is mutated, spore viability decreases dramatically due to improper segregation of nuclear DNA (Limbo, *et al.*, 2007).

The mammalian counterpart, called CtIP in humans, was initially identified as an interactor for CtBP11 and the tumor suppressor proteins RB1 (Fusco, *et al.,* 1998) and BRCA1 (Yu, *et al.*, 1998) that is recruited to DNA damage and complexes with BRCA1 to control the G2/M DNA-damage checkpoint (Yu, *et al.,* 2006; Yu, *et al.*, 2004). CtIP also promotes ATR activation and homologous recombination by cooperating with MRN upon DNA damage (Greenberg, *et al.*, 2006; Sartori, *et al.*, 2007) to mediate DSB resection. It furthermore facilitates single-stranded DNA (ssDNA) formation and is phosphorylated by the checkpoint kinase ATM. Its mutation leads to the death of knockout mice in an early embryonic stage (Chen, *et al.*, 2005) and hypersensitivity of cells towards DSB-inducing agents (Sartori, *et al.*, 2007). Taken together, these results show that CtIP/COM1/SAE2 is required for meiotic DSB repair and resistance to DSB-inducing agents. These findings place the mammalian gene right next to *AtCOM1/GR1*, the plant homologue of Com1/Sae2.

AtCOM1 was first described in 2000 as a gene being highly upregulated after ionizing irradiation and first called *AtGR1* (*A.thaliana gamma response gene 1*) (Deveaux, *et al.*, 2000). Back then, neither its relation to COM1/SAE2, nor its role in meiosis was recognized. It took another seven years until its role in somatic and meiotic DNA repair in plants and its connection to homologues in other organisms was recognized and published.

AtCOM1 confers resistance against the potent intrastrand-crosslinking and alkylating agent Mitomycin C and is essential for male and female meiosis. If it is mutated, plants are sterile due to severe DNA fragmentation but the vegetative development under non-stress conditions is not affected. In the *Atspo11-1-1 Atcom1-1* double mutant there is almost no more DNA fragmentation indicating that the *Atcom1-1* phenotype is generated by the inability to repair meiotic DSBs. Furthermore, AtCOM1 is essential for the regular turnover of AtSPO11-1 and subsequent processing of DSBs (Uanschou, *et al.*, 2007).

4.8 Objectives

This thesis is about the analysis of the promoter of *AtCOM1*. In general, a promoter analysis may comprise different questions. It is fundamental to assess when and in which tissue and developmental context the promoter is active and what kinds of exogenous influences are translated into and combined with endogenous controlling factors which affect its activity. If the results of these general questions are about to be used for the creation of artificial promoters a subset of additional experiments has to be arranged.

There were three main objectives for this specific project. (1) The dynamics of promoter induction where analyzed. It was already known before that *AtCOM1* expression is strongly induced after treatment with ionizing radiation (Deveaux, *et al*., 2000) and needed for resistance against the intrastrand crosslinking agent Mitomycin C (Uanschou, *et al.*, 2007) but there was no information on the reaction to other genotoxic agents on the mRNA level. To get a better understanding of the effects of different DNA damaging substances on the promoter activity of *AtCOM1*, their potential to activate it was analyzed. In order to investigate the dynamics of *AtCOM1* promoter induction, quantitative real time PCR (qPCR) was the method of choice. This technique is, once established, a convenient method for the analysis of mRNA levels, and therefore for the rate of transcription of the gene of interest in different parts of the plant body or variably stressed tissues.

(2) The differential requirements for somatic and meiotic promoter induction were analyzed experimentally in parallel to the search for the *cis*- and *trans*-regulatory factors controlling the promoter. A promoter deletion analysis was conducted: various fragments of the promoter region were generated and cloned in front of a reporter gene to visualize their ability to drive this gene. Reporter gene activity was investigated in transgenic cell suspension culture cells and whole plants carrying the promoter fragment-reporter construct.

(3) The last aim was to deploy the findings of the analysis of required *cis*-regulatory factors to eventually develop artificial promoters specifically upregulated upon genotoxic stress or specific for meiotic expression. The availability of the latter would be enormously beneficial for scientists working on meiosis that are in the need of expressing transgenes specifically during meiosis or have to isolate high amounts of meiocytes. This could be achieved by combining a meiosis-specific artificial promoter fragment with a fluorescent reporter gene. If there is enough fluorescent signal in the cells, they could be

sorted out by a flow cytometry method called fluorescence activated cell sorting (FACS). Thereby it would be possible to create cell suspensions that only contain meiocytes, facilitating for example the isolation of higher amounts of meiosis-specific proteins.

Artificial promoters that are strongly upregulated after exposure to genotoxic stress could be used in plants in conjunction with phenotypic marker genes to establish cheap and easy-to-use screening tools for environmental perils. The idea is to grow transgenic plants carrying the artificial and stress-inducible promoter fragment in conjunction with a phenotypic reporter gene next to, for instance, nuclear power plants. In case of the emersion of a hazardous amount of radiation the plants could serve as alert signals by changing their color.

5 Results

5.1 *AtCOM1* expression in wild-type plants

In order to understand the dynamics of a putatively differentially regulated promoter, it is important to know the state of expression under normal growth conditions in comparison to conditions that potentially stimulate promoter activity. The expression levels of *AtCOM1* in different wild-type (wt) plant tissues were analyzed by quantitative real time PCR (qPCR), a convenient method for gene expression analyses.

This technique allows the quantification of any polymeric DNA based on conventional polymerase chain reaction (PCR) together with the use of fluorescent dyes like "SYBR Green", TaqMan probes, LightCycler probes or molecular beacons. SYBR Green (used during this study) only fluoresces if bound to double-stranded DNA and thereby allows measuring the increase of fluorescence that is proportional to the increase of the amount of PCR products. In contrast to conventional PCR methods, quantification is done in real time after each amplification cycle and not at the end of the reaction; gel-electrophoresis to assess the amount of product is no longer necessary. As it is not possible to distinguish the fluorescence of the specific PCR product from that of primer dimers or other unspecific dsDNA molecules, a melt curve analysis is done following the PCR reaction. In doing so, temperature is raised continuously from 50°C to 90°C. If the specific melting temperature of the PCR product is reached, DNA strands are separated and there is a significant decrease in fluorescence. Unspecific PCR products give additional signals thereby allowing readout of reaction specificity by melt curve analysis.

To allow accurate comparison of the expression of the gene of interest in every sample, all values are normalized to the expression level of a reference gene, an actin gene (*ACTIN* 2/7 in this study), that is assumed to be homogenously expressed in all tested tissues. The use of a reference gene accounts for loading differences or variations in the absolute amount of DNA.

The critical value for calculation is the threshold cycle (C_T), describing the PCR cycle during that the level of fluorescence significantly exceeds background fluorescence for the first time. This value depends on the number of DNA templates at the beginning of the reaction and allows relative quantification according to the $\Delta\Delta$Ct method. The amount of target, determined by normalization to the reference gene and relative to the control (cDNA from

untreated seedlings), is $2^{-\Delta\Delta Ct}$. $\Delta\Delta$Ct is the substraction of the Ct value of the control from the Ct value of the sample (Lafarge, *et al*., 2003).

Only DNA can be quantified by qPCR. As the starting material for expression analyses is RNA, it has to be converted to copy DNA (cDNA). This is achieved during a reaction called reverse transcription PCR where an RNA-dependant reverse transcriptase is used to convert mRNA to cDNA. Reverse transcription is primed with oligo (dT)s that are complementary to the poly-A tail of mRNA molecules. After that a standard PCR reaction is initiated in the same tube, thereby creating dsDNA molecules that correspond to the mRNA templates. This PCR reaction can either be primed with gene-specific primers or random hexamer oligonucleotides. The advantage of the use of the latter is that the same cDNA sample can be used for the amplification of more than one target in subsequent qPCR reactions.

Stems, cauline leaves, rosette leaves, siliques and inflorescences were cut off wt-plants, frozen in liquid nitrogen and ground to a fine powder. This powder was used as substrate for an RNA extraction kit that yields whole RNA. This was converted to cDNA with a cDNA synthesis kit containing random hexamer primers and subjected to qPCR analysis.

All data were normalized to *ACTIN* 2/7, a so-called housekeeping gene that is homogenously expressed in every part of the plant.

All values are given relative to the expression level in stem that was set to "1" and in arbitrary units of "normalized fold expression". It could be shown that *AtCOM1* is expressed in all tested plant tissues with the highest levels in inflorescences and siliques (Figure 8).

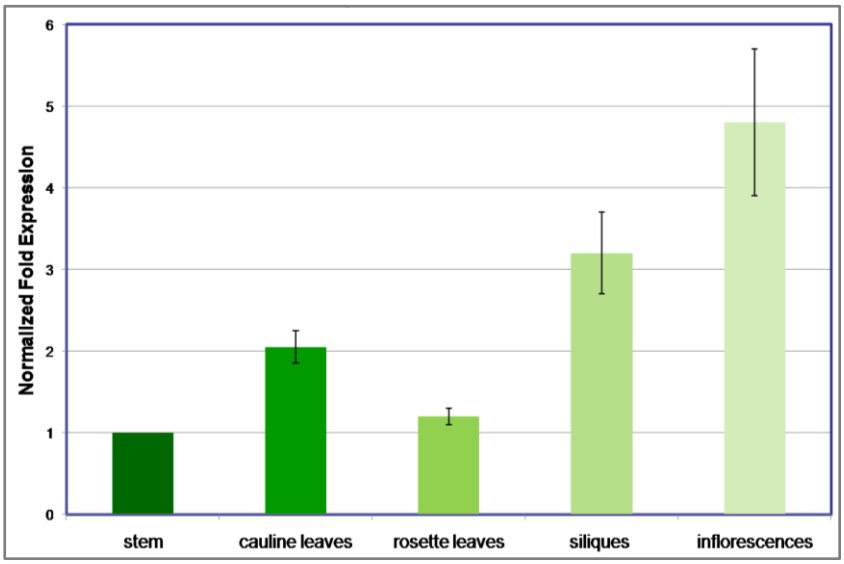


Figure 8 – Expression levels of *AtCOM1* in wt plant tissues; qPCR experiment. RNA of different wild-type plant parts was extracted and converted to cDNA that was subjected to qPCR analysis. Values are relative to the amount of expression in stem that was set to "1" and given in arbitrary units of "normalized fold expression" and normalized to the expression of the homogenously

expressed gene *ACTIN* 2/7. *AtCOM1* is expressed in all tested plant tissues with the highest levels in inflorescences and siliques.

The highest expression in inflorescences (about 5-fold compared to stem), where meiosis happens, seems to be statistically relevant. As you will see later, *AtCOM1* expression is indeed about 90-fold increased after treatment with ionizing radiation. But, in experiments where there was no promoter induction, variation of expression levels was on average lower than 5-fold.

5.2 *AtCOM1* is upregulated by genotoxic stress

AtCOM1 is known to be part of the DNA DSB repair machinery but until today there were no data about its specific reaction to different DNA damaging substances. It was only shown before that *AtCOM1* is upregulated upon treatment with gamma-radiation (Deveaux, *et al*., 2000).

Wild-type seeds were sown on ARA plates and after about two weeks, seedlings were exposed to gamma-radiation or infiltrated with the genotoxic drugs Mitomycin C, Bleomycin, Hydroxyurea and Methylmethane-sulfonate, respectively. Ionizing radiation was applied directly to the plant plates, whereas infiltration was performed in 24-well plates containing liquid infiltration medium supplemented with the genotoxics. After different timepoints, the medium was carefully removed and seedlings were frozen in liquid nitrogen, ground to a fine powder and stored at -80°C. RNA was extracted from the frozen powder, converted to cDNA and subjected to qPCR analysis.

Ionizing radiation leads to a variety of DNA lesions. It induces damage including oxidized bases, abasic sites, interstrand crosslinks, single-strand breaks (SSBs) and DSBs and the generation of free H_2O radicals that can further damage the cell. These lesions trigger signaling cascades that lead to a stop or delay in cell cycle progression and upregulation of various genes of the repair machinery. In the case of DSBs, the effect is dependent on AtATM (and to a lesser extent to AtATR) (Ricaud, *et al.*, 2007) and induces the expression of *AtCOM1* over 90-fold. Corresponding to the findings mentioned above, *AtCOM1* induction is not detectable in plants deficient in AtATM (Figure 9) and diminished in *Atatr* mutants (Ricaud, *et al.*, 2007). The effect of the mutation of ATR on *AtCOM1* expression was shown in the course of a large scale microarray experiment by Ricaud *et al*. They claim that *AtCOM1* showed attenuated expression in *Atatr* with high statistical significance. The ATM-dependant upregulation of *AtCOM1* after treatment with ionizing radiation was also shown in the same study (Ricaud, *et al.*, 2007).

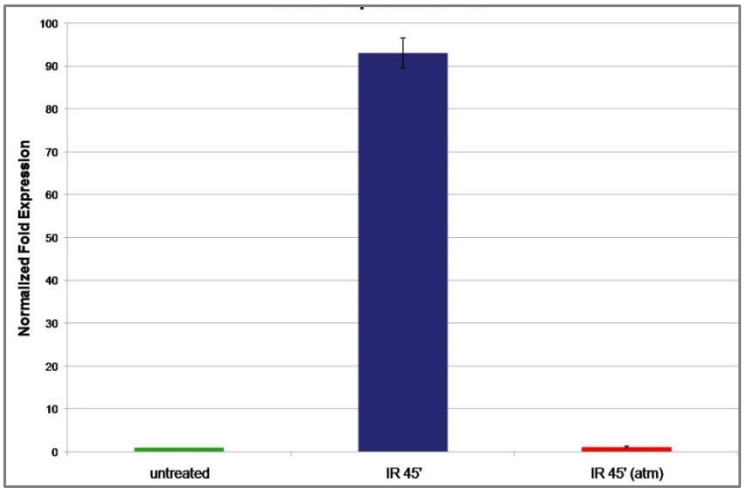


Figure 9 – *AtCOM1* expression 45 minutes after treatment with gamma-radiation (100Gy; 42Gy/min) in wild-type and *Atatm* mutant seedlings as compared to wt. The experiment was not conducted in *Atatr* mutants because this was done recently by Ricaud *et al*., showing that *AtCOM1* is only weakly upregulated in the mutant. qPCR experiment.

Seedlings for the untreated control were treated exactly as irradiated ones, except that they were not exposed to radiation.

Mitomycin C (MMC) is a natural cytotoxic agent used in clinical anticancer chemotherapy due to its function as a potent intrastrand crosslinker and N-alkylating agent. Guanine residues are mono- and bifunctionally alkylated specifically at 5'-CpG-3' sequences (Paz, *et al.*, 1999). MMC-treatment does not directly introduce DSBs but it is known that chromosome breaks occur later, as the result of the cell reaching mitosis without having fully completed DNA replication (Sognier, *et al*., 1986).

Seedlings treated with MMC only show strong upregulation (~40-fold) of *AtCOM1* expression after overnight incubation. This might also be explained by the idea that DSBs occur after the replication machinery falls off due to the introduced intrastrand crosslinks, followed by incomplete repair implying that the cell needs some time to reach S-phase and even M-phase until the effect is perceivable (see discussion). The stimulation of *AtCOM1* expression by MMC treatment could not be detected in *Atatm* mutant plants and was severely diminished in *Atatr* (Figure 10), where the induction was reduced to one sixth of that in wild-type seedlings.

AtCOM1 is needed for the resistance against MMC but A*tcom1* mutant plants were not more sensitive to other genotoxic treatments (Bleomycin, Aphidicolin, Cisplatin, Hydroxyurea, IR, UV B light) than wild-type plants (Uanschou, *et al.*, 2007). Therefore it was not expected that Bleomycin would induce *AtCOM1* expression.

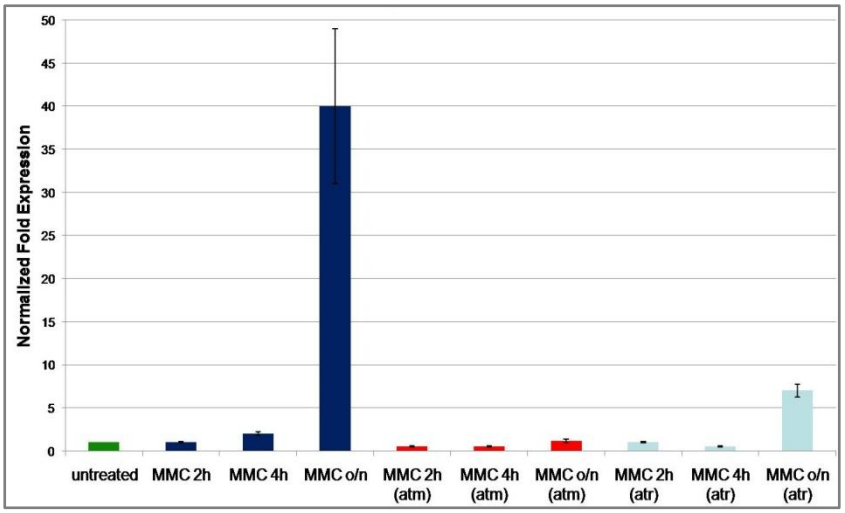


Figure 10 – *COM1* expression in seedlings at various timepoints after treatment with 40µM MMC. Induction is highest after overnight incubation, maybe because MMC does not directly introduce DSBs. There is no AtCOM1 induction in *Atatr* mutants and the induction is reduced to about one sixth in *Atatr*. qPCR experiment. Untreated control plants were infiltrated with medium only and harvested after overnight incubation.

Bleomycin, another anticancer drug, is a strand scission agent that is, dependent on metal ions and oxygen, capable of directly introducing DSBs and SSBs. Strand scission is initiated through the exclusive abstraction of the H at C4', mainly at pyrimidine nucleotides of sites containing GC and GT sequences (Claussen, *et al.*, 1999; D'Andrea, *et al.*, 1978).

Treatment of seedlings with Bleomycin leads to an about 60-fold induction of *AtCOM1* expression, already after two hours. This effect could be completely abolished by the mutation of *AtATM* and reduced to a fifth of the wild-type level in *Atatr* mutants (Figure 11).

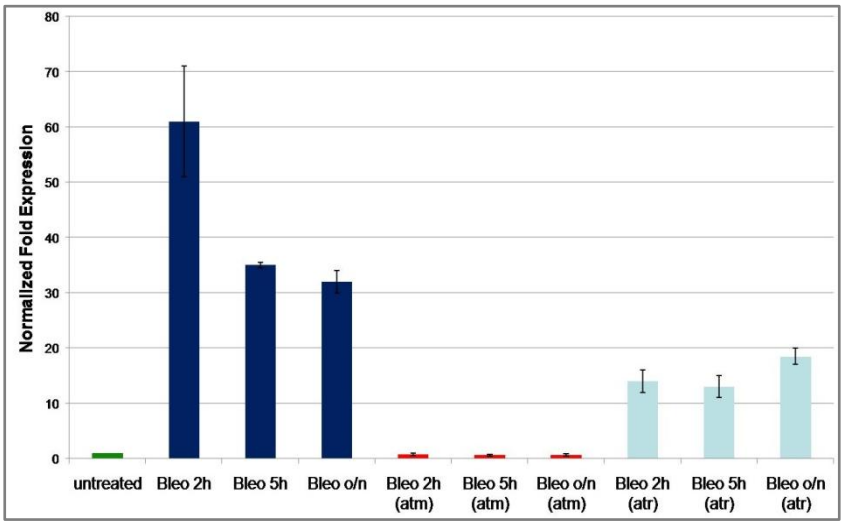


Figure 11 - *AtCOM1* expression in seedlings treated with Bleomycin (15µg/ml) is highest (~60-fold) already two hours after treatment. Lack of ATM stops *COM1* induction. Lack of ATR reduces the induction to a fifth of the wild-type level. qPCR experiment. Untreated control plants were infiltrated with medium only and harvested after overnight incubation.

The other tested genotoxic agents, Cisplatin, Hydroxyurea (HU) and Methylmethane-sulfonate (MMS) had no effect on the expression of *AtCOM1* – at

least during the observed period of time but there was no control for effective infiltration. However, these chemicals had no effect on the development and growth of wild-type and *Atcom1* mutant seedlings (Uanschou, *et al.*, 2007). Cisplatin is a chemotherapeutic drug. Its cytotoxicity is mediated by the formation of Cisplatin-DNA adducts that may inhibit DNA replication and transcription. Cisplatin also forms intrastrand crosslinks between adjacent purines and might introduce other adducts that are repaired mainly by nucleotide excision (Moggs, *et al.*, 1997; Zamble, *et al.,* 1995).

Hydroxyurea selectively inhibits ribonucleotide reductase, an enzyme of the deoxyribonucleotide synthesis pathway. This inhibition leads to a dramatic decrease in intracellular deoxyribonucleoside triphosphate pools, thereby slowing down or even blocking replication (Medscape Portals, 2000). This might result in the occurrence of stalled replication forks but no direct DSBs are introduced. MMS is a DNA alkylating agent that has been used for many years as a DNA damaging agent to induce mutagenesis and in recombination experiments. It modifies guanine (to 7-methylguanine) and adenine (to 3-methlyladenine), thereby causing base mispairing and replication blocks, respectively. As it is the case for DNA lesions introduced by Hydroxyurea, DSBs might be introduced as repair intermediates but there is no direct DSB induction (Beranek, 1990; Lundin, *et al.*, 2005).

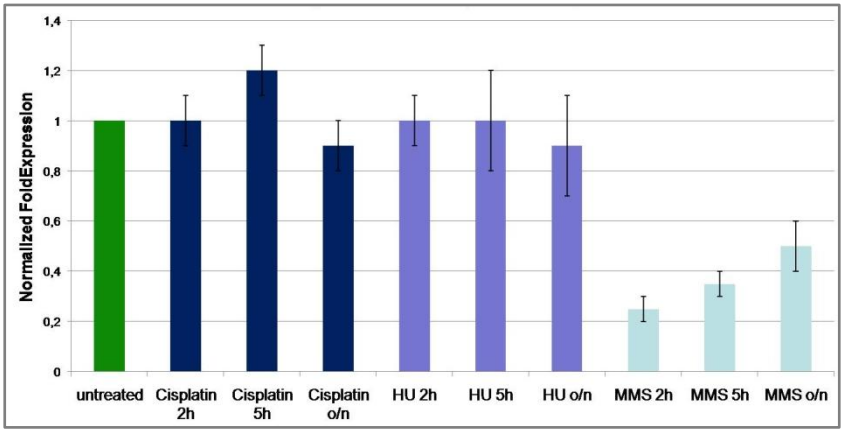


Figure 12 – Treatment of seedlings with Cisplatin, HU and MMS had no effect on the expression of *AtCOM1*. Untreated control plants were infiltrated with medium only and harvested after o/n incubation.

Expression of *AtCOM1* in plants treated with MMS even seemed to have decreased. Further experiments and repetitions will show whether this decrease is statistically significant.

Taking all experiments together, it could be shown that the induction of *AtCOM1* expression depends on the nature of the DNA lesions. Treatment with ionizing

radiation and Bleomycin, both directly inducing DSBs, leads to an early and strong induction of *AtCOM1* expression, whereas agents that induce other DNA lesions have no effect. After treatment with MMC, induction was only seen after overnight incubation, supporting the theory that DSBs are introduced but not immediately and only after cells have reached S phase – maybe explaining the delay in promoter induction. Additionally, it is known that chromosome breaks might occur after treatment with MMC as the result of cells reaching mitosis without having fully completed DNA replication (Sognier, *et al*., 1986), indicating that the DNA lesions introduced by MMC might be very special.

5.3 Promoter Deletion Analysis

After the stimuli that activate the *AtCOM1* promoter were known the next step was the search for *cis*- and *trans*-regulatory factors: the promoter analysis started with an *in silico* promoter prediction that involved screening of the web-tools SCOPE and WEEDER (Pavesi, *et al.*, 2004; Carlson, *et al.*, 2007) for consensus sequences of transcription factor binding sites (Uanschou, unpublished data) (Figure 13).

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 transcription factor binding sites (-644; -524), three putative bZIP transcription factor binding sites (-587; -536; -517) and one putative E2F transcription factor binding site (-311). The positions of the binding sites relative to the ATG are given in parentheses. The *AtCOM1* 5' untranslated region (UTR) is 284 bp long and interrupted by an intron. The *AtCOM1* gene itself is 1,8 kilobases (kb) in length and consists of two exons that are separated by a 147bp intron. The 3' UTR is 82bp long.

The approach to study the promoter was a deletion analysis combined with a GUS reporter assay.

Various fragments of the promoter were created by PCR and combined with a reporter gene that allows visualization of the activity of the promoter fragments.

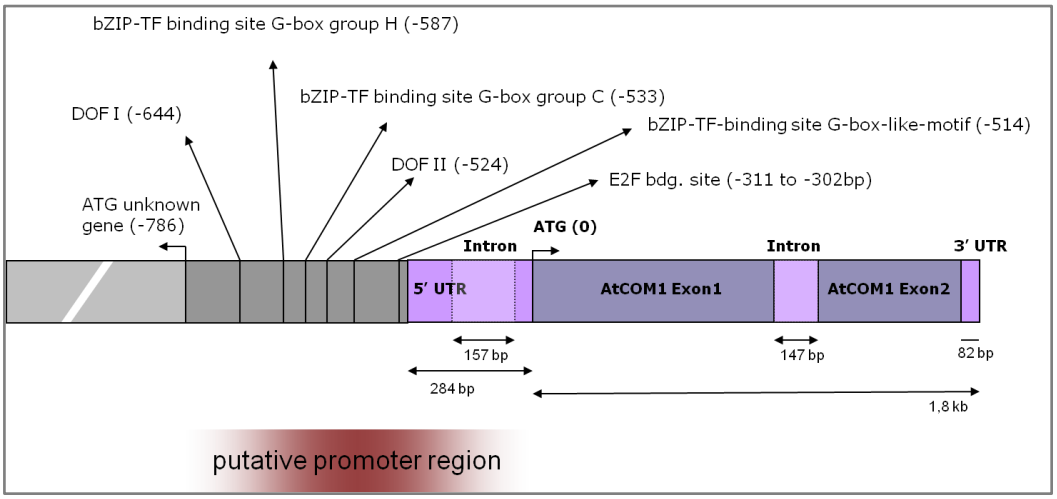


Figure 13 – in silico promoter prediction. The *AtCOM1* gene is 1,8kb in length and consists of two exons that are separated by a 147bp intron. The 3' UTR is 82bp long. The putative promoter region lies upstream of the 284bp 5' UTR, is about 1kb and reaches into an unknown gene of opposite orientation. Consensus sequences of three bZIP, two DOF and one E2F transcription factor binding sites were found in the putative promoter region.

Several down-primers carrying a PstI restriction site were designed and combined with two different up-primers with an XbaI site to create different fragments of the promoter region. One of the up-primers (com1_p1_up) lies right upstream of the ATG of *AtCOM1,* the other (com1_p2_frame_up) starts 99 bp downstream of the ATG and is in frame for the GUS gene in the context of the test vector pCBK04 (see below). The thereby created difference in length on the 3' ends of the promoter fragments allows testing of the regulatory importance of the first part of Exon 1.

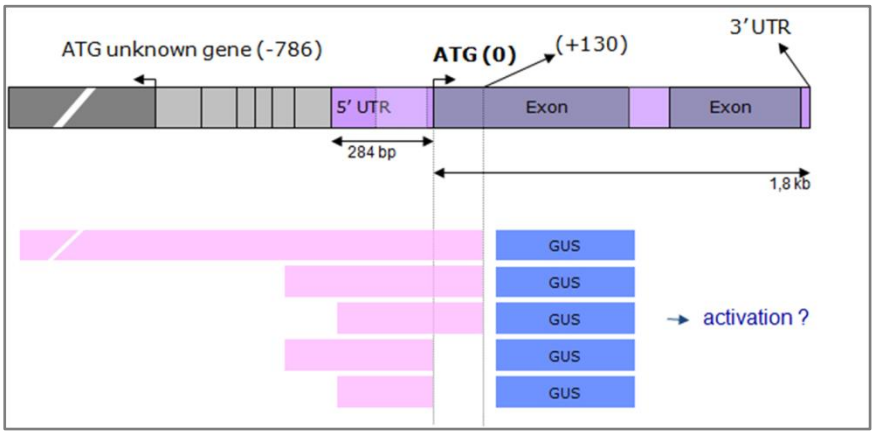


Figure 14 - Strategy of the promoter deletion analysis: different promoter fragments were generated and tested for their potential to drive the GUS reporter gene.

The artificial promoter fragments were PCR-generated with a high-fidelity polymerase and cloned into the TA cloning vector pCR2.1. After amplification and restriction analyses, the fragments were cut out via the introduced PstI and XbaI restriction sites and ligated into the vector pCBK04 instead of its original

constitutively expressed CaMV 35S promoter (Figure 15) that had been cut out via the PstI and XbaI sites before.

pCBK04 is a binary plant vector (see Figure 16) that can be used to transform plant cells or plants via *Agrobacterium tumefaciens*. If the inserted promoter fragment is active, it should drive 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 coloring 5,5'-dibromo-4,4'-dichloro-indigo that is directly visible without further treatment. This is a convenient and widely-used method to assay the activity of promoters.

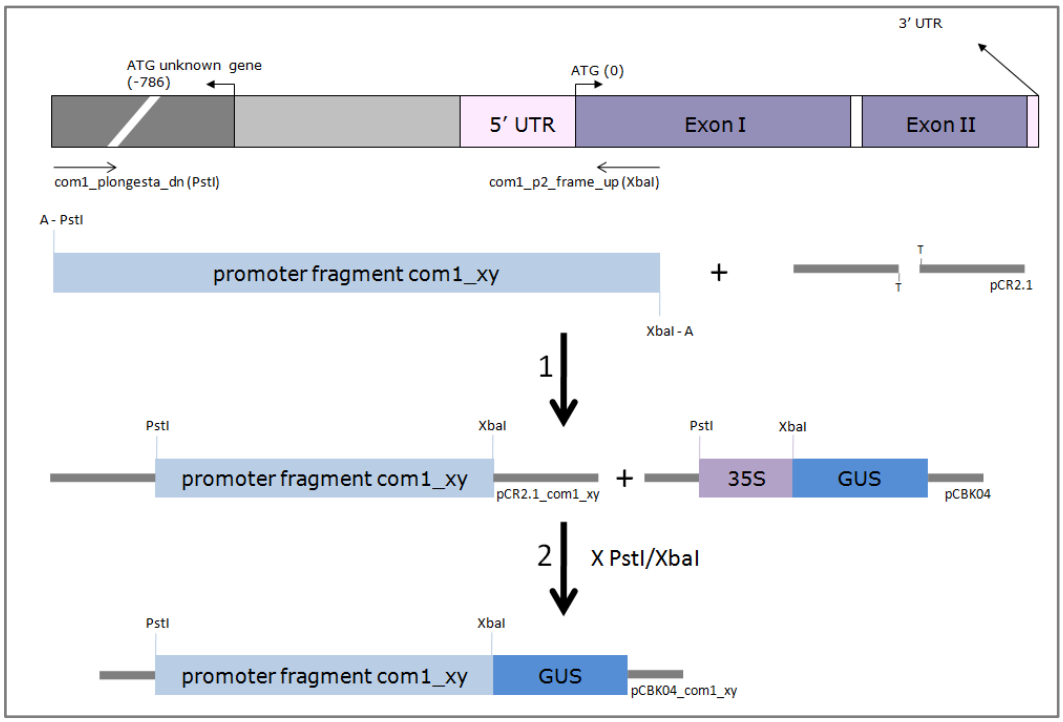


Figure 15 – Cloning strategy for the construction of the GUS reporter constructs. Promoter fragments were PCR-generated with down-primers containing a PstI site and up-primers with an XbaI site (only the primer pair for the construct of the longest promoter fragment is shown) and cloned into the TA cloning vector pCR2.1 via its T-A cloning site (1). The resulting vector was digested with PstI and XbaI, releasing the promoter fragment. Analogously, the binary plant vector pCBK04 was cut, thereby releasing the 35S promoter. Ligation of these fragments created derivatives of pCBK04 where the 35S promoter was substituted by the promoter fragments of AtCOM1 (2). Complete vectors are shown in Figure 16.

After the construction of all promoter::GUS fragments (Table 1) and their verification via sequencing they were transformed into *Agrobacteria* that were in turn first used to transform plant cell suspension culture cells. The experiments were performed in cell suspension because of its advantages compared to plants.

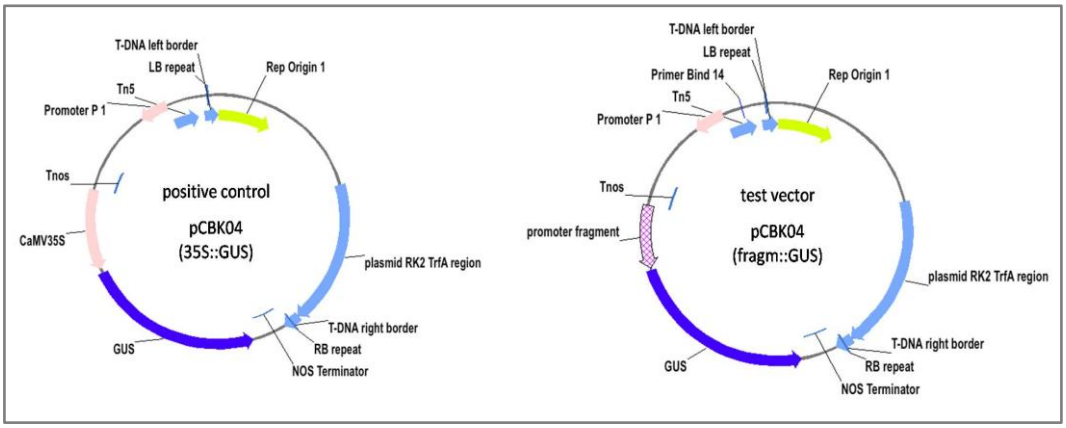


Figure 16 – Schematic drawing of the binary plant vector pCBK04. Left: original pCBK04 vector with a CaMV 35S driven GUS reporter gene, serving as a positive control for the GUS assays. Right: pCBK04 carrying an *AtCOM1* promoter fragment driving the GUS gene instead of the 35S promoter.

A suspension culture is easy to maintain, easy to transform, space-saving and results can be obtained after a few days. It takes only ten days from transformation of the reporter gene constructs until reporter gene activity can be assayed. In plants, a single experiment would last about 14 weeks. Only adult (~3 weeks old) plants can be transformed before it takes another 2-3 weeks until seeds can be harvested and sown again to grow the T_0 generation. Seedlings have to be selected and seeds of the surviving plants harvested (~6 weeks after sowing). These seeds produce the T_1 generation, where the first experiments can be performed after 1-4 weeks, depending on the desired developmental stage of the plants. But there is another decisive advantage of using cell suspension culture cells. Many genes are upregulated in a plant cell suspension culture, rendering the use of stimulators of gene expression (like genotoxic agents) unnecessary.

5.4 The activity of artificial *AtCOM1* promoter fragments is influenced by an E2F transcription factor binding site

All promoter fragments were tested for their potential to drive the reporter gene by transformation into cell suspension culture cells and GUS assays. Soon it became apparent that promoter fragments lacking the putative E2F transcription factor binding site never activated the GUS gene in this experimental setup. All fragments containing the E2F site showed deep blue staining in the performed assays.

name	dn-primer	up-primer	length (bp)
com1_1	com1_plongesta_dn	com1_p1_up	1616
com1_2	com1_plongesta_dn	com1_p2_frame_up	1742
com1_3	com1_p2_dn	com1_p1_up	818
com1_4	com1_p2_dn	com1_p2_frame_up	944
com1_5	com1_p3_dn	com1_p1_up	652
com1_6	com1_p3_dn	com1_p2_frame_up	778
com1_7	com1_p4_dn	com1_p1_up	593
com1_8	com1_p4_dn	com1_p2_frame_up	719
com1_9	com1_p6_dn	com1_p1_up	484
com1_10	com1_p6_dn	com1_p2_frame_up	610
com1_11	com1_pcore_dn	com1_p1_up	301
com1_12	com1_pcore_dn	com1_p2_frame_up	427
com1_13	com1_pleast_dn	com1_p1_up	152
com1_14	com1_pleast_dn	com1_p2_frame_up	278

Table 1 – Promoter fragments: names, primers for construction and lengths are indicated. The primer com1_p1_up is located right upstream of the ATG of *AtCOM1*, whereas the primer com1_p2_frame_up lies ~130 bp downstream of the ATG. The latter primer was designed to create promoter fragments that are in frame for the GUS gene when inserted into the vector pCBK04. All fragments were cloned in front of the GUS gene to test their potential to drive the reporter in GUS assays. For a schematic view of the promoter fragments, see Figure 18.

The 127 bp differences in length on the 3' end of the fragments that was created by the use of different up-primers (com1_p1_up and com1_p2_frame_up) had no effect on GUS expression. This leads to the assumption that the first about 100bp of Exon1 that are missing in fragments constructed with the first primer have no effect on promoter activity.

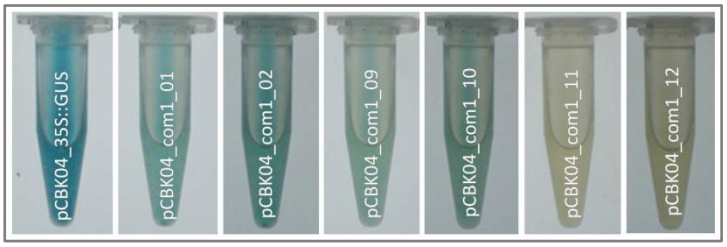


Figure 17 – Example of GUS assays of plant cell suspension culture cells, transformed with the assigned vectors. Blue staining stands for the active GUS reporter gene. The original pCBK04 vector served as positive control. Fragments com1_11 and com1_12 that lack the E2F transcription factor binding site never activated the reporter gene, while fragments com1_01, 02, 09 and 10, containing the E2F site always did.

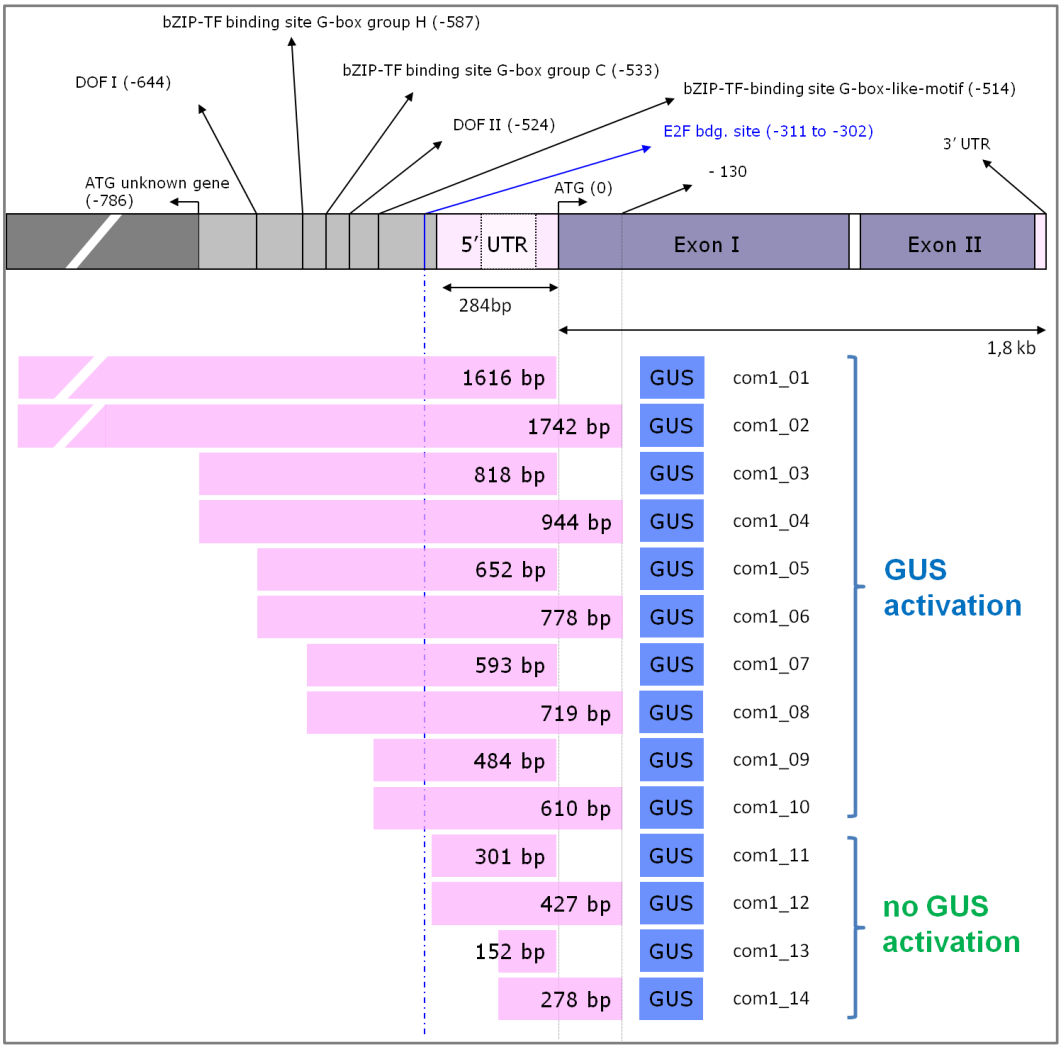


Figure 18 – Schematic view of the constructed *AtCOM1* promoter test vectors. *AtCOM1* promoter fragments were PCR-generated and cloned into a binary plant vector in front of a GUS gene in order to assess their potential to activate this reporter gene. After transformation into plant cell suspension culture cells GUS assays were performed. Only fragments containing the E2F binding site (com1_01-10) activated the GUS gene. Fragments lacking the E2F site (com1_11-14) never showed staining in GUS assays. The 127bp difference in length on the 3' end created by the use of different up-primers had no effect on the expression of the reporter gene (see also Figure 17).

To further investigate the importance of the E2F site, more promoter fragments, derived from fragment com1_10 that showed activity in GUS assays, were generated. Therefore, primers were constructed that allowed the generation of fragments that start with the E2F site on their 5' end and don't contain stretches longer than 25bp upstream of this putative transcription factor binding site. In addition, the E2F site was mutated to be sure that it is the decisive factor for promoter activity.

name	dn-primer	up-primer	length
com1_10a1	com1_10a_dn1	com1_p2_frame_up	473
com1_10a1_short	com1_10a_dn1	com1_p1_up	347
com1_10a2	com1_10a_dn2	com1_p2_frame_up	458
com1_10a2_short	com1_10a_dn2	com1_p1_up	332

Table 2 – Additionally created promoter fragments to further assess the role of the E2F transcription factor binding site: up-primers are the same as used for the construction of the original com1_10 promoter fragment but the new down-primers create fragments that just contain the E2F site on their 5' end.

Fragments com1_10a1 and com1_10a1_short start 25 bp upstream of the E2F site, fragments com1_10a2 and com1_10a2_short only 10 bp. They are derived from the promoter fragment com1_10 that starts 162bp upstream of the E2F site and were created to rule out other upstream sequences than the E2F site as regulating factors.

Figure 19 – 5' sequence of the fragments com1_10a1 and com1_10a1_short (first panel) and com1_10a2 and com1_10a2_short (second panel). These fragments were created from com1_10 that starts 162bp upstream of the E2F site and activated the GUS gene in the previously described experiments. The newly created fragments start only 25 and 10bp upstream of the transcription factor binding site to rule out other upstream sequences than the E2F site as regulating factors. The E2F site is highlighted in blue.

All of the newly synthesized promoter fragments activated the GUS gene in cell suspension culture cells, except com1_10a1_short that after sequencing showed to have a T to C mutation in the 5' UTR that destroyed promoter function. The site of the mutation will have to be further characterized and may be part of the core promoter.

Taken together, all this data speak for the E2F site to be one of the major regulating factors in the *AtCOM1* promoter. To further support this theory, the E2F site was mutated in fragments com1_10 and com1_9 that had always shown reporter gene expression in GUS assays by a bridging PCR. The original consensus sequence CGC GCG was altered to TAA TTT. The resulting fragments were subjected to GUS assays and did no longer activate the GUS reporter gene.

Figure 20 - Sequences of the natural E2F binding site found in the AtCOM1 promoter (top) and the mutated version that was introduced in an artificial promoter fragment. Mutation of the E2F site led to a loss of promoter activity.

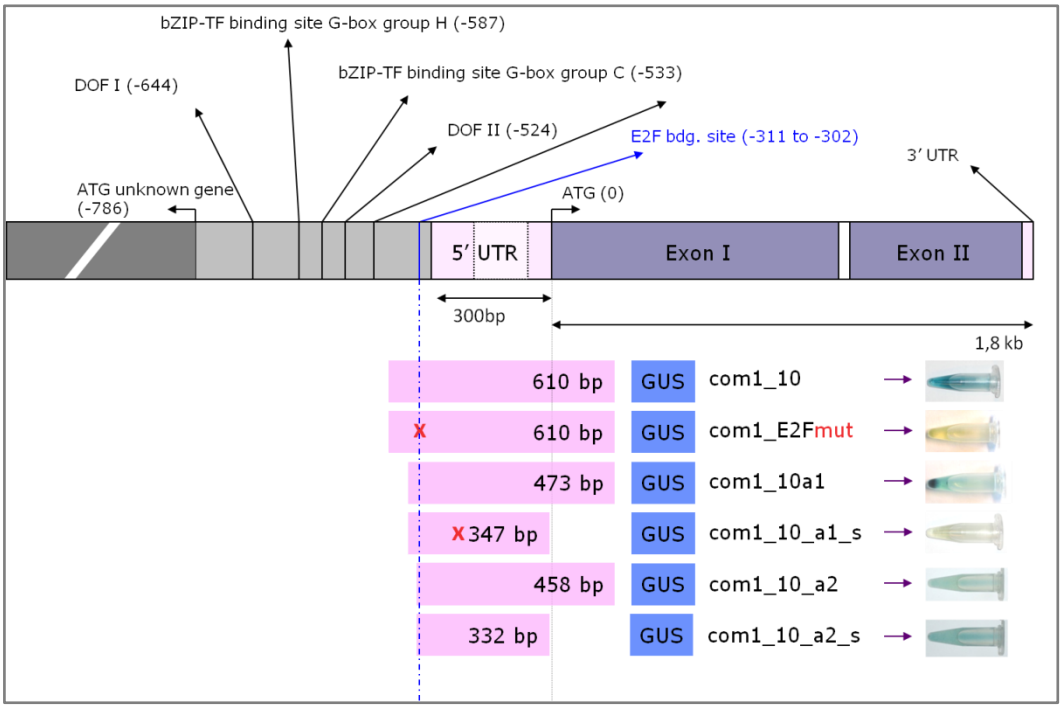


Figure 21 – Promoter fragments derived from com1_10. The E2F site is essential for the activation of the GUS gene in the context of pCBK04. All artificial promoter fragments of the *AtCOM1* promoter could drive the GUS reporter gene as long as they contain the E2F transcription factor binding site, even if it lies at the outermost end of the fragments, except com1_10a1_short that after sequencing showed to have a T to C mutation in the 5' UTR that destroyed promoter function. GUS staining was lost after mutation of the E2F site in promoter fragments that had been active before, The eppendorf tubes shown on the right side contain cell suspension culture cells that have been subjected to GUS assays. Blue coloring stands for an active promoter fragment. The differences in the darkness of the staining are a result of variations in cell number.

In total it could be shown that artificial promoter fragments of the *AtCOM1* promoter drive the GUS reporter gene as long as they contain the E2F transcription factor binding site, even if it lies at the outermost end of the fragments. The mutation of the E2F site leads to a loss of promoter activity. This is strong evidence that the E2F site plays an important role in controlling the *AtCOM1* promoter and is essential for artificial promoter fragments to drive the GUS reporter gene in this experimental setup.

5.5 The GUS reporter gene under the control of artificial *AtCOM1* promoter fragments is differentially expressed in cell suspension culture and whole plants

After analysis in cell suspension culture, the reporter gene constructs in pCBK04 carrying the fragments com1_02, com1_10 or com1_12 were transformed into wild-type plants. This was done to assess their activities in plants and not in cell

culture, where most genes are constantly upregulated. It was expected that there would not be somatic activity without the application of genotoxic stresses.

com1_02, the longest and most wild-type like promoter fragment was chosen because it was of big interest to see if it behaves like the wild-type promoter in meiosis and is inducible by genotoxic stress (see qPCR experiments above). This would be essential, concerning the intention to create screening tools for genotoxic stress and cells undergoing meiosis, respectively. com1_10, containing the E2F transcription factor binding site and com1_12 without it were chosen to discover, if the presence of this site has the same effect on reporter gene expression in plants as it could be seen in cell suspension culture cells.

Wild-type plants were transformed, seeds harvested and positive transformants selected by using the BASTA resistance gene carried by pCBK04. About twenty plants per line survived. To reduce the amount of work, seeds of only 6 surviving plants per transformed promoter fragment were harvested, sown on soil and plants of the T_1 were used for analysis. Leaves of up to 24 individuals of each plant line were harvested into 96-well plates and GUS staining was performed.

fragment	line ID	# of plants	stained	not stained
com1_02	127	20	9	11
com1_02	128	24	12	12
com1_02	129	24	21	3
com1_02	130	24	2	22
com1_02	131	15	13	2
com1_02	132	24	19	5
com1_10	57	24	1	23
com1_10	58	24	7	17
com1_10	59	24	8	16
com1_10	60	24	12	12
com1_10	61	24	13	11
com1_10	62	24	18	6

Table 3 - Wild-type plants were transformed with reporter gene constructs in pCBK04 carrying the fragments com1_02, com1_10 or com1_12. Up to 24 – if available - leaves of 6 lines per genotype were subjected to GUS assays. The number of plants that showed staining in their leaves per plant line is shown. None of the plants carrying the com1_12::GUS construct showed staining in leaves. Only lines 58 and 59 seem to be 3:1 segregating, meaning that they carry only one insertion. Subsequent generations will be further analyzed.

All plant lines carrying fragments com1_02 and com1_10 contained individuals that showed GUS staining in leaves, although no genotoxic stresses had been applied (Table 3). The different amount of stained individuals per line is normal for the generation T_1 as there is no information on multiple insertions of the transgene. Only 2 lines were possibly 3:1 segregating, meaning that they could carry – after Mendelian laws - only one insert of the transgene. The existence of only one copy of the transgene is beneficial for further crossings and experiments, as genotypes can be clearly assigned.

Seeds of all plant lines were harvested and subsequent generations will be further analyzed.

Analogous to the results of GUS assays in transgenic cell suspension culture cells, the plants carrying the com1_12::GUS reporter construct never showed staining in leaves.

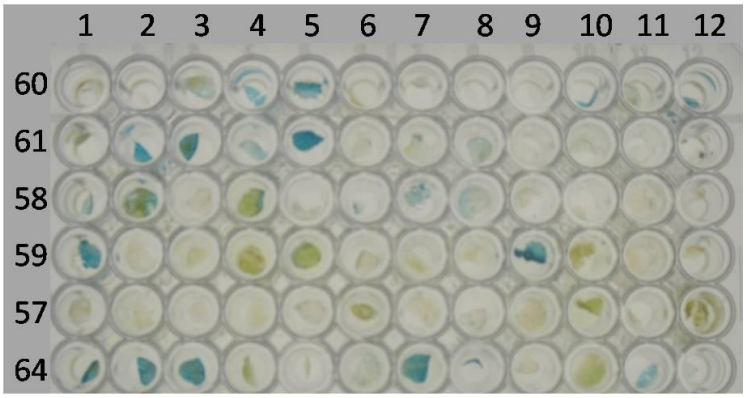


Figure 22 – Example of a GUS assay on parts of leaves of transgenic plants in a 96-well plate. Results for fragment com1_10, lines 57-62, individuals 1-12 are shown. Plants from all lines show GUS activity there are only differences in the number of stained individuals.

As stated above, it should also be tested if the activity of the promoter fragments was inducible. Therefore, leaves of individuals that had shown staining before were collected and half of them infiltrated with Bleomycin, a genotoxic drug that lead to strong activity of the wild-type promoter (see 5.2). GUS assays were performed on treated and untreated leaves in parallel but there was no visible effect on the intensity of the staining. Quantitative assays will be performed (see discussion).

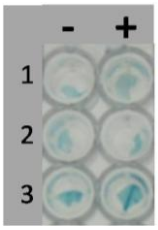


Figure 23 – GUS assay on leaves of transgenic plants carrying the com1_02::GUS reporter gene construct. Leaves on the left are untreated (-), leaves on the right (from same individuals) were infiltrated with 15µg/ml Bleomycin (+). Leaves from individuals 1-3 from the plant line 129 are shown. There is no visible difference in the intensity of the staining leading to the assumption that the promoter fragment cannot be induced by treatment with Bleomycin. But there was no control for the successful uptake of Bleomycin. The same results were obtained for plant lines carrying the com1_10::GUS transgene. Quantitative assays are in preparation.

To further analyze the activity of the promoter fragments – especially in meiosis – also inflorescences of all plants of all three genotypes were harvested and subjected to GUS staining. In cell suspension culture cells GUS activity was only seen if the reporter gene was driven by a promoter fragment carrying the E2F binding site (like com1_02 and 10). In contrast to that, there were strong signals in mature anthers of plant lines carrying the com1_12 promoter fragment (no E2F binding site) that had not given any staining in leaves before. The inflorescences revealed quite different GUS staining patterns for the three tested reporter gene constructs. Plant lines, carrying the com1_02 promoter fragment, showed faint GUS staining in the female reproductive organs (gynoecium) of young and mature flowers and strong staining in leaves.

Comparably, the gynoecium (young and mature flowers) and leaves of plants with com1_10 were stained but then together with the anthers (of mature flowers), the male reproductive organs. As stated above, in plants carrying com1_12, the shortest tested promoter fragment without the E2F site, strong GUS staining was exclusively observed in anthers of mature flowers and in no other parts of the plants.

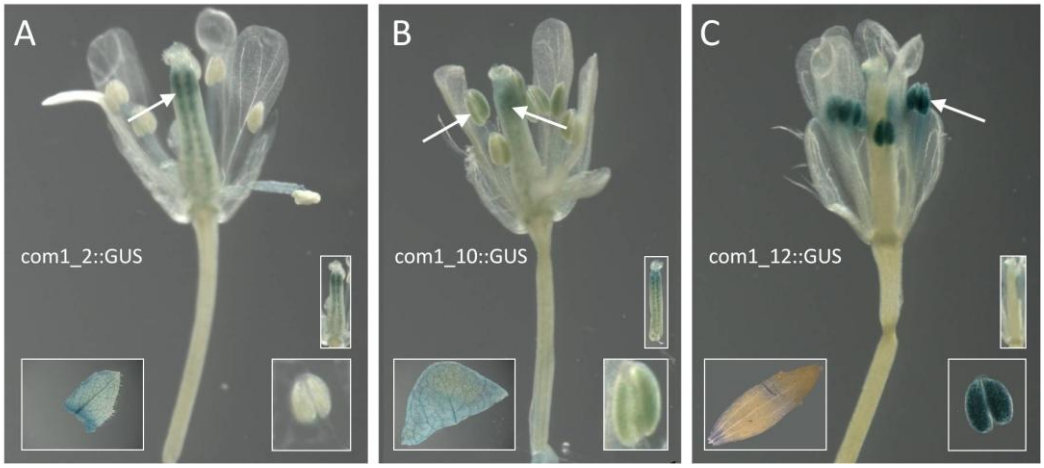


Figure 24 – GUS staining of plants transformed with pCBK04_com1_02, 10 and 12. Plants that express the longest promoter fragment in conjunction with the GUS reporter gene (com1_02::GUS; A) show GUS staining in leaves and the female reproductive organs (gynoecium). Plants with com1_10::GUS give signals in leaves, in the gynoecium and in anthers, the male reproductive organs. Plants expressing com1_12::GUS, the promoter construct lacking the E2F site, give a strong GUS signal in mature anthers and none in leaves. Stained parts of inflorescences are indicated by arrows. The boxes below show pictures of leaves, anthers and the gynoecium.

Figure 25 - GUS staining of smaller buds of a plant carrying the pCBK04_com1_02 transgene. The gynoecium is faintly stained.

These results indicate that *AtCOM1* is, at least partly, regulated by the E2F transcription factor binding site found in its promoter region. The presence or absence of this site influences the expression of a reporter gene driven by artificial fragments of the *AtCOM1* promoter. As stated above, the binding site is essential for promoter activity in cell suspension culture cells.

In plants, the absence of the E2F site leads to strong GUS activity in the male reproductive organs of mature flowers. As GUS staining could not be observed in smaller buds that are usually used for analyses of meiotic processes it is assumed that the com1_12 promoter fragment is active only after meiosis has completed. Promoter fragments carrying the E2F binding site activated the GUS gene in somatic tissues (even without genotoxic treatment) and the female reproductive organs of young and mature flowers. The shorter fragment, com1_10, also drove the reporter in the anthers of mature flowers, leading to the

assumption that there might be negative *cis*-regulatory elements in the *AtCOM1* promoter region that distinguishes the fragments com1_02 and com1_10.

6 Discussion

This study aimed to reveal the dynamics of somatic and meiotic *AtCOM1* promoter activity. The influences of exogenous factors like genotoxic stress and endogenous regulators like transcription factors on the promoter were analyzed.

6.1 AtATM and AtATR play a role in the regulation of *AtCOM1*

In humans, ATR is generally thought to be activated by stalled replication forks or repair intermediates like single-stranded DNA (ssDNA) while ATM is activated by double-strand breaks (DSBs) (Cuadrado, *et al.,* 2006). They play partially redundant roles in the induction of cell-cycle responses to DNA damage but ATR is suggested to be specially required for the long-term maintenance of the response by maintaining the phosphorylated state of the downstream targets (Culligan, *et al.*, 2006). ATM is one of the major checkpoint kinases and essential for cellular response to DNA damage. As CtIP, the AtCOM1/SAE2/GR1 homologue, is phosphorylated by ATM in humans (Li, *et al.,* 2000), the ATM homologue Tel1 is known to phosphorylate Com1/Sae2 in *S. cerevisiae*. There, phosphorylation of Com1/Sae2 by Mec1, the yeast homologue of ATR, is also known to be essential for Com1/Sae2 to exert its repair and recombination functions (Baroni, *et al.,* 2004).

Together with ATR, ATM is among the earliest signaling molecules to initiate the cellular response at DNA damage sites (Cuadrado, *et al.*, 2006). In contrast to ATM-deficient plants that are hypersensitive to ionizing radiation, *Atatr* mutant plants are only mildly sensitive to ionizing radiation, leading to the assumption that ATR plays a secondary role in response to DSBs (Culligan, *et al.*, 2004). This is concordant with the observation that *AtCOM1* induction after introduction of DSBs is only reduced in *Atatr* mutants but not completely abolished as in *Atatm* (see 5.1).

6.2 *AtCOM1* is upregulated by a subset of DNA lesions

The effects of different DNA damaging substances were analyzed. The strongest and fastest promoter induction was achieved by treatment with ionizing radiation

that induces a variety of damages, among them SSBs and DSBs. An about 90-fold induction was observed after only 45 minutes. The activation is dependent on AtATM and drops down to zero in *Atatm* mutant plants. In contrast, activation is only reduced in *Atatr*.

The second strongest activator was Bleomycin, a strand scission agent, that lead to an about 60-fold increase of *AtCOM1* transcription after two hours that was not observed in *Atatm* mutant plants. If AtATR is missing, *AtCOM1* expression is diminished to about one-fourth and did not significantly change over the observed time span. Treatment with MMC lead to a 40-fold induction of gene expression but only after overnight incubation. This corresponds to the theory that treatment with MMC in fact leads to DSBs but not directly and as the result of the cell reaching mitosis without having fully completed DNA replication (Sognier, *et al.,* 1986). MMC did not stimulate *AtCOM1* expression in Atatm mutants, whereas mutation of ATR only diminished it to a level of about 6-fold induction and with kinetics comparable to the wild-type situation.

The other tested genotoxic chemicals (HU, MMS and Cisplatin) had no effect on *AtCOM1* expression – at least over the observed time. The DNA lesions that are introduced by these agents are manifold but do not include direct double-strand breaks and might be repaired by an AtCOM1-independent pathway. It is true that DSBs might be introduced as repair intermediates but these seem to have no effect on the expression of *AtCOM1*.

Interestingly, it could be shown before that only the presence of MMC leads to strong inhibition of *Atcom1* mutant seedling growth, whereas treatment with IR, Bleomycin and the other genotoxic chemicals did not affect the development of the mutants compared to wild-type plants (Uanschou, *et al.*, 2007).

It is possible that ATM-dependent signaling cascades lead to broad induction of genes of the DNA repair machinery but that it depends on the very special type of damage which of the corresponding proteins is in fact needed for repair.

6.3 E2F transcription factors play a role in *AtCOM1* control

Different fragments of the *AtCOM1* promoter were generated and cloned into a binary vector in front of a GUS reporter gene. GUS activity, and thereby the activation potential of the promoter fragments, was then analyzed after transient transformation of plant cell suspension culture cells and stable transformation in plants. In cell suspension culture, GUS activity could be observed in all fragments

containing the E2F binding site. There was no staining in culture plant cells transformed with fragments lacking the E2F binding site and also no staining in cells transformed with promoter fragments carrying a mutated E2F binding site. Our data indicate that *AtCOM1* could be, at least partly, regulated by one of the 6 E2F proteins found in *A. thaliana*, which would fit into their function as key components of cell cycle control in higher eukaryotes. Furthermore, it is known that the human homologue of *AtCOM1*, CtIP, is also regulated by E2F transcription factors (Liu, et al., 2006).

But, the assumption that a single transcription factor regulates a gene that is differentially expressed in meiotic and somatic tissues, was too naïve.

Figure 26 – Sequences of the E2F binding site. Top: consensus sequence (Vandepoele, *et al*., 2005); Middle: Sequence found in the AtCOM1 promoter; Bottom: Mutated version of the E2F binding site that was introduced into an artificial promoter fragment.

After transformation in plants, the following pattern of GUS staining was observed. com1_02, the longest and wt-like fragment showed strong staining in leaves and faint staining in the gynoecium (of young and mature flowers), the female reproductive part of flowering plants. com1_10, the shorter fragment with the E2F site, showed strong staining in leaves, and faint in the gynoecium (of young and mature flowers) and mature anthers, the male reproductive organs. com1_12, the fragment without the E2F site showed absolutely no staining in somatic tissues, but gave a very strong signal in mature anthers. This was an undesirable result, as one objective of this thesis was to generate a meiosis-specific promoter but the promoter-fragment activity seems to be reserved to mature anthers and therefore post-meiotic. More experiments are needed to assess the exact point in time of expression in relation to meiosis (see 6.7).

6.4 *Cis*- and *trans*-regulatory factors

Central to this study was to identify *cis*- and *trans*-regulatory factors that positively or negatively influence *AtCOM1* promoter induction. It is clear that the E2F binding site plays an important role in controlling the promoter but there seem to be other factors as well, as the promoter fragments com1_02 (wild-type like) and com1_10 (shorter fragment containing the E2F site) showed different activity in anthers. The shorter fragment activated the GUS gene in anthers, whereas the longest fragment did not, leading to the assumption that there might

be other *cis*-regulatory sequences in the *AtCOM1* promoter region besides the E2F transcription factor binding site. The *in silico* promoter prediction revealed that there are three putative bZIP binding domains and two putative DOF transcription factor binding sites and maybe one of these also exerts controlling functions on the promoter (see Figure 13). The DOF transcription factor family is putatively specific to plants and they are suggested to play a role in the regulation of processes exclusive to plants, like photosynthetic carbon assimilation, light-regulated gene expression, germination, dormancy, and so on (Moreno-Risueno, *et al.*, 2007). Until today there is no report about any function in DNA repair and meiosis leading to the assumption that these are no likely controlling factors of the analyzed promoter. The other group of transcription factor binding sites that were found in the promoter are bZIP binding sites. The respective transcription factors are known to regulate processes including seed maturation, flower development, pathogen defense and light signaling – but also stress signaling (Jakoby, *et al.*, 2002). This puts them in an interesting light regarding this promoter analysis.

6.5 Generation of artificial promoters

Furthermore, during the course of the project, we intended to create artificial promoters. Until now, they could not be established. com1_12, the shortest fragment so far analyzed in plants, leads to strong reporter gene activity in anthers but obviously acts too late in meiosis or even after it and is therefore not applicable for screening tools for meiotic cells. This could have helped scientists working on meiosis in two ways: transgenes could be specifically expressed and studied during meiosis and meiotic cells could be labeled. The latter could be achieved by combining a meiosis-specific artificial promoter fragment with a fluorescent reporter gene. This should give fluorescent signal in meiocytes, allowing them to be sorted out by a flow cytometry method called fluorescence activated cell sorting (FACS). Thereby it would be possible to collect specifically meiocytes, facilitating, for example the isolation of higher amounts of meiosis-specific proteins.

The other fragments, com1_02 and com1_10 might not be inducible by genotoxic stress or not sensitive enough to sense genotoxic stresses in the environment. The experiments on the wild-type promoter were performed with 100 Gray, a high dose of radiation and until now an induction of the activity of the promoter fragments could not be achieved in whole plants – GUS staining was already

detectable under non-stress conditions and could not be obviously intensified by the application of Bleomycin. Quantitative experiments to further address this problem are in preparation.

The promoter fragment has to be well inducible in order to follow the plan to use it in plants in conjunction with phenotypic marker genes to establish cheap and easy-to-use screening tools for environmental perils. The idea behind that is to plant transgenic plants carrying the promoter fragment in conjunction with a phenotypic reporter gene around, for example, nuclear power plants. In case of the emersion of a hazardous amount of radiation the plants could serve as alert signals by changing their color, for example.

Cloning of *AtPAP1* (kindly provided by Tsvi Tzfira) is currently underway. This gene of the anthocyanin synthesis pathway will be tested for its suitability as potential marker gene. It would be used analogously to the GUS gene described in section 5.3 and is supposed to turn plant parts from green to a reddish or brownish color – like leaves usually change color in autumn.

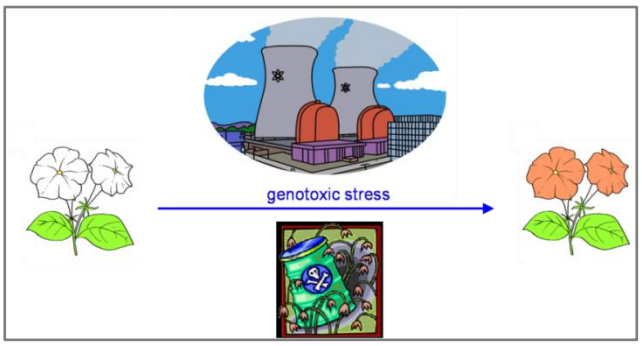


Figure 27 – Schematic drawing of the potential use of the artificial promoters as monitoring tools for genotoxic hazards in our environment. Transgenic plants carrying the reporter gene construt could be planted around, for example, nuclear power plants. By changing their color if exposed to genotoxic stress, they could serve as alert signals for the accidental emersion of ionizing radiation. Tools like that could be cheap, easy-to-handle, fast, safe and accurate.

6.6 Model of *AtCOM1* Promoter Induction

Figure 28 shows the model of *AtCOM1* promoter control in somatic, meiotic and post-meiotic tissues. In somatic cells, DSBs are sensed via AtATM (and to a lesser extent by AtATR) that phosphorylates a variety of downstream targets, including cell cycle regulators, transcription factors and DNA repair proteins. This leads to a cell cycle arrest or delay, giving the in parallel activated repair machinery enough time to exert its effects before the cell reaches M-phase. It is not known whether ATM acts directly on AtCOM1 or via other signaling molecules. E2F genes are also part of this ATM-dependent signaling cascade, supporting the notion that *AtCOM1* is upregulated after DSB formation by one of the 3 known transcriptional activators of the E2F family that are in turn regulated by ATM.

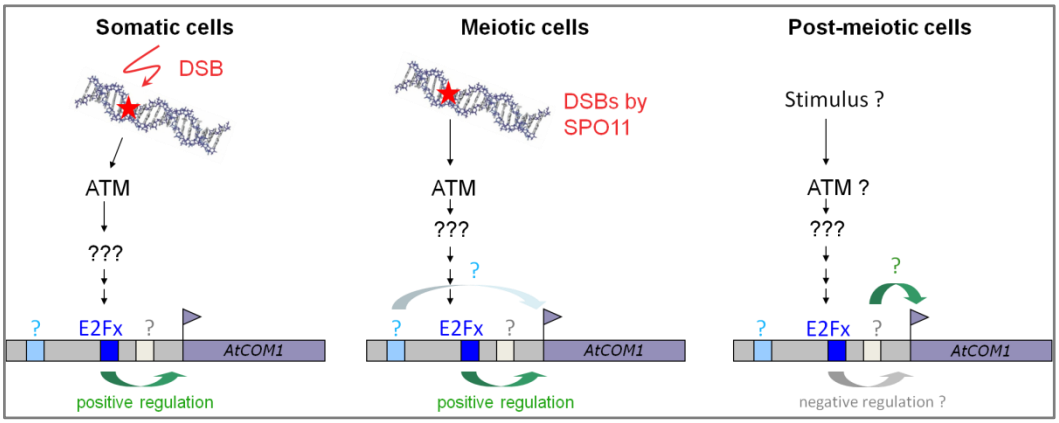


Figure 28 – Model of *AtCOM1* promoter control. In somatic cells, DSBs are believed to be sensed by AtATM that phosphorylates a variety of downstream targets including cell cycle regulators and transcription factors thereby leading to cell cycle arrest or delay and activation of the DNA repair machinery. It is not known if AtCOM1 is a direct target of ATM but this model supports the notion that the checkpoint kinase exerts its effect on the *AtCOM1* promoter via one of the known E2F proteins. In meiotic cells, where DSBs are introduced by AtSPO11-1, regulation might follow similar pathways but maybe under control of additional upstream factors. In post-meiotic cells, where promoter activity was highest when the E2F site was missing, one of the E2F proteins might act as transcriptional repressor. If E2F-mediated repression is missing, other factors might stimulate gene expression.

The full length construct, resembling the wt promoter, is tightly regulated in meiosis, leading to only faint staining of the gynoecium that could be observed in younger inflorescences. Surprisingly, these plants showed strong somatic promoter activity, even without genotoxic treatment. If there in fact is misregulation of the promoter has to be clarified by quantitative assays (in preparation) as the qualitative GUS assays already performed might not be exact enough to answer this question. Nevertheless, the E2F site seems absolutely required for somatic expression, because there was no staining in culture plant cells or somatic tissues of plants transformed with fragments lacking the E2F binding site. There was also no staining in cells transformed with promoter fragments carrying a mutated E2F binding site.

Taken together, these results indicate that *AtCOM1* regulation in meiosis might follow similar pathways as in somatic tissues but maybe under additional control of other upstream factors.

In post-meiotic cells, promoter activity is strongest when the E2F site is missing indicating that it naturally acts as a repressor on the *AtCOM1* promoter after meiosis has been completed. This is possible, as three of the known E2F transcription factors are known to act as transcriptional repressors via the same consensus sequence as their activating counterparts. If E2F-mediated repression is missing due to the lack of the transcription factor binding site, other factors might stimulate gene expression.

The E2F site may recruit – together with other factors - positive or negative regulatory players, depending on the tissue context but the exact mechanisms of *AtCOM1* promoter control remains elusive.

6.7 Experimental Outlook

It was possible to set up a preliminary model of *AtCOM1* promoter control but more experiments have to be considered to ask all open questions.

In Planta Analysis of More Promoter Fragments

Currently, transformation of the reporter gene constructs with the promoter fragments com1_01, 09, 11 and 14 is underway. Com1_01 and 09 carry the E2F site, whereas com1_11 and 14 do not (Figure 18). These experiments will show whether the shortest fragment, com1_14, can activate reporter gene transcription in whole plants, or the shortening of the fragments on their 3' end (as in com1_01, 09, 11) has any effect on their activity. Additionally, the fragments with mutated E2F binding sites will be transformed into plants as well.

Analysis of *AtCOM1* Expression in Different E2F Backgrounds

Various research materials concerning the E2F factors, like E2F and DP overexpression lines, mutants if available (E2F-e, f), antibodies (α-AtE2Fa, b, c) and tagged versions of the proteins have been requested and obtained. It will be analyzed by qPCR if there is any change in *AtCOM1* expression in plant lines overexpressing one or more of the E2F genes or in e2f mutant plants. The antibodies will be used in biochemical assays. These experiments will shed more light on the interactions between E2F transcription factors and *AtCOM1* expression.

Electrophoretic Mobility Shift Assay (EMSA)

This gel-shift assay is a common technique to study protein-DNA interactions. The DNA sequence of interest is mixed with nuclear extract and run on a gel. If a protein is able to bind the assayed DNA sequence, the complex will migrate less far on the gel as the DNA alone will, leading to the visible "gel-shift" effect. EMSA is planned to be performed with putative transcription factor binding sites lying upstream of the E2F binding site to isolate other potential *trans*-acting factors controlling the *AtCOM1* promoter. These factors are supposed to lie somewhere in the *AtCOM1* promoter region that distinguishes the fragments com1_02 and

com1_10. com1_10::GUS lead to staining in the gynoecium and anthers, whereas com1::02 only showed staining in the gynoecium, leading to the assumption that the fragment com1_02 carries negative regulators that are missing in com1_10.

Additionally, EMSA will be performed with the tagged proteins mentioned above, to confirm the interaction of E2F proteins with the putative E2F binding site in the *AtCOM1* promoter.

Elucidating the exact timely manner of activity of the promoter fragment com1_12 in respect of meiosis

In order to reveal when the smallest tested promoter fragment is exactly active in relation to meiosis it would be useful to correlate stained buds to flower stages. Immunofluorescence analysis with an α-GUS antibody on squashed anthers was performed but the first results were not encouraging due to enormous background staining – this experiment will be repeated and improved. In addition, buds of same size from one inflorescence will be subjected to GUS staining and cytological analysis that can easily reveal the developmental state of the flower. The occurrence of GUS staining could then be correlated with the exact stage of flower development.

Does Genotoxic Stress Induce the Activity of Promoter Fragments in planta?

Ionizing radiation and Bleomycin lead to a strong upregulation of AtCOM1 in wild-type plants. In contrast, Bleomycin had no visible effect on GUS activity when applied to plants carrying the com1_10::GUS or com1_02::GUS transgene. As the GUS assays were maybe not exact enough and there was no positive control for Bleomycin uptake, inducibility of the promoter fragments will be tested as follows.

Seedlings carrying pCBK04_com1_02, 10 and 12 were exposed to 100 Gy of ionizing radiation and will be treated with Bleomycin. qPCR analysis will be performed analogously to that in wild-type plants except using primers for the GUS gene. Primers have already been designed and the experiment will be set up in the near future. This experiment will reveal to what extent the activity of the promoter fragments differs from that of the wild-type promoter. In order to create the mentioned screening tools for genotoxic stress, the promoter fragments have to be inducible as it was shown for the wild-type promoter.

Does the Mutation of ATM Abolish Promoter Fragment Activity *In Planta*?

Plant lines carrying the pCBK04_com1_02, 10 and 12 reporter constructs were crossed to *atm* mutant plants. Plants of the F_0 were selected for the reporter gene construct using BASTA and seeds of the surviving plants were harvested. Generation F_2 will be screened for plants that are homozygous for *atm* and carry the reporter gene construct. Leaves and inflorescences of these plants will be subjected to GUS assays to answer the question if the mutation of *Atatm* leads to a loss of activity of the promoter fragments.

Promoter Analysis of *AtBRCA1*

In order to be able to generate artificial promoters that might be more sensitive to genotoxic stress, the putative promoter region of *AtBRCA1* will be analyzed in an analogous manner to the promoter deletion analysis presented in this study.
AtBRCA1 is an orthologue of the human breast cancer susceptibility gene1 and strongly induced by gamma-radiation. It was shown to be upregulated even after low doses of ionizing radiation. Maximal induction of 140-fold was observed after treatment with 100Gy but a more than 20-fold induction was already reached at the low doses of 1-3Gy (Lafarge, et al., 2003), making the AtBRCA1 promoter a promising candidate for the generation of screening tools for genotoxic stress.

Is *AtPAP1* an appropriate reporter gene?

As it is intended to create screening tools for genotoxic hazards, an appropriate reporter gene has to be selected. As depicted in Figure 27, activation of the reporter gene should lead to an easily detectable change of the plant phenotype. The change of leaf or petal colour would perfectly suit this demand. The *AtPAP1* gene is part of the anthocyanin synthesis pathway is supposed to turn plant parts from green to a reddish or brownish color – like leaves usually change color in autumn. *AtPAP1* is currently being cloned into pCBK04 instead of the GUS gene. This potential new reporter gene construct will be tested in plant cell suspension culture and in whole plants to see whether it is appropriate.

7 Materials and Methods

7.1 Media

Plant Media

ARA medium: 4,3g/l MS basal salt; 0,5g/l MES; 10g/l Sucrose and 1x Gamborg's vitamin solution; pH5,8 (calibrated with 1M KOH); 6g/l plant agar; autoclave

Cell suspension culture medium: 4,33g/l MS basal salt; 2x Gamborg's vitamin solution; 30g/l sucrose; 0,5mg/l 2,4-Dichloro-phenoxyacetic acid; 2mg/l Indole-3-acetic acid; 0,5mg 6(γ,γmethylallylamino)-purine riboside [all Duchefa]; pH5,8 (calibrated with 1M KOH); filter sterilize

Bacterial Media

LB: 10g/l tryptone; 5g/l yeast extract; 10g/l NaCl; pH7.0 (calibrated with 1N NaOH); 15g/l agar for plates

E. coli bacteria were selected on media containing 50mg/l Ampicillin [Duchefa] or 25mg/l Kanamycin [Duchefa]. *A.tumefaciens* GV3101 bacteria were selected on media containing 50mg/l Gentamycin [Duchefa] and 25mg/l Kanamycin or 50mg/l Ampicillin. Stock solutions were prepared for all antibiotics and stored at -20°C.

7.2 Plant Work

Growth Conditions

Plants were grown under long day conditions (16h light, 8h dark, 21°C; 60-80% humidity, 5800 LUX, 4x Philips TLD 36W and 2x Sylvana GroLUX 36W).

All lines were *Arabidopsis thaliana*, ecotype Columbia (col-0).

Seed Sterilization

About hundred seeds were covered with 1ml sterilization solution (5g Ca(OCl)2 added to 100 ml dH2O; 0.02% Triton X-100; used after one day; prepared fresh every 14 days) and mixed vigorously. After that seeds were agitated at room temperature for 20 minutes, washed twice with 1ml of sterile water after short centrifugation steps and dried in the laminar flow for at least one day before they were sown on ARA plates.

Transformation of Agrobacterium tumefaciens

100µl of electro-competent *Agrobacteria*, strain GV3101, were mixed on ice with 1-3µl plasmid. Electroporation was performed in a pre-cooled 1mm electroporation kuvette at 400, 25µF and 1,8kV (pulse length of 5-8ms). Cells were incubated for 1h at RT after addition of 1ml LB medium. 100µl of the cell suspension were spread on a plate containing 50mg/l Kanamycin and 50mg/l Gentamycin and incubated for 2-3 days at RT or 1-2 days at 30°C.

Stable Transformation *In Planta*

3ml LB medium supplemented with 50mg/l Gentamycin and 25mg/l Kanamycin were inoculated with a single colony of *Agrobacterium tumefaciens* (strain GV3101) and incubated at 28°C overnight under constant shaking. 500ml of LB containing the same antibiotics as before were inoculated with 3ml of overnight culture and again incubated at 28°C overnight under constant shaking. The cells were harvested by centrifugation (25', 3000rpm, RT). Cells were then washed with 5% sucrose (10min, 3000rpm, RT), the supernatant was discarded and cells were re-suspended in 300ml 5% sucrose supplemented with 0.02% Silwet L-77. Inflorescences were dipped into the bacterial suspension for 30 seconds, and left in a box covered with saran foil for 2 days under light before being returned to normal growth conditions.

BASTA selection on soil

Seeds were sown on soil (100-200 per pot) and left at 4°C for two days for pre-germination. After about one week, when the first true leaves have developed, seedlings were sprayed with 150µg/ml BASTA (200g/l Glufosinate-Ammonium; Bayer). Spraying was repeated every two days until most seedlings were dead (three to four times).

Maintenance of Plant Cell Suspension Culture

Every seven days, 5ml of cell suspension were transferred to a sterile 100ml Erlenmeyer flask and 20ml of cell suspension culture medium were added. Cultures were kept at 21°C in the dark under constant shaking.

Transient Transformation of Plant Cell Suspension Culture

One day after sub-cultivation of the cell suspension *A. tumefaciens* bacteria (strain GV3101), containing the plasmid of interest, were plated from glycerol-stock on a fresh LB plate. On the third day after sub-cultivation the bacteria were

inoculated into 50ml LB supplemented with 50mg/l Gentamycin and 25mg/l Kanamycin and grown at 28°C under constant shaking at 130rpm. The next day, bacteria were harvested by centrifugation and resuspended in 1ml of cell suspension culture medium. The concentrated bacteria were then added to the plant cell suspension cultures together with 2µl of 0,2M Acetosyringone (kept as a stock solution diluted in sterile water at 4°C in the dark). One week after sub-cultivation, cells were washed three times (5min, 2600rpm, no break) with 10ml fresh cell suspension culture medium. After the last centrifugation step, 25ml medium were added and cells were resuspended carefully. 5ml of this solution were transferred to a fresh flask, together with 20ml fresh medium, supplemented with 250mg/l Amoxicillin [Duchefa]. Two days later, cells were ready for analysis.

GUS staining of Cell Suspension Culture

Cells from 1ml of cell suspension culture were collected by centrifugation and incubated in 1ml GUS buffer (1g X-Gluc dissolved in 2ml DMSO; 1ml Triton X-100; 997ml 0,1M phosphate buffer pH7; stored at -20°C) at 37°C for at least one and up to 24 hours. The reaction is stopped by washing with dH_2O. If the GUS reporter gene is transcribed, cells show a dark blue coloring.

GUS staining of Plant Material

Leaves or inflorescences were harvested and put into a 24-well plate, containing 2 ml ice-cold fixing solution (2% formaldehyde; 1mM EDTA; 0.1% TritonX-100) per well. A short vacuum was applied and the material was fixed at 4°C for 30 minutes.

The material was washed twice with 100mM Na-phosphate buffer pH7 and 1ml staining solution (100mM Na-phosphate buffer pH7; 10mM EDTA; 1mM X-Gluc; 0.1% TritonX-100) was added. The plates were incubated overnight at 37°C. After washing with dH_2O the chlorophyll was extracted from the material at room temperature with 70% ethanol or a mixture of 3:1 absolute ethanol:glacial acetic acid.

Genotoxic Treatments

Seedlings of col-0 seeds were grown under long day conditions (16h light, 8h dark, 21°C) on ARA plates. A few days after the development of the first true leaves, seedlings were γ-irradiated (Co^{60}; 100Gy, 42Gy/minute) or infiltrated in 24-well plates containing 2ml of infiltration medium (4,3g/l MS basal salt; 5% sucrose; 0.02% Silwet L-77) supplied with genotoxic agents (40µM Mitomycin C

[Sigma Aldrich]; 30µM Cisplatin [Sigma Aldrich]; 1mM Hydroxyurea [Sigma Aldrich]; 15µg/ml Bleomycin; 100ppm Methylmethane sulfonate [Fluka]). After incubation for 2h, 4h (or 5h) and overnight, seedlings were removed from the medium, frozen in liquid nitrogen and stored at -80°C.

7.3 DNA Work

DNA preparation from *E.coli*

1.5ml of an overnight culture of *E. coli* were centrifuged in an Eppendorf tube for 1 minute at 14000 rpm. The supernatant was discarded and the cells were re-suspended in 200µl GTE buffer (50mM glucose; 25mM Tris-Cl pH8; 10mM EDTA). After adding 200µl of freshly prepared Alkali-SDS solution (0.2N NaOH; 1% SDS) the tube was gently inverted several times. 200µl acetate solution (3M KoAc; 11.5% acetic acid) were added and the tube was inverted again. The sample was centrifuged for 5 minutes at 14000 rpm at room temperature and 500 µl of the supernatant were mixed with 500µl isopropanol. The DNA was precipitated by centrifugation (15 min, 14000rpm, RT), washed with 500µl 70% ethanol (20min, 14000 rpm, 4°C), dried and re-suspended in 50µl of 1x TE (10mM Tris-Cl pH8; 1mM EDTA pH8) or sterile water.

Pure Grade DNA preparation from *E.coli*

DNA was extracted from 1,5ml of an overnight culture with the QIAGEN Plasmid Mini Kit (Cat.No. 12125) according to the manufacturer's protocol.

DNA preparation from *A. thaliana*

One to three young leaves or inflorescences were harvested in Eppendorf tubes and 200µl extraction buffer (200mM Tris pH7,5; 250mM NaCl; 25mM EDTA; 0,5% SDS) were added. The plant material was homogenized using plastic pestles. After centrifugation (5min, 14000 rpm, RT) the supernatant was mixed with 1 volume of isopropanol and incubated at room temperature for 5 to 10 minutes. The DNA was precipitated by centrifugation (5min, 14000 rpm, RT). After removal of the supernatant the pellet was washed with 300µl 70% ethanol, dried, re-suspended in 50µl 1x TE or sterile water and incubated at 65°C for 10 minutes.

Transformation of chemically competent *E.coli*

100µl of chemically competent E.coli strains XL-1 Blue or TOP10F' were thawn on ice and mixed with 1-5µl of plasmid. Following incubation on ice for 10 minutes, cells were kept at 42°C for 2 minutes. After addition of 1ml of LB medium, cells were incubated at 37° under constant shaking. Cells were concentrated by short centrifugation at 13000 rpm and the supernatant was discarded, leaving approximately 100µl. The cell pellet was resuspended and plated on selection plates that were incubated overnight at 37°C.

Standard PCR

A standard reaction mix contained 2µl 10x PCR buffer (500mM KCl; 100mM Tris-Cl pH 9; 1% Triton X-100), 1.5mM $MgCl_2$, 200µM dNTPs, 20pmoles of each primer, 0.2µl of homemade Taq polymerase (1:10 dilution of initial preparation), 1µl DNA sample and dH_2O to a final volume of 20 µl. A standard program comprised 1 cycle of 94°C for 1 minute, 40 cycles (94°C for 15 seconds, the required annealing temperature for 15 seconds and 72°C elongation (1min per 1kb)) and 1 final cycle of 72°C for 5 minutes.

PCR Amplification and Cloning of the Promoter Fragments

These reactions were performed with a high-fidelity ExTaq [Takara] polymerase. The reaction mix contained 5µl 10x buffer (supplied), 4µl $MgCl_2$ (25mM), 4µl dNTPs (supplied), 1µl of template, 1µl of each primer, 0,25µl ExTaq and dH_2O to a final reaction volume of 50µl. The PCR program comprised 1 cycle of 94°C for 30 seconds, 30 cycles (94°C for 30 seconds, 50°C for 1 minute and 72°C elongation (30 seconds per 1kb length of amplified fragment)) and 1 final cycle of 72°C for 5 minutes. As every Taq polymerase, Ex Taq adds one or more As to the ends of the amplified fragments, thereby facilitating the following TA cloning step.

The reaction mix was loaded onto an agarose gel and the respective fragments were cut out and ligated into pCR2.1 by using the Invitrogen TA Cloning Kit according to the manufacturer's protocol.

After verifying the fragments by sequencing they were cut out via the introduced PstI and XbaI restriction sites. Analogously, the vector pCBK04 was cut, thereby relieving the 35S promoter. The digestion mixes were separated on an agarose gel, the fragments and cut vectors eluted and ligated together, yielding the constructs that were later subjected to GUS assays (see Figure 15).

fragment	elongation time
com1_01	60"
com1_02	60"
com1_03	60"
com1_04	60"
com1_05	30"
com1_06	30"
com1_07	30"
com1_08	30"
com1_09	20"
com1_10	20"
com1_11	15"
com1_12	15"
com1_13	15"
com1_14	15"
com1_10a1	20"
com1_10a1_short	20"
com1_10a2	20"
com1_10a2_short	20"

Table 4 – Elongation times for PCR-generation of all promoter fragments. Reactions were performed with Takara ExTaq; reaction mix: 5µl 10x buffer (supplied), 4µl $MgCl_2$ (25mM), 4µl dNTPs (supplied), 1µl of template, 1µl of each primer, 0,25µl ExTaq and dH_2O to a final reaction volume of 50µl; PCR conditions: 1 cycle of 94°C for 30 seconds, 30 cycles (94°C for 30 seconds, 50°C for 1 minute and 72°C elongation (times are given in the table)) and 1 final cycle of 72°C for 5 minutes

Mutagenesis PCR (Bridging PCR)

For the in vitro mutagenesis of the E2F binding site of the fragment com1_09 new primers were designed that contain an altered version of the E2F binding site (the original sequence CGC GCG was altered to TAA TTT) and have 22bp in common (E2F_mut_up/dn).

The bridging PCR itself involved two subsequent rounds of PCR reactions. The first round of PCRs comprised two PCR reactions with the primer combinations com1_p6_dn/E2F_mut_up, yielding a product of 189bp in length and E2F_mut_up/com1_p1_up, yielding a product of 327bp in length. The vectors pCR2.1_com1_9 served as templates.

As the used primers share 22 bp the ends of the newly synthesized fragments should be able to anneal to each other.

In the second round of PCRs, the 189bp fragment was mixed with the 327bp fragment (together serving as template for the PCR reaction), and the primers com1_p6_dn and com1_p1_up were only added after the first cycle of the PCR reaction. This was done to let the two fragments with the 22bp overlap anneal to each other, before the new fragment of 494bp, containing the mutated E2F site could be synthesized.

Another mutagenesis PCR was performed analogously with the promoter fragment com1_10 and the up-primer com1_p2_frame_up, yielding the fragment com1_10 with a mutated E2F binding site.

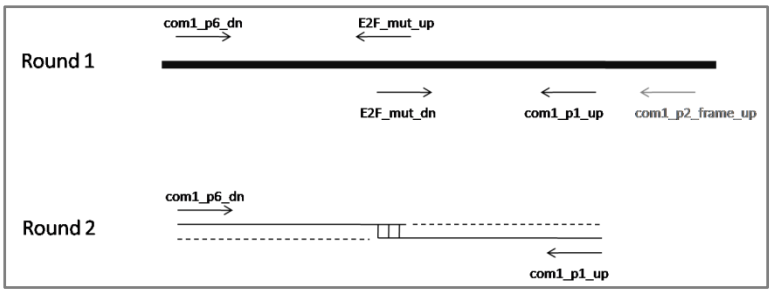


Figure 29 – Scheme of the two rounds of the bridging PCR. During the first round, two fragments are generated in parallel PCR reactions that are composed of the half of the template fragment and the mutated E2F binding site on their 5' or 3' ends, respectively. The second round involves the annealing and elongation of the two newly synthesized fragments during the first PCR cycle via the 22bp overlap created by the mutation primers E2F_mut_up and E2F_mut_dn (indicated by dashed lines). After addition of the primers com1_p6_dn and com1_p1_up the whole fragment containing the mutated E2F binding site is synthesized.

DNA Gel-Electrophoresis

Standard agarose gels for DNA analysis were prepared with 1% Standard Electrophoresis Agarose [genXpress] in 1x TAE buffer ((50X: 242g/l Tris, 57,1ml/l glacial acetic acid, 100ml/l 0,5M EDTA). For fragments less than 300bp in length, the amount of agarose was increased to 2%.

DNA Gel Extraction

The bands containing the DNA fragments of interest were cut out of the gel and DNA was eluted with the OMEGA E.Z.N.A. Gel Extraction Kit according to the manufacturer's protocols.

RNase Treatment

1/10 of total reaction volume RNase A (10mg/ml) was added to the DNA in solution and incubated for 30 minutes at 37°C. The DNA was then precipitated as follows.

DNA precipitation

$^{1}/_{10}$ volume 3M NaOAc pH5,2 and 2 ½ volumes 96% ethanol were added to the DNA sample and mixed vigorously. The mixture was incubated at least 20 minutes or overnight at -20°C. After centrifugation (15min, 14000rpm, 4°C) the supernatant was removed and the pellet was washed with 500µl 70% ethanol. The DNA pellet was dried and re-suspended in an appropriate amount of 1xTE or sterile water.

Sequencing

DNA was extracted as described in 0. Approximately 350 ng of DNA were used for the sequencing reaction containing DNA, 1,5µl 2,5x Sequencing Buffer, 1µl

Big Dye, 2µl Primer (2µM) and sterile water to a total volume of 10µl. The sequencing program comprised 1 cycle of 96°C for 2 minutes and 25 cycles (96°C for 30 seconds, 45°C for 15 seconds and 60°C for 4 minutes). If necessary the DNA was precipitated. DNA sequence was determined at the Institute of Botany,1030, Vienna.

7.4 RNA Work

Total RNA Extraction from *A. thaliana*

Plant material was harvested, ground to a fine powder in liquid nitrogen and stored at -80°C. For total RNA extraction a small amount of powder, covering the tip of a spatula, was processed with the Promega SV Total RNA Isolation System (Cat.No. Z3100) according to the manufacturer's protocol.

RNA Gel-Electrophoresis

After isolation, RNA quality and amount were analyzed on a denaturing RNA gel, prepared as follows. For up to ten samples 0,7g Standard Electrophoresis Agarose [genXpress] were dissolved in 50ml DEPC.H_2O (add 450µl DEPC to 1l sterile water, incubate for at least one hour at room temperature, autoclave). After cooling down 5,58ml 10xMOPS (41,8g MOPS; 16,6ml 3M NaOAc pH4,8; 20ml 0,5M EDTA; ad 1000ml DEPC.H_2O) and 1,75ml formaldehyde were added.

cDNA Synthesis

Gene expression studies via qPCR require cDNA as template. This was synthesized with the BioRad iScript cDNA Synthesis Kit (Cat.No. 170-8891) according to the manufacturer's protocol (1µg RNA, 4µl 5x reaction mix, 1µl reverse transcriptase, nuclease-free water to a final volume of 20µl).

Quantitative Real Time PCR (qPCR)

Primers for qPCR were designed using the online application Primer3 (http://frodo.wi.mit.edu/), according to the recommendations of the manufacturer of the used qPCR kit, the iQ SYBR Green Supermix [BioRad] (annealing temperature 60°C; amplicon length 100-300bp). They were chosen to bind in 2 different exons of the gene to make contaminations by genomic DNA (gDNA) easily visible – final amplicon length was 194bp for *AtCOM1* and 180bp for the reference gene *ACTIN 2/7* (in case of gDNA contaminations, the respective fragments would be 340bp and 278bp).

Expression analyses were performed using cDNA samples obtained from genotoxically treated seedlings and control plants. After RNA isolation from seedlings, RNA was quantified on an RNA gel and quantified. After cDNA synthesis, a conventional PCR was performed on the samples to rule out gDNA contaminations.

The final reaction mix was prepared using the iQ SYBR Green Supermix according to the manufacturer's protocol but with a total reaction volume of 25µl (12,5µl 2x reaction mix, 2,5µl of each primer; 2,5µl template and 5µl dH_2O). *ACTIN 2/7* (At5g09810) was used as reference gene. Reactions were performed in the BioRad iQ5 Cycler and results were calculated according to an improved ΔΔCt-method with the gene expression analysis tool of the BioRad iQ5 software. The amount of target, determined by normalization to the reference gene and relative to the control (cDNA from untreated seedlings), is $2^{-\Delta\Delta Ct}$. ΔΔCt is the substraction of the Ct value of the control from the Ct value of the sample (Lafarge, *et al*., 2003). This calculation allows evaluating target concentration in cDNA samples to infer messenger RNA levels and thereby the level of gene expression.

All experiments concerning qPCR analysis were performed with special diligence to avoid contaminations. This involved wearing of gloves, use of a special set of newly calibrated pipettes, use of filter tips and use of autoclaved and filter-sterilized dH_2O only.

Every sample was analyzed twice in parallel and every distinct experiment was repeated twice.

8 Supplementary Data

8.1 Primers

qPCR

All primers are listed in 5' to 3' orientation.

actin_ampl3_dn: TTGCTGACCGTATGAGCAAAGA
actin_ampl3_up: TCGATGGACCTGACTCATCGT
com1_ampl1_dn: TTCACCAAAGCAGCCTTGAG
com1_ampl1_up: GGAAGTGATAGGTGTCTGCACTG
GUS_ampl_dn: GGCACAGCACATCAAAGAGA
GUS_ampl_up: CTGATAGCGCGTGACAAAAA

Cloning

com1_plongest_a_dn: AAACTGCAGTTCCAAGTCATGAACAGAGTAAGGC
com1_p2_dn: AAACTGCAGGCCAAGAAATCGAACCAT
com1_p3 _dn: AAACTGCAGGGAGAAGACGAATGTAACC
com1_p4_dn: AAACTGCAGTCTCTTGTTACCGTTGCAGC
com1_p6 _dn: AAACTGCAGGAGCTTATTGGGCCATAAC
com1_pcore_dn: AAACTGCAGGAAAATCACCGCGACAATC
com1_pleast_dn: AAACTGCAGAAAGTTGTTACACAGATCTC
com1_p1_up: GCTCTAGACTTCAAGCTGTAATCAGAAAACG
com1_p2_frame_up: GCTCTAGACTGGCTACAAAAAATATACTCGAT
com1_10a_dn1: ATACTGCAGTTTATAAGTCGCATCTCAAC
com1_10a_dn2: ATACTGCAGTCAACGCGCGAAAATTCCG
com1_p220_up: GCTCTAGAGATTGTCGCGGTGATTTTC

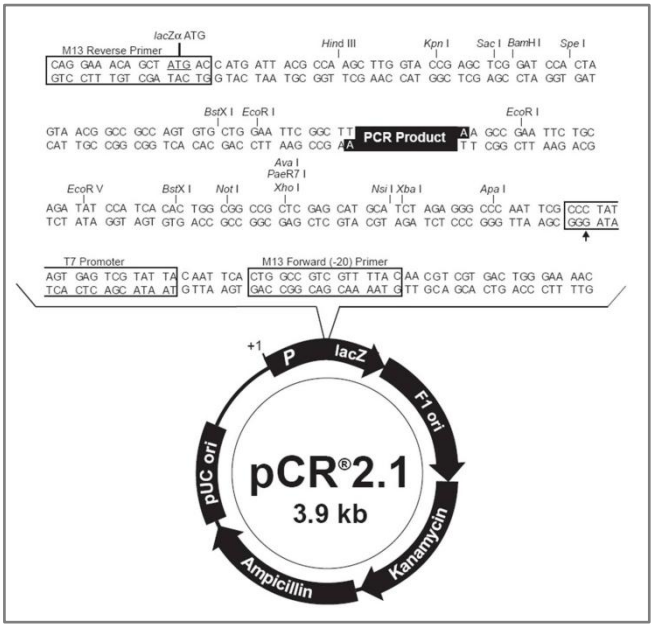
E2F_mut_dn: CATCTCAATAATTAAAATTCCGAAATTCCGAAATT
E2F_mut_up: CGGAATTTTAATTATTGAGATGCGACTTATAAATTTTC

PAP1_dn: GCTCTAGAATGGAGGGTTCGTCCAAAGGGCTGC
PAP1_up: GCGAGCTCCTAATCAAATTTCACAGTCTCTCCA

8.2 Vectors

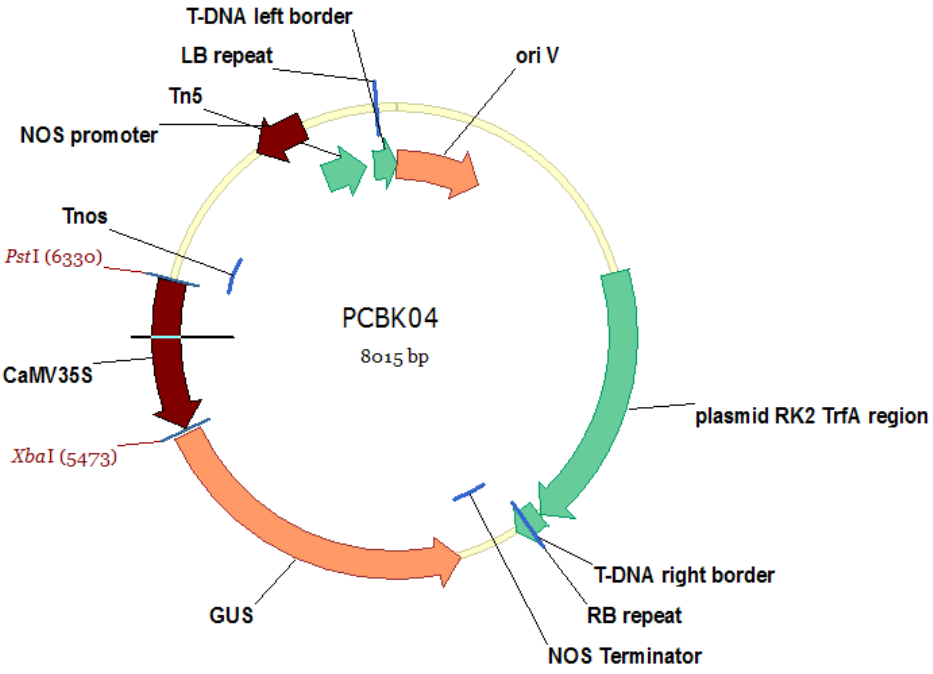
pCR2.1

This vector is part of the Invitrogen TA cloning kit.



pCBK04

This binary vector was kindly provided by Karel Riha.



8.3 Sequence of *AtCOM1*

The sequence of the putative promoter region including 2098 bp upstream of the ATG of *COM1* followed by *AtCOM1* (At3g52115) full-length genomic according to the TAIR database (http://www.arabidopsis.org) (in **bold** letters, introns in *italics*); the sequence of the longest promoter-fragment is highlighted in orange; the shortest promoter fragment is indicated by smaller font size; the E2F binding site is marked in blue; UTRs are underlined.

TTCTCAGAGAAGCATTGAGGAGATGGTGGGTTTGTCCTGATTTGCTGCAGG
AATCGTTCGTGTCCTTTGTTCTTTATAGTCTCCAGTTTTAGAGGAACTGGGAT
GTTCTCTGCATCATCCATTCTCCTAAAAAAAATCCTCACAGAATCCAAAGTTT
AGTACTTTACGCACTAAACTCATCAATTGTATACATATCTAAGGCTCAAACAT
ATAAAGGAACTTACTTCTTTCTTCTACCCTCCTCTCCTTTGTTTTTTACAGTCT
CCACATTAAGTGAGACAGGAATGTTCTCTGCATCATCCACTCTCCTGCAAAT
CATCATTAGAAGTTTAGTAAAGTCACTTCTATGTAGGTTGTGCATAACACTAT
TTTACTGAAAAAATGACAAAACTTACTTCTTTCTTCTACGGTTCTCTTTAAATG
TGCTTCCTTTAACATCCGTGGACTCAGGGAGCTTATCTTTGCTTACTTCAAG
CAGTGGCTTTCTTTCCTTTTTCAATTCCAAGTCATGAACAGAGTAAGGCTGAT
TTACTATTTCACCCACTTGATGACTCTCCTTCACTTCAGATTTCCCAGTATCT
TCTCTCTCAGTAACCCTTGTGGCTAATCTCTTCAATTCATAGCAGAAATCTGA
GATATGACCAAATATGTCTCTCCCTGAAAACAGATTTCTAGCTGATTGTGTG
CTCTTTAGTCTTGGAACTTCTTTATTCATCTTCTTGACAGAAGTTGATTTAATT
GCTGCTCTCCATGATTTTGCTTGGTAGCTTGGAGGTGTGGCTAAGTTCGGG
TTCTGATTTTCACTTTCTTGGTTGTTTGGAGTAGAAGGTGATAAAGCATATCC
CCTCCTCCTACAAGTAAATTGAAACAGAGTGTGAACCAAAAAGCATTCAAAG
GCTATAGCAAAGAATTGAGATTGGAGATCCTTAAGTTAGAGATCAGTACCTC
TGATTCTGCTCTGTTAGAGTCTCATTCGTATCCCTCAGACTTGGATGCTTCG
AAACAGAAAACGCAGGAAATTATTAAAGGTGAAACCTTTGTGATTGTCAAGA
AACTGTAATTTCATATCTTTATGAGGAAACAAGAAAGTAAATAAGAAGGGAGA
AACAAACCTTGGAAGAAGTCGGCGTTGAGAAAAAGTCCTCAGGTCTCTTGG
GATGATTACATTCTGGAGTTGAAGATAAAACCAGAATCAGTAAAGAACGATA
GAATAGAAGCAGAGATACAAGAATAGTTTCTTTATTTACCAGGTTTGCAGAA
CCAAGCATCGTCGTCAATGGAGTCAAAATGGGTAGGGGCCAAGAAATCGAA
CCATTTAGGAGCTCTGATGTGTTCGTAAAAAACGTCTTCAACAAAACGAGAA
TCGATTCTCTTCCAATCGATATGAGCAGGCTCCATGAGAGAAGAATACACAG
AGATACAGAGAGAAAGGATGGAGTTGTTGGTTGGAGAGAAAAAGAAAAGGA
GAAGACGAATGTAACCGTTGTAGTGAACGTTACAGGTCCATGTGGACACGT

GGCTTTCTCTTGTTACCGTTGCAGCTTTATTTAATAACACTAGCCAATCCACG
TGGAAAAAGAAACGAGACGTGGGAAGGGAATGAATGAGTTTCTTTATTACG
GGCCATAAACGAGCTTATTGGGCCATAACTGATCTGAGTTCAGGCTGCATTG
GGTCGGACCAGCGAGTTATCCGCTCCAACAACTTGATAACGGGTCGGGTCT
CCCAGCAAAAGCTTCTCGGGTAAGCCTCCACTGAAACTTGAAAATTTATAAG
TCGCATCTCAACGCGCGAAAATTCCGAAATTATAGAGGGAAAATCACCGCG
ACAATCTAAAATTTTGGACACTCAAAATCCCAAATTAGTCTTGTTGCTTTCAT
CACCGGAATCACTCAAGCTTCTTCGATCTCTCTCGCGTCGTTTCGTTTCCAA
AT*GTTAGTTTTCTTCTATTCATTTTTTCTTT*AAAGTTGTTACACAGATCTCAAAACTAGA
TCGAATTGTAAGAAATCGAAATTTATTGTAATGTTTAGGTAGGAGAAAACCCAGTTATTGAATCG
*TTGAATTGAGTCCATTTACGTTTTCTGATTACAG*CTTGAAG**ATGGGAGATGAAACTGTT**
GACGGTATCGAAGCCAAGTATATATCTGGTCTCAGTACTATAATGGTTGCT
ACAATTCAAGAAGCCAAGGATAGAATTTCTCAGATCGAGTATATTTTTTGT
AGCCAGTTATTTCCAAATTTTCAATCCAAATCCAAAGCTTTTGAAAAAGTTT
ATTCAGAAGCTCGTCTTGCTGCTTGTGATACTTGGAAAGATAGAGAGAAAA
GTTTGTTAGATCAGATTGAAGAGCTTAAGGTAGAGAATCAACAGATTAAGT
CAGATAAGGAGAAGTTAGCTGAAGAACTTGGTAAAACTGCTTCTATGCCTC
TTAGATTGACGAGTCTACAAGGCTACATCGATCATTTGAAAAAGAAGATGA
AGAGTAGATCTAAGATGGTTGGTGATGCAAGAGATTTGTATTATAGGTTAG
TTGAGTTGTTGCAGGTTAAGGGTTTGGATGAGCTTAGCGAAGATGGGATA
AATATGATTGTTTCGGAAGTGAAGAGCCTTAAGATGAAGACTGAATTTCTT
CAGGAGGAACTCTCAAAGAAGACATTGGTGACGGAGAATCTGCTGAAGAA
ACTGGAGTACTTGTCTACAGAAGCTGCAGATGGTGAAAGGAAGTTGAGCA
GCGTTGAAGAAGAGAAACAAAGATTGAAGACTAGGTTGCAGGTTTTTGAG
GAGAATGTCGGTAGACTTGAAGAGATACTTAGGCAAAAGACCGATGAGGT
TGAGGAAGGAAAGACAGCTCTTGAGGTTTTGCAGGGTAAACTCAAATTGA
CCGAGAGGGAAATGTTGAATTGTAAACAAAAGATTGCAGATCATGAGAAA
GAAAAGACAGTGGTTATGGGAAAAGCAAAGGATGATATGCAGGGCAGAC
ACGGAAGCTACTTGGCTGACTTAGAAGCTTTACGTTGCCAGAGTGAAGAG
AAATCATTTGAGTTAGCTATGGAGATAAAGAAAAATAAAGAACTCTCTCGT
ACTTGCAAAAAGTGGAAGTCCCAACATACTTTTCTCTGCAAAAGGTTTAAT
TTTACTCCTGACAGTGTTCTTCACCAAAGCAGCCTTGAGGATGAGAATAAA
GAAATTGGTCAGCATGAGAAATCAGCAATCTCGAGTT*GTAAGTATTAGCCG*
TCTCTGACCTAGACTACTTTGAACTACCTCTCGCGTTAAATTACTTGCTATAG
TCAGTCATCCTTTAGCTGCAGGTTTCTTATTGGTATTAGTAAACTCACCCAGC
*TTGATTTTTTACTCTCTCTATTACAG***ATCTTGAAAGGAAACACTCAGAAACT**
GCTGAAGGAGCAGACAAAGTGAGAATTGGTACTGGCTCGAGTGGCAACA

ACTATGAGAAAGAGAGTATAATCAAAACAGTGCAGACACCTATCACTTCC
ATCTCACCAATTGTGCGGTCACCTGGTGCTGCAAAACCTCCACAGCTAAG
CGGATTAAAGCGTCCAGCTTCCATTTGGAGAGACACAAGATCTCGTCAAT
CTCCTGGAGGACATGATCCTCATGATGATTTTCTAGATACTCCCATTGAGA
ATGTCAAAAGGGTCGCTGGGGAAGAAAAACATGTTCATGATGTTGCTAAG
AAAGATGATTCAGACGACGAGACACAGGACATGAATCCTAAACCGAGTCC
ATCACGACAGCGAATTCAAATTGCAGAAACGAGTAAAAAGAGTTTCAAGC
ATGTGGAGTCAGTGAGGAAAAAAGCCGAGAGGGAGAATTTGAAAGGTAT
AGAATGTAAACAATGCAAGAAATTCTATGATGCTGTTCATCCTGAGAATGA
AGGAAATGGTAACAAAAGCTTGAGATGTGAACATCATGAAGGTGTGTCGA
GGCATCGTTACAGATACGCTCCTCCAATGACTCCTGAAGGGTTTTGGAACA
TCGGGTTTGAATCCGAAATGTAATTCTGTATCTTGACATTCTTTGCATCTAC
GTTCTTTGGATCGAAAGACTATACAATAAAGTTTTTCTCTGCTTTCTCGAAGA

8.4 Abbreviations

bp	base pairs
cDNA	copy DNA
CO	crossover
DAPI	4'-6-diamidino-2-phenylindole
dHJ	double Holliday-junction
DNA-PK	DNA-dependent protein kinase
DNA-PKc	catalytic subunit of DNA-PK
DSB	double-strand break
dsDNA	double-stranded DNA
EMSA	electromobility shift assay
FA	Fanconi anemia
gDNA	genomic DNA
GTF	general transcription factor
GUS	β-glucuronidase
HR	homologous recombination
HU	Hydroxyurea
kb	kilobases
MMC	Mitomycin C
MMS	methyl methanesulfonate
MRX	Mre11-Rad50-Xrs2 complex
MRN	MRE11-RAD50-NBS1 complex
mRNA	messenger RNA
NCO	non-crossover
NHEJ	non-homologous end-joining
PCR	polymerase chain reaction
Pol	polymerase
qPCR	quantitative real time PCR
RPA	replication protein A
rRNA	ribosomal RNA
SC	synaptonemal complex
SDSA	synthesis-dependent strand annealing
SSB	single-strand break
ssDNA	single-stranded DNA
TF	transcription factor
tRNA	transfer RNA
UTR	untranslated region
X-Gluc	5-Bromo-4-chloro-3-indoxyl-beta-D-glucuronide cyclohexylammonium salt

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